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Free Fatty Acid Receptors

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Free Fatty Acid Receptors

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Preface

Recent times have seen food-derived products and metabolites, including free fatty acids, highlighted as activators of a substantial range of cell surface G protein-coupled receptors. Four members of this broad receptor family, FFA1-FFA4, are now fully accepted as receptors for which the natural ligands are either short chain (FFA2/FFA3) or both saturated and unsaturated longer chain (FFA1/FFA4) fatty acids. A further G protein-coupled receptor, GPR84, is also widely recognized as being activated by a group of medium chain fatty acids. Moreover, given that effects of different fatty acids are viewed as being either broadly beneficial or detrimental to health, recognition of the effects of various fatty acids via these G protein-coupled receptors has resulted in considerable interest in the potential to target their effects therapeutically by either activating or blocking one of more members of the free fatty acid receptor group with synthetic small molecule ligands. To date, the major foci of interest in clinical settings have centred on either activating the long chain fatty acid receptors to enhance insulin release from pancreatic islets, to improve insulin sensitivity and to provide potential anti-inflammatory input in metabolic diseases, or antagonizing short- and medium-chain fatty acid receptors to limit neutrophil and other white cell chemotaxis and to assess if this might provide a means to limit the development or maintenance of inflammatory conditions of the lower bowel. Although no medicines that target one of more of the free fatty acid receptors have yet been approved for patient treatment, the progress of the FFA1 agonist fasiglifam into a phase III clinical trial before this trial was halted due to deleterious effects on liver transporter systems, provided strong validation of activation of this receptor as a means to provide glycaemic control. Whilst no FFA4 ligands have entered clinical trials to date, activation of this receptor is also viewed as a conceptually attractive means to improve metabolic function. Neither the FFA2 antagonist GLPG0974 nor the GPR84 antagonist GLPG1205 proved effective for the treatment of ulcerative colitis, but recent publications on both of these receptors suggest a broader range of other potential therapeutic areas that might be targeted, whilst publications on aspects of free fatty acid regulation of cancer development and progression, both positive and negative,

and its effects on adipocyte versus bone differentiation also highlight different roles of the long chain fatty acid receptors.

The group of reviews by leading experts that comprise this volume of *Handbook of Experimental Pharmacology* therefore covers topics that range from basic underpinning knowledge of the biology and physiology regulated by members of the free fatty acid receptor family, through the chemistry and generation of synthetic ligands, both tool compounds and the development of drug like-molecules, to the clinical potential of such ligands, and well as analysis of the opportunities and challenges that remain. With a single atomic level structure of a free fatty acid receptor available to date, then the incorporation of efforts to provide both homology models and mutagenic analysis of the modes of binding of free fatty acid receptor ligands and whether such ligands bind in a manner that is competitive with that of the endogenous fatty acids is also explored. From initial de-orphanization in the earliest years of the century and rather modest numbers of reported studies in the early days, there has been a rapid acceleration of interest in and knowledge of the free fatty acid receptors that reflects the still growing appreciation of their roles and their potential for therapeutic regulation. It is therefore an ideal time to consider and review the progress to date and prospects for the future.

Glasgow, UK — G. Milligan
Tokyo, Japan — I. Kimura
October 2016

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Ligands at Free Fatty Acid Receptor 1 (GPR40)

Takafumi Hara

Contents

Abstract

FFA1 is a G protein-coupled receptor activated by medium- to long-chain fatty acids. FFA1 plays important roles in various physiological processes such as insulin secretion and energy metabolism. FFA1 expressed on pancreatic β-cells and intestine contributes to insulin and incretin secretion, respectively. These physiological functions of FFA1 are interesting as an attractive drug target for type II diabetes and metabolic disorders. A number of synthetic FFA1 ligands have been developed and they have contributed to our current understanding of the physiological and pathophysiological functions of FFA1 both in in vitro and

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in vivo studies. In addition, these synthetic ligands also provided information on the structure–activity relationships of FFA1 ligands. Further, FFA1 protein crystallized with one of the high affinity agonist leads provided useful insights for the development of more effective ligands. Among FFA1 ligands, several compounds have been further investigated in the clinical trials. Thus, FFA1 ligands have great potential as drug candidates. In this section, recent progress about FFA1 ligands and the possibility of their clinical use are described.

Keywords
Diabetes • Fatty acids • FFA1 ligand • Free fatty acid receptor 1 (FFA1) • G protein-coupled receptor • Metabolic disorder • Structure–activity relationships

1 Introduction

G protein-coupled receptors (GPCR) are the major target for approved clinical medicines of various diseases. The human genome project revealed that a large number of GPCRs are encoded in the genome; however, there are still a significant number of orphan GPCRs that are considered as attractive drug targets (Civelli et al. 2013).

Deorphanization of GPCRs identified a group of GPCRs activated by free fatty acids (Briscoe et al. 2003; Hirasawa et al. 2005; Itoh et al. 2003) that are now defined as free fatty acid receptors (FFARs) (Stoddart et al. 2007; Davenport et al. 2013). To date, four FFARs are characterized and defined by differences of the carbon chain length of fatty acid ligands. FFA1 and FFA4 are activated by medium- to long-chain fatty acids, while FFA2 and FFA3 are activated by short-chain fatty acids. FFARs therefore act as sensors for fatty acids.

FFA1 is highly expressed in intestine and pancreatic β-cells. FFA1 activation induces incretin and insulin secretion from intestinal endocrine cells and pancreatic β-cells, respectively. Therefore, synthetic compounds that can interact with FFA1 selectively are considered as potential drug candidates for the treatment of metabolic disorder such as type 2 diabetes. To date, a number of synthetic compounds have been developed and structure–activity relationships of these FFA1 agonists have also been investigated (Defossa and Wagner 2014). In addition, FFA1 crystal structure with the selective agonist TAK-875 ([(3S)-6-({2′,6′-dimethyl-4-′-[3-(methylsulfonyl)propoxy]biphe-nyl-3-yl}meth-oxy)-2,3-dihydro-1-benzofuran-3-yl]acetic acid hemi-hydrate) has been reported (Negoro et al. 2010), which provided great insights into the process of developing the ligands more efficiently in terms of the selectivity of the ligands and of pharmacodynamics and pharmacokinetics. Several ligands developed by pharmaceutical companies have entered clinical trials. In this chapter, FFA1 ligands and their therapeutic utility are described together with recent progress in this area.

2 Natural Ligands

Several groups reported that medium- to long-chain fatty acids activated $[Ca^{2+}]_i$ responses in FFA1 expressing cells (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003). Since FFAs are known to have numerous biological effects on intracellular signaling, high-throughput screening using inducible or stable expression of the receptor in cell lines was applied to evaluate FFA1 specific responses.

Agonistic activity of fatty acid ligands of FFA1 was measured by $[Ca^{2+}]_i$ responses. The rank order of the agonistic activities of fatty acid ligands are as follows: docosahexaenoic acid (DHA, C22:6) > α-linolenic acid (α-LA) (C18:3) > oleic acid (C18:1) > palmitic acid (C16) > lauric acid (C12) > capric acid (C10) > caprylic acid (C8) (Christiansen et al. 2015; Itoh et al. 2003). Almost all fatty acids ligands can activate FFA1 in the submicromolar range (Christiansen et al. 2015).

On the other hand, short-chain fatty acids including acetic acid (C2), butyric acid (C4), caproic acid (C6), and methyl linoleate could not activate FFA1 signaling, which indicated that the carbon chain length and carboxylate group in the fatty acid structure would be critical for agonistic activity at FFA1. Several compounds which have different structures from fatty acids have also been reported as natural ligands, for example, conjugated linoleic acid (Schmidt et al. 2011) known as a dietary component associated with anticarcinogenic effects, showed agonistic activity in FFA1 expressing cells. Since fatty acid ligands were considered to exhibit their biological function via some other signaling pathways, including fatty acid binding proteins (Furuhashi and Hotamisligil 2008), FFA1 signaling could provide a better explanation for some of the biological processes of FFAs.

3 Synthetic Ligands

3.1 Agonists

To develop high affinity and selective ligands is of great interest not only for exploring pharmacological functions of FFA1 but also for developing potential candidates for clinical use, because physiological functions of FFA1 are strongly related to glucose homeostasis and energy metabolic processes such as secretion of insulin and incretins and therefore regulation of blood glucose levels. Several research groups including pharmaceutical companies have developed various synthetic compounds (Fig. 1) and characterized them as novel FFA1 ligands by using in vitro and in vivo studies (Garrido et al. 2006; Humphries et al. 2009; Krasavin et al. 2016; Li et al. 2016; Tikhonova et al. 2008; Yang et al. 2016). Various studies have used the synthetic compound GW9508 (4-[[(3-Phenoxyphenyl)methyl]amino] benzenepropanoic acid) as a reference compound. GW9508 also activated FFA4 signaling, although it showed approximately 100-fold selectivity compared to FFA4 (Briscoe et al. 2006; Tikhonova et al. 2008). Some studies have also utilized the FFA1 antagonist GW1100. GW1100 showed inhibitory effects on glucose-stimulated insulin secretion (GSIS) from the mouse insulinoma cell line MIN6

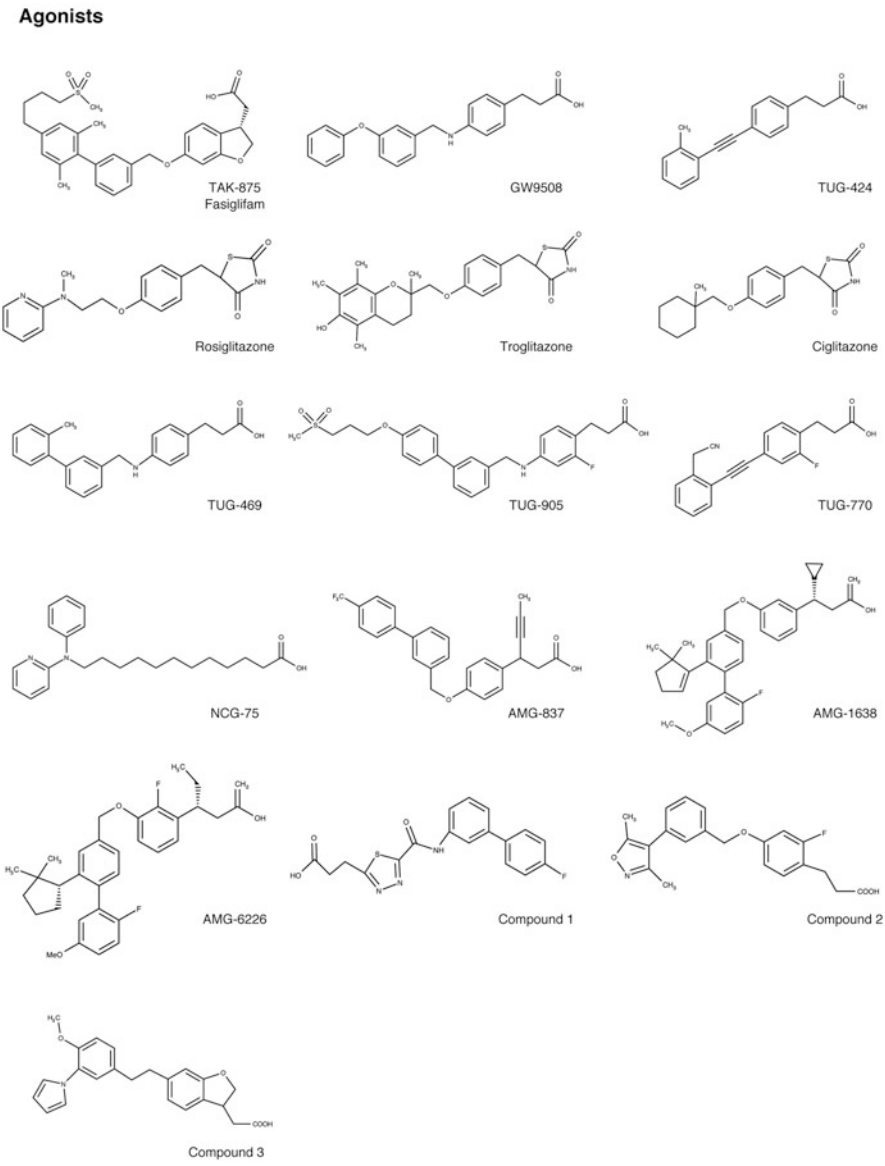

Fig. 1 Representative FFA1 ligands. The structures of representative FFA1 selective ligands are shown

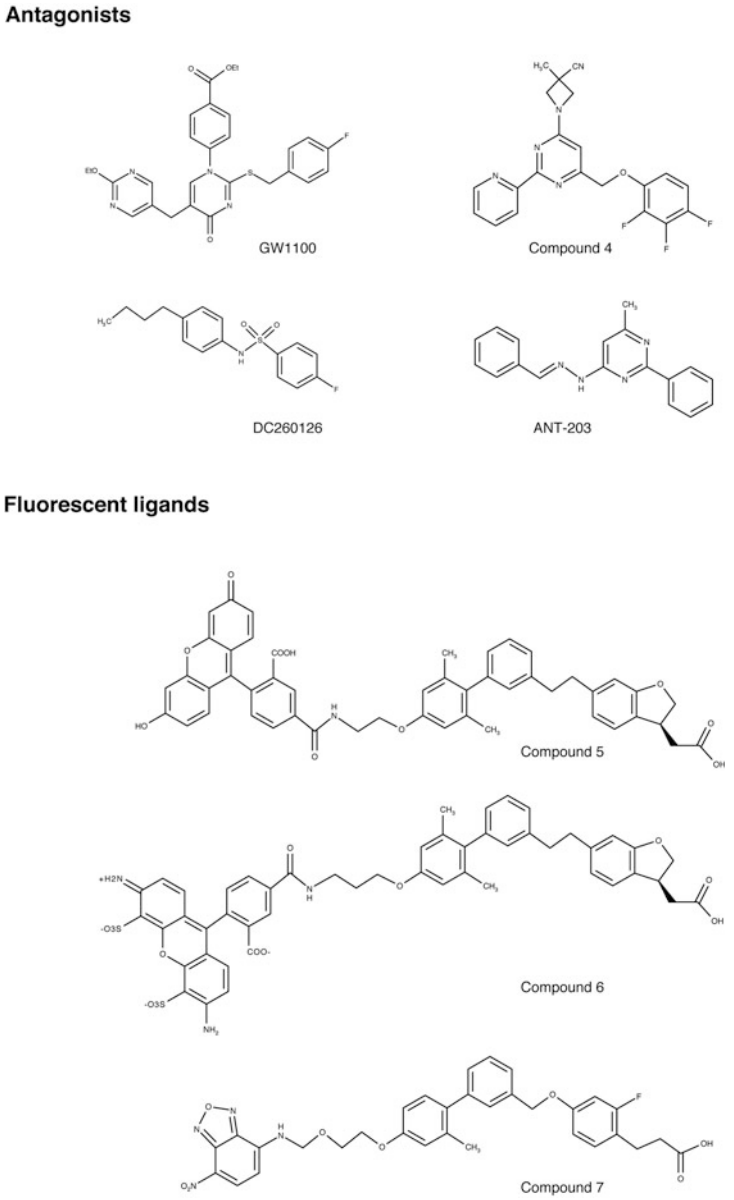

Fig. 1 (continued)

induced by GW9508 (Briscoe et al. 2006). Some research groups reported that thiazolidinediones including peroxisome proliferator-activated receptor-γ (PPARγ) agonists: rosiglitazone, troglitazone, and ciglitazone, those are known as anti-diabetic compounds, showed agonistic activity at FFA1 (Kotarsky et al. 2003; Smith et al. 2009; Stoddart et al. 2007). A series of 4-phenethynyldihydrocinnamic acids showed agonistic activity at FFA1. Among these compounds, TUG-424 showed GSIS in INS-1 cell line and pancreatic islets isolated from wild type mice, but not from FFA1 knock out mice. TUG-770, which was developed based on the structure of TUG-424 and has improved metabolic stability and short plasma half-life, showed a potent effect on glucose tolerance in diet-induced obesity mice, a situation that was sustained after 29 days of chronic dosing (Christiansen et al. 2008, 2013). Further structural optimizations were to lower lipophilicity and increase metabolic stability of the ligand. Combining features of TAK-875 and TUG-469 was explored and resulted in TUG-905, which showed lower lipophilicity and higher metabolic stability while preserving potency to activate FFA1 (Christiansen et al. 2010, 2012).

TAK-875 was developed based on phenylpropanoic acid derivatives. Although these compounds appeared to be susceptible to β-oxidation at the phenylpropanoic acid moiety, cyclization of the phenylpropanoic acid moiety, which produced a series of fused phenylalkanoic acids, showed favourable pharmacokinetic profiles (Negoro et al. 2010). Although prolonged exposure of FFA ligands showed lipotoxicity in the INS-1 cell line and primary pancreatic β-cells, prolonged treatment of TAK-875 did not impair GSIS and insulin content (Tsujihata et al. 2011; Yashiro et al. 2012). TAK-875 showed high selectivity for FFA1 compared to other FFARs including FFA4 ($EC_{50} = 14$ nm in human FFA1 and >10 μm in human FFA4) (Negoro et al. 2010; Srivastava et al. 2014). In addition, TAK-875 acts as partial agonist that binds to an allosteric binding site of FFA1 and increases the agonistic activity of endogenous fatty acid ligands (Srivastava et al. 2014).

Our group developed a synthetic ligand, NCG75, using *in silico* docking simulations of FFA1. NCG75 showed potent agonistic activity in ERK1/2 and $[Ca^{2+}]_i$ assays. NCG75 promoted insulin secretion from mouse insulinoma MIN6 cell line, which expresses FFA1 endogenously (Takeuchi et al. 2013). AMG-837 that was developed by the modification of a series of β-substituted phenylpropanoic acids was identified and characterized as an FFA1 partial agonist (EC_{50} = approximately 0.1 μm) (Houze et al. 2012; Lin et al. 2011; Yazaki et al. 2011). Although both TAK-875 and AMG-837 exhibited anti-hyperglycemic effects, these two compounds did not increase incretin levels in in vivo experiments. AM-1638 and AM-6226 which were designed by modification of AMG-837 showed potent effects on both insulin and incretin secretion (Luo et al. 2012).

In 2016, some further synthetic compounds have been reported as FFA1 agonists. Krasavin et al. reported that Compound 1, containing 1,3,4-thiadiazole-2-carboxamide group showed excellent plasma and metabolic stability with FFA1 selectivity compared to other FFARs. Yang et al. reported that Compound 2 containing 3,5-dimethylisoxazole moiety showed agonistic activity with EC_{50}

value of 15.9 nm and exhibited glucose excursion to approximately 25% at 30 mg/kg of oral administration. Most FFA1 agonists bearing a common biphenyl scaffold had low water-solubility and metabolic toxicity (Takano et al. 2014). In order to improve these properties, Li et al. explored compounds with a non-biphenyl scaffold and developed Compound 3 that showed potent and orally bioavailable agonistic activity without the risk of hypoglycaemia.

3.2 Antagonists

Some compounds have been reported as FFA1 antagonists. GW1100 was first reported as an FFA1 antagonist that inhibited GW9508 and linoleic acid-induced FFA1 signals (Briscoe et al. 2006). DC260126 containing a sulfonamide structure showed inhibitory effects on $[Ca^{2+}]_i$ responses induced by FFA ligands in FFA1 expressing CHO cells (Hu et al. 2009). DC260126 was examined in in vivo experiments with diabetic model mice and rats. Eight weeks treatments of DC260126 decreased insulin levels and improved insulin tolerance in obese Zucker rats (Zhang et al. 2010). Three weeks treatments of DC260126 significantly inhibited GSIS and serum insulin levels in db/db mice. In addition, DC260126 also reduced the apoptotic rate of pancreatic β-cells (Sun et al. 2013).

The pyrimidinylhydrazone ANT-203 was identified in high-throughput screening and showed anti-apoptotic effect on MIN6 cell line (Kristinsson et al. 2013). A series of 2-(pyridinyl)pyrimidines were reported as potent antagonists of FFA1. Among the series of compounds, Compound 4 showed moderate antagonistic activity ($pIC_{50} = 6.2$) in FFA1 stably transfected cells and reduced plasma insulin level which was elevated by β_3-agonist-induced plasma non-esterified fatty acids in Zucker fa/fa rats (Waring et al. 2015). Further studies might reveal the precise binding mode of these antagonists in FFA1.

3.3 Fluorescent Ligands

To assess the pharmacology of FFA1, some fluorescent FFA1 ligands have been reported. Although fluorescent-labeled FFAs containing the BODIPY structure are commercially available, their high lipophilicity and high EC_{50} value (submicromolar) limited their use in studying interactions with FFA1 (Hara et al. 2009). To overcome these problems, compound 5, 6 and 7 were developed based on either TAK-875 or TUG-905 (Bertrand et al. 2016; Christiansen et al. 2016; Ren et al. 2016). Especially, Compound 7 was demonstrated as a useful tracer for bioluminescence resonance energy transfer (BRET)-based binding assays, which allowed for the characterization of binding affinities of various known FFA1 agonists (Christiansen et al. 2016). Hence, these compounds are useful pharmacological tools not only to examine the binding mode of known FFA1 ligands but also to conduct high-throughput screening.

4 Structure–Activity Relationships

FFA1 is involved in the activation of insulin release from pancreatic β-cells and, as such, is considered an attractive therapeutic target for the treatment of diabetes. Many groups have tried to develop compounds which can activate FFA1 efficiently and selectively. To understand ligand recognition in FFA1 protein and how the receptor transduces its signals is helpful for the development and rational design of efficient ligands with high affinity and selectivity. Using *in silico* docking simulations with FFA1 homology models based on identified protein structure of GPCRs and amino acid sequence of FFA1, some research groups, including pharmaceutical companies, have attempted to develop novel FFA1 ligands. Information about the chemical structure and the recognition mechanism of the compound is important to evaluate binding affinity of ligand. In certain class A GPCRs, ionic locks at the extracellular surface of the receptor are considered key components in the activation of the receptor; however, how the ionic lock is involved in detail in ligand binding remains unclear. FFA1 has two such ionic locks between residues of the second extracellular loop and one of the transmembrane domains (Sum et al. 2009). In addition to the importance of such ionic locks, aromatic, hydrophilic and hydrophobic amino acid residues of FFA1 contribute to the ligand binding is defined in mutagenesis studies. Such a mutagenesis study revealed that Arg183, Asn244 and Arg258 were involved in the interaction with the carboxylate group of ligand, whilst His86, Tyr91 and His137 were involved in the interaction with aromatic or hydrophobic properties of the ligand. In addition to these amino acid residues, the binding mode of NCG75 analysed by *in silico* docking simulations showed that Val141, Ala146 and Ala173 in FFA1 might stabilize ligand interactions through hydrophobic interactions (Takeuchi et al. 2013). These findings therefore indicated that amino acid residues, which were expected to be essential for ligand interactions, were different in each ligand binding to FFA1. Since an FFA1 structure crystallized with TAK-875 has been reported recently (Srivastava et al. 2014), the results of *in silico* docking simulations combined with the information obtained from the crystal structure analysis provide great opportunity for further development of potential drug candidates (see Chapter "Homology Modeling of FFA Receptors" organized by Dr. Tikhonova IG).

5 Crystal Structure of FFA1 with TAK-875

Srivastava et al. reported a high-resolution structure of human FFA1 receptor bound to the allosteric agonist TAK-875 (Srivastava et al. 2014). The crystal structure was analysed at 2.3 Å resolution. TAK-875 showed a unique binding property, which suggested that the binding site of TAK-875 was different from that of fatty acid ligands. The binding pocket of TAK-875 that was identified by the crystal structure analysis is located in between helix3 and helix4 (Fig. 2a, b). TAK-875 might enter

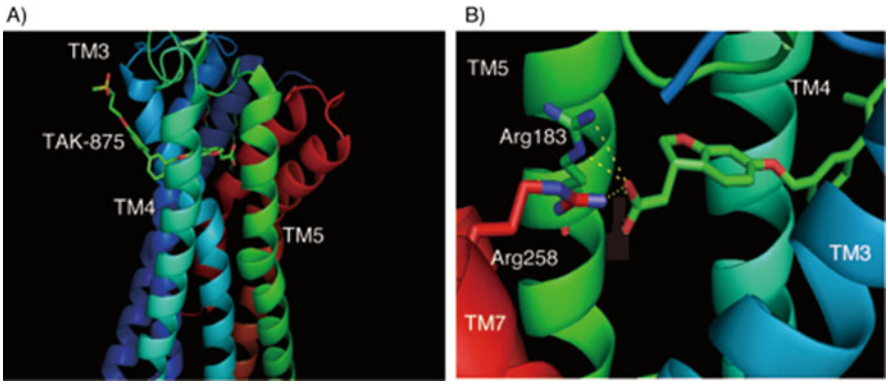

Fig. 2 Docking mode of TAK-875 in a human FFA1 crystal structure. (**a**) Overview of FFA1 structure crystallized with TAK-875. TAK-875 binds to an allosteric binding site located between TM3 and TM4. (**b**) Expansion of the binding mode of TAK-875 in the binding pocket. The interactions between the carboxylate element in TAK-875 with Arg183 and Arg258 are shown as *yellow dotted lines*. Structural information of FFA1 crystallized with TAK-875 (10.2210/pdb4phu/pdb) was derived from PDB database (http://www.rcsb.org.com). The structural data was analysed by MacPyMOL software

this binding pocket via the lipid bilayer from the extracellular side. In addition to the binding pocket for TAK-875, another potential binding pocket located between helix 1 and helix 7 was also predicted. Ligand binding assays with mutated FFA1 protein supported this model. Further, biological assays monitoring Ca^{2+} flux indicated that the effect of γ-linoleic acid was enhanced in a positively cooperative manner by addition of TAK-875 and mutagenesis studies confirmed different amino acid residues required for Ca^{2+} flux via FFA1 (Ito et al. 2013; Lin et al. 2012). These reports supported that more than one binding pocket would be present in FFA1. The FFA1 structure with TAK-875 bound was potentially an inactive state. Thus, FFA1 signaling induced with the ligand binding to the receptor might be modified by another allosteric ligand. Further analysis would be useful to examine if both of the expected binding pockets can be occupied with distinct ligands simultaneously. It will also be interesting to know if both binding pockets are occupied simultaneously if this alters the structure of the receptor and which signaling pathways are engaged.

6 Biased Agonism of FFA1 Ligand

FFA1 is reported to signal mainly via Gq/11 and Gs pathways which are mediated by IP3 and Ca^{2+} modulations and cAMP elevation, respectively. Hauge et al. reported that FFA1 signals were produced via both Gq and Gs upon binding of certain but not all agonists (Hauge et al. 2015). They evaluated the pharmacological profile of FFA1 agonists by measuring IP3 and cAMP levels, and found that

several ligands increased both IP3 and cAMP levels. Interestingly, in receptor binding assays with Gq-only (L358) and both Gs and Gq (Gs + Gq) agonists (AM-1638) showed that L358 binding to FFA1 was increased with co-incubation of the Gs + Gq agonist, and AM-1638 binding also increased in the presence of the Gq selective ligand. However, in silico docking simulations suggested that both the Gq specific and the Gq + Gs ligand could be docked into the same ligand binding site as TAK-875. Further work is therefore required to understand the basis of this differential engagement with G proteins. In vitro studies using primary intestinal cells showed that both Gq specific and the Gq + Gs ligands-induced incretin secretion. However, the effect of Gq + Gs ligands on incretin secretion was higher than that of Gs specific ligands. A similar tendency of these agonists was also shown in in vivo experiment measuring incretin levels.

Manicini et al. reported that FFA1 can be coupled to both G protein and β-arrestin signaling pathways (Mancini et al. 2015). These two pathways were both associated with regulation of insulin secretion. β-arrestin pathways are considered as essential for receptor internalization and desensitization; however, a number of recent studies have revealed that β-arrestin pathways are also key signaling routes for GPCRs (DeWire et al. 2007). Among the FFARs, FFA4, also known as GPR120, has been reported to engage both Gprotein and β-arrestin pathways and that both play important roles in the physiological functions of FFA4 (Ichimura et al. 2012; Oh and Walenta 2014; Oh et al. 2010, 2014). FFA4 expressed on adipocytes was coupled via Gq-protein pathways and contributed to glucose uptake. On the other hand, FFA4 expressed on macrophages is mainly coupled via β-arrestin pathways and contributes to regulation of inflammatory responses. Hence, biased ligands, which can selectively activate only one of the signaling pathways, will be useful to evaluate precise receptor functions, not only in in vitro but also in in vivo studies.

Ligand-induced β-arrestin interactions with FFA1 were evaluated in BRET-based studies (Mancini et al. 2015). TAK-875 produced a potent effect on the recruitment of β-arrestin compared to endogenous FFA ligands, while this compound showed only partial agonistic activity on Gq/11 pathways. Although FFAs-induced insulin secretion was inhibited by treatment with the highly selective Gq/11 inhibitor UBO-QIC, TAK-875-induced insulin secretion was only weakly attenuated by β-arrestin siRNAs. FFA1 is therefore able to engage at least three signaling pathways (Gq/11, Gs and β-arrestin). Further studies of biased agonism at FFA1 might address the specific contribution of each pathway to the physiological functions of FFA1 and assist in tailoring these components to optimize ligands for pre-clinical and clinical assessment.

7 Clinical Trials

A small number of FFA1 ligands have entered clinical trials (Table 1). TAK-875, developed by Takeda progressed to phase III clinical trials. In a phase I, placebo-controlled, double blind trial with healthy subjects, oral administration of TAK-875

Table 1 FFA1 agonists subjected to clinical trials

Compounds	Phase of clinical trials	Companies
TAK-875 (Fasiglifam)	Phase III (-2013) (discontinue due to liver toxicity)	Takeda
JTT-851	Phase II (ongoing)	Japan Tobacco
P11187	Phase I (-2013) (completed → no further information)	Piramal Enterprises
AMG-837	Phase I (-2013) (completed → discontinue)	Amgen
LY2881835	Phase I (-2011) (completed → discontinue)	Eli Lilly
ASP5034	Phase I (-2012) (discontinue)	Astellas Pharma

was examined in terms of pharmacodynamics and safety (Naik et al. 2012). With a single oral administration, TAK-875 was safe and well tolerated. Phase II, randomized, double-blind, multicenter parallel group studies were conducted in type 2 diabetes patients whose symptoms showed tolerability to diet or metformin treatment. Two weeks oral administration of TAK-875 showed efficacy and tolerability (Araki et al. 2012; Kaku et al. 2015). In addition, once daily oral administration for 12 weeks showed an anti-hyperglycemic effect with low risk of hypoglycemia. However, a subsequent phase III trial was discontinued because of the potential of liver injury (Kaku et al. 2015). Li et al. have reported that TAK-875 inhibited the efflux transporter multidrug resistance-associated protein 2 (Mrp2), bile acid transporters, Na(+)/taurocholate co-transporting polypeptide (Ntcp) and the organic anion transporter protein (OATP), which may cause hyperbilirubinemia and hepatotoxicity (Li et al. 2015). Although the mechanism of this adverse effect of TAK-875 remains to be reported in detail, early stage analysis of potential FFA1 ligand effects on such transporters will clearly be essential for progress.

Eli Lilly completed a phase I trial of LY2881835 in 2011 (Defossa and Wagner 2014; Watterson et al. 2014). LY2881835 showed agonistic activity on human FFA1 with moderate potency ($EC_{50} = 230$ nm); however, significant side effects were observed in participants in a phase I trial, resulting in no further development of this compound.

ASP5034 was developed by Astellas. The chemical structure, which was undisclosed, was based on oxadiazolidinediones. In 2012, a phase I clinical trial of ASP5034 for type 2 diabetes was initiated; however, this was stopped from further evaluation based on a comprehensive review of the results and the competitive landscape (Astellas Pharma 2013).

P11187 developed by Piramal Enterprise Ltd. entered a phase I trial (Mancini and Poitout 2015; Watterson et al. 2014). Although the chemical structure has not been disclosed (Defossa and Wagner 2014) and some patent documents indicate the compound likely to be phenyloxetanylacetic acid-based. AMG-837 was developed by Amgen. In a phase I clinical trial, AMG-837 increased plasma insulin level, but did not lower glucose levels (Mancini and Poitout 2015). Further development of this compound has been abandoned for undisclosed reasons (Oh and Olefsky 2016).

JTT-851, also with undisclosed chemical structure, was developed by Japan Tobacco. A phase II trial for type 2 diabetes has progressed in Japan and USA (Japan Tobacco 2013) but further information is lacking at this time.

8 Conclusions

More than a decade has passed since FFA1 was identified as a receptor for medium- to long-chain fatty acids. As discussed elsewhere, identification of cell membrane G-protein coupled receptors for free fatty acids including FFA2 and FFA3 for short-chain fatty acid receptors, and FFA4 for medium- to long-chain fatty acids has resulted in a paradigm shift for fatty acid biology towards drug development. Even though the overlapping endogenous ligand spectrum of FFA1 and FFA4 has caused difficulties defining the specific function of FFA1, development of ligands with high affinity and selectivity has successfully overcome this problem. Structural information of FFA1 ligand interactions provided by both structure–activity relationships and crystal structure analysis have assisted further development of new ligands. A small number of compounds have entered clinical trials; however, almost all of these compounds were terminated due to either publically acknowledged adverse effects or in internal company reviews. Questions remain about the precise binding mode of distinct FFA1 ligand classes, and better knowledge of this is required for structure-based, rational drug design, and to understand more fully the relationships between signaling pathways activated and the biological functions of FFA1.

Taken together, as FFA1 remains a potential drug target, further comprehensive studies of FFA1 may address these issues and lead to the identification of therapeutic candidates for the treatment of type II diabetes and metabolic disorders.

Conflicts of Interest The author declares no conflict of interest associated with this manuscript.

References

Araki T, Hirayama M, Hiroi S, Kaku K (2012) GPR40-induced insulin secretion by the novel agonist TAK-875: first clinical findings in patients with type 2 diabetes. Diabetes Obes Metab 14:271–278

Astellas Pharma (2013) https://www.astellas.com/en/ir/library/pdf/1q2013_pre_en.pdf

Bertrand R, Wolf A, Ivashchenko Y, Löhn M, Schäfer M, Brönstrup M, Gotthardt M, Derdau V, Plettenburg O (2016) Synthesis and characterization of a promising novel FFAR1/GPR40 targeting fluorescent probe for β-cell imaging. ACS Chem Biol 11:1745–1754

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT et al (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C et al (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 148:619–628

Christiansen E, Urban C, Merten N, Liebscher K, Karlsen KK, Hamacher A, Spinrath A, Bond AD, Drewke C, Ullrich S et al (2008) Discovery of potent and selective agonists for the free fatty acid receptor 1 (FFA(1)/GPR40), a potential target for the treatment of type II diabetes. J Med Chem 51:7061–7064

Christiansen E, Due-Hansen ME, Urban C, Merten N, Pfleiderer M, Karlsen KK, Rasmussen SS, Steensgaard M, Hamacher A, Schmidt J et al (2010) Structure-activity study of dihydrocinnamic acids and discovery of the potent FFA1 (GPR40) agonist TUG-469. ACS Med Chem Lett 1:345–349

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schröder R, Hudson BD, Milligan G, Cawthorne MA, Kostenis E, Kassack MU et al (2012) Free fatty acid receptor 1 (FFA1/GPR40) agonists: mesylpropoxy appendage lowers lipophilicity and improves ADME properties. J Med Chem 55:6624–6628

Christiansen E, Hansen SVF, Urban C, Hudson BD, Wargent ET, Grundmann M, Jenkins L, Zaibi M, Stocker CJ, Ullrich S et al (2013) Discovery of TUG-770: a highly potent free fatty acid receptor 1 (FFA1/GPR40) agonist for treatment of type 2 diabetes. ACS Med Chem Lett 4:441–445

Christiansen E, Watterson KR, Stocker CJ, Sokol E, Jenkins L, Simon K, Grundmann M, Petersen RK, Wargent ET, Hudson BD et al (2015) Activity of dietary fatty acids on FFA1 and FFA4 and characterisation of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. Br J Nutr 113:1677–1688

Christiansen E, Hudson BD, Hansen AH, Milligan G, Ulven T (2016) Development and characterization of a potent free fatty acid receptor 1 (FFA1) fluorescent tracer. J Med Chem 59:4849–4858

Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, Schiöth HB (2013) G protein–coupled receptor deorphanizations. Annu Rev Pharmacol Toxicol 53:127–146

Davenport AP, Harmar AJ (2013) Evolving pharmacology of orphan GPCRs: IUPHAR Commentary. Br Pharmacol 170:693–695

Defossa E, Wagner M (2014) Recent developments in the discovery of FFA1 receptor agonists as novel oral treatment for type 2 diabetes mellitus. Bioorg Med Chem Lett 24:2991–3000

DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007) Beta-arrestins and cell signaling. Annu Rev Physiol 69:483–510

Furuhashi M, Hotamisligil GS (2008) Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. Nat Rev Drug Discov 7:489

Garrido DM, Corbett DF, Dwornik KA, Goetz AS, Littleton TR, McKeown SC, Mills WY, Smalley TL, Briscoe CP, Peat AJ (2006) Synthesis and activity of small molecule GPR40 agonists. Bioorg Med Chem Lett 16:1840–1845

Hara T, Hirasawa A, Sun Q, Koshimizu T, Itsubo C, Sadakane K, Awaji T, Tsujimoto G (2009) Flow cytometry-based binding assay for GPR40 (FFAR1; free fatty acid receptor 1). Mol Pharmacol 75:85–91

Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di Salvo J, Weinglass AB, Engelstoft MS, Madsen AN, Lückmann M et al (2015) GPR40 (FFAR1) – combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo. Mol Metab 4:3–14

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94

Houze JB, Zhu L, Sun Y, Akerman M, Qiu W, Zhang AJ, Sharma R, Schmitt M, Wang Y, Liu J et al (2012) AMG 837: a potent, orally bioavailable GPR40 agonist. Bioorg Med Chem Lett 22:1267–1270

Hu H, He LY, Gong Z, Li N, Lu YN, Zhai QW, Liu H, Jiang HL, Zhu WL, Wang HY (2009) A novel class of antagonists for the FFAs receptor GPR40. Biochem Biophys Res Commun 390:557–563

Humphries PS, Benbow JW, Bonin PD, Boyer D, Doran SD, Frisbie RK, Piotrowski DW, Balan G, Bechle BM, Conn EL et al (2009) Synthesis and SAR of 1,2,3,4-tetrahydroisoquinolin-1-ones

as novel G-protein-coupled receptor 40 (GPR40) antagonists. Bioorg Med Chem Lett 19:2400–2403
Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K et al (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 483:350–354
Ito R, Tsujihata Y, Matsuda-Nagasumi K, Mori I, Negoro N, Takeuchi K (2013) TAK-875, a GPR40/FFAR1 agonist, in combination with metformin prevents progression of diabetes and β-cell dysfunction in Zucker diabetic fatty rats. Br J Pharmacol 170:568–580
Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H et al (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422:173–176
Japan Tobacco (2013) https://www.jt.com/media/news/2013/pdf/20130131_04.pdf
Kaku K, Enya K, Nakaya R, Ohira T, Matsuno R (2015) Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial. Diabetes Obes Metab 17:675–681
Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B (2003) A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem Biophys Res Commun 301:406–410
Krasavin M, Lukin A, Zhurilo N, Kovalenko A, Zahanich I, Zozulya S, Moore D, Tikhonova IG (2016) Novel free fatty acid receptor 1 (GPR40) agonists based on 1,3,4-thiadiazole-2-carboxamide scaffold. Bioorg Med Chem 24:2954–2963
Kristinsson H, Smith DM, Bergsten P, Sargsyan E (2013) FFAR1 is involved in both the acute and chronic effects of palmitate on insulin secretion. Endocrinology 154:4078–4088
Li X, Zhong K, Guo Z, Zhong D, Chen X (2015) Fasiglifam (TAK-875) inhibits hepatobiliary transporters: a possible factor contributing to fasiglifam-induced liver injury. Drug Metab Dispos 43:1751–1759
Li Z, Pan M, Su X, Dai Y, Fu M, Cai X, Shi W, Huang W, Qian H (2016) Discovery of novel pyrrole-based scaffold as potent and orally bioavailable free fatty acid receptor 1 agonists for the treatment of type 2 diabetes. Bioorg Med Chem 24:1981–1987
Lin DC-H, Zhang J, Zhuang R, Li F, Nguyen K, Chen M, Tran T, Lopez E, Lu JYL, Li XN et al (2011) AMG 837: a novel GPR40/FFA1 agonist that enhances insulin secretion and lowers glucose levels in rodents. PLoS One 6
Lin DC-H, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J et al (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859
Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ et al (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7, e46300
Mancini AD, Poitout V (2015) GPR40 agonists for the treatment of type 2 diabetes: life after "TAKing" a hit. Diabetes Obes Metab 17:622–629
Mancini AD, Bertrand G, Vivot K, Carpentier É, Tremblay C, Ghislain J, Bouvier M, Poitout V (2015) β-Arrestin recruitment and biased agonism at free fatty acid receptor 1. J Biol Chem 290:21131–21140
Naik H, Vakilynejad M, Wu J, Viswanathan P, Dote N, Higuchi T, Leifke E (2012) Safety, tolerability, pharmacokinetics, and pharmacodynamic properties of the GPR40 agonist TAK-875: results from a double-blind, placebo-controlled single oral dose rising study in healthy volunteers. J Clin Pharmacol 52:1007–1016
Negoro N, Sasaki S, Mikami S, Ito M, Suzuki M, Tsujihata Y, Ito R, Harada A, Takeuchi K, Suzuki N et al (2010) Discovery of TAK-875: a potent, selective, and orally bioavailable GPR40 agonist. ACS Med Chem Lett 1:290–294
Oh DY, Olefsky JM (2016) G protein-coupled receptors as targets for anti-diabetic therapeutics. Nat Rev Drug Discov 15:161–172

Oh DY, Walenta E (2014) The role of omega-3 fatty acid receptor GPR120 in insulin resistance. Int J Obes Suppl 4:S14–S16

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698

Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR, Sasik R, Hah N, Chi TJ, Cox JM et al (2014) A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med 20:942–947

Ren X-M, Cao L-Y, Zhang J, Qin W-P, Yang Y, Wan B, Guo L-H (2016) Investigation of the binding interaction of fatty acids with human G protein-coupled receptor 40 using a site-specific fluorescence probe by flow cytometry. Biochemistry (Mosc) 55:1989–1996

Schmidt J, Liebscher K, Merten N, Grundmann M, Mielenz M, Sauerwein H, Christiansen E, Due-Hansen ME, Ulven T, Ullrich S et al (2011) Conjugated linoleic acids mediate insulin release through islet G protein-coupled receptor FFA1/GPR40. J Biol Chem 286:11890–11894

Smith NJ, Stoddart LA, Devine NM, Jenkins L, Milligan G (2009) The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1. J Biol Chem 284:17527–17539

Srivastava A, Yano J, Hirozane Y, Kefala G, Gruswitz F, Snell G, Lane W, Ivetac A, Aertgeerts K, Nguyen J et al (2014) High-resolution structure of the human GPR40 receptor bound to allosteric agonist TAK-875. Nature 513:124–127

Stoddart LA, Brown AJ, Milligan G (2007) Uncovering the pharmacology of the G protein-coupled receptor GPR40: high apparent constitutive activity in guanosine 5'-O-(3-[35S]thio) triphosphate binding studies reflects binding of an endogenous agonist. Mol Pharmacol 71:994–1005

Sum CS, Tikhonova IG, Costanzi S, Gershengorn MC (2009) Two arginine-glutamate ionic locks near the extracellular surface of FFAR1 gate receptor activation. J Biol Chem 284:3529–3536

Sun P, Wang T, Zhou Y, Liu H, Jiang H, Zhu W, Wang H (2013) DC260126: a small-molecule antagonist of GPR40 that protects against pancreatic β-cells dysfunction in db/db mice. PLoS One 8, e66744

Takano R, Yoshida M, Inoue M, Honda T, Nakashima R, Matsumoto K, Yano T, Ogata T, Watanabe N, Toda N (2014) Discovery of 3-aryl-3-ethoxypropanoic acids as orally active GPR40 agonists. Bioorg Med Chem Lett 24:2949–2953

Takeuchi M, Hirasawa A, Hara T, Kimura I, Hirano T, Suzuki T, Miyata N, Awaji T, Ishiguro M, Tsujimoto G (2013) FFA1-selective agonistic activity based on docking simulation using FFA1 and GPR120 homology models. Br J Pharmacol 168:1570–1583

Tikhonova IG, Sum CS, Neumann S, Engel S, Raaka BM, Costanzi S, Gershengorn MC (2008) Discovery of novel agonists and antagonists of the free fatty acid receptor 1 (FFAR1) using virtual screening. J Med Chem 51:625–633

Tsujihata Y, Ito R, Suzuki M, Harada A, Negoro N, Yasuma T, Momose Y, Takeuchi K (2011) TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats. J Pharmacol Exp Ther 339:228–237

Waring MJ, Baker DJ, Bennett SNL, Dossetter AG, Fenwick M, Garcia R, Georgsson J, Groombridge SD, Loxham S, MacFaul PA et al (2015) Discovery of a series of 2-(pyridinyl) pyrimidines as potent antagonists of GPR40. Med Chem Commun 6:1024–1029

Watterson KR, Hudson BD, Ulven T, Milligan G (2014) Treatment of type 2 diabetes by free fatty acid receptor agonists. Front Endocrinol 5

Yang L, Zhang J, Si L, Han L, Zhang B, Ma H, Xing J, Zhao L, Zhou J, Zhang H (2016) Synthesis and biological evaluation of GPR40/FFAR1 agonists containing 3,5-dimethylisoxazole. Eur J Med Chem 116:46–58

Yashiro H, Tsujihata Y, Takeuchi K, Hazama M, Johnson PRV, Rorsman P (2012) The effects of TAK-875, a selective G protein-coupled receptor 40/free fatty acid 1 agonist, on insulin and glucagon secretion in isolated rat and human islets. J Pharmacol Exp Ther 340:483–489

Yazaki R, Kumagai N, Shibasaki M (2011) Enantioselective synthesis of a GPR40 agonist AMG 837 via catalytic asymmetric conjugate addition of terminal alkyne to α, β-unsaturated thioamide. Org Lett 13:952–955

Zhang X, Yan G, Li Y, Zhu W, Wang H (2010) DC260126, a small-molecule antagonist of GPR40, improves insulin tolerance but not glucose tolerance in obese Zucker rats. Biomed Pharmacother 64:647–651

Ligands at the Free Fatty Acid Receptors 2/3 (GPR43/GPR41)

Graeme Milligan, Daniele Bolognini, and Eugenia Sergeev

Contents

Abstract

A large number of reviews and commentaries have highlighted the potential role of the short-chain fatty acid receptors GPR41 (FFA3) and, particularly, GPR43 (FFA2) as an interface between the intestinal microbiota and metabolic and inflammatory disorders. However, short-chain fatty acids have very modest potency and display limited selectivity between these two receptors, and studies on receptor knockout mice have resulted in non-uniform conclusions; therefore, selective and high-potency/high-affinity synthetic ligands are required to further explore the contribution of these receptors to health and disease. Currently no useful orthosteric ligands of FFA3 have been reported and although a number of orthosteric FFA2 agonists and antagonists have been described, a lack of affinity of different chemotypes of FFA2 antagonists at the mouse and rat orthologs of

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this receptor has hindered progress. Selective allosteric regulators of both FFA2 and FFA3 have provided tools to address a number of basic questions in both in vitro and ex vivo preparations, but at least some of the positive modulators appear to be biased and able to regulate only a subset of the functional capabilities of the short-chain fatty acids. Significant further progress is required to provide improved tool compounds to better assess potential translational opportunities of these receptors for short-chain fatty acids.

Keywords
Diabetes • Free fatty acids • Inflammation • Microbiota

1 The Short-Chain Fatty Acid Receptors

In 2003 three distinct groups demonstrated that two seven-transmembrane domain, G protein-coupled receptors, GPR41 and GPR43, were activated by short-chain fatty acids (SCFAs) (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003). Initially, Brown et al. (2003) reported that each of a number of basic peptide preparations was able to activate GPR43 but noted that the common feature amongst them was that they were all formulated as acetate salts. They went on to show that acetate (C2) alone was sufficient to activate the receptor and that this was mimicked by other SCFAs including propionate (C3) and butyrate (C4). This effect required the presence of the receptor, and the closely related receptor GPR41 was also activated by the same group of ligands. In parallel, the studies of Le Poul et al. (2003) also showed acetate as the common feature of the salts of a number of disparate peptides that appeared to activate the receptors and that both C2 and C3 promoted chemotaxis of human neutrophils, which express GPR43. Nilsson et al. (2003) were the first to suggest the designation of free fatty acid receptor 2 for GPR43, based on the earlier identification of the related sequence GPR40 as a receptor for longer-chain fatty acids, that they designated free fatty acid receptor 1. These initial studies also highlighted that there was a clear structure-activity relationship (SAR) associated with fatty acid chain length for activation of the receptors (see Milligan et al. 2009 for review) and that, although the potency of these ligands was modest, it was commensurate with levels of the SCFAs present in the gut and peripheral circulation to allow occupancy and activation of both GPR41 (subsequently renamed FFA3) and GPR43 (FFA2) (Stoddart et al. 2008a).

Subsequently, almost 100 studies have reported on aspects of the expression pattern and profile of these two receptors, their possible roles in nutrient homeostasis, particularly of glucose; in the regulation of white cell function and obesity; in aspects of inflammation biology, including the regulation of release of inflammatory mediators and, although to a lesser extent, in cancer biology (Bolognini et al. 2016a; Milligan et al. 2016). Many of these studies have used specific SCFAs directly to activate FFA2 and/or FFA3 or employed FFA2/FFA3 knockout

or transgenic overexpressing mice to attempt to define specific roles for each of these two receptors (Bolognini et al. 2016a; Milligan et al. 2016). The effectiveness of these approaches has been mixed. The low potency of the SCFAs at both these receptors, as well as potential pleiotropic 'off-target' effects of such ligands can challenge interpretation in the absence of effective and selective antagonist ligands. Moreover, in at least one case, knockout of GPR41/FFA3 has been reported to also reduce levels of expression of GPR43/FFA2 (Zaibi et al. 2010). Given that these receptor sequences are encoded in mouse in close proximity on chromosome 7, then genetic manipulation to eliminate expression of one might affect expression of the other. Moreover, a number of the studied knockout lines have been generated on distinct genetic backgrounds (see http://www.informatics.jax.org/marker/MGI:2441731 and http://www.informatics.jax.org/marker/MGI:2685324 for details of genotypes and associated phenotypes), and, in concert with likely variation in the microbiota in mice maintained in different facilities, these factors may be sufficient to account for the differences recorded.

2 How Do SCFAs Interact with FFA2 and FFA3?

Initial efforts to define the basis of recognition of FFA2/3 by the SCFAs were based on sequence alignment of the human orthologs with the longer-chain fatty acid receptor FFA1. Earlier work on ligand recognition by FFA1 had identified a pair of arginine residues, located at positions 5.39 and 7.35 in the Ballesteros and Weinstein (1995) numbering system that, in concert with Asn 6.55, acted to anchor the carboxylate of one of the earliest described synthetic agonists, GW9508 (Sum et al. 2007). Interestingly, mutation of either of these arginine residues to alanine had a much less dramatic effect on the ability of the polyunsaturated fatty acid, linoleic acid (C18:2), to signal (Sum et al. 2007), whilst for the saturated fatty acid, lauric acid (C12:0), no signal was recorded at such mutants (Smith et al. 2009). Each of these arginine residues is conserved in both FFA2 and FFA3. Mutation of either of these residues to alanine in both FFA2 and FFA3 eliminated responses to either C2 or C3 (Stoddart et al. 2008b). Moreover, replacement of the histidine at position 6.55 (the equivalent residue is asparagine in FFA1) also resulted in large reduction in function of the SCFAs. However, efforts to restore function by charge reversal, in which the arginine at residue 5.39 was converted to glutamate whilst acetate was substituted by acetamide or propionate with propionamide, were not successful (Stoddart et al. 2008b). A number of small, non-SCFA, carboxylic acids are also able to activate FFA2 and/or FFA3, and Schmidt et al. (2011) were able to demonstrate a degree of selectivity and predict and confirm that those containing substituted sp(3)-hybridised α-carbons would preferentially activate FFA3, whereas ligands with sp(2)- or sp-hybridised α-carbons would prefer FFA2. Within these studies anchorage of the carboxylate to arginines 5.39 and 7.35 was shown, and this demonstrated the basic similarity of recognition with that of the SCFAs. Modelling of the binding pocket of both FFA2 and FFA3 provided a degree of

understanding of this selectivity, and by generating a mutant form of FFA2 in which key variations were introduced to make the binding pocket more akin to FFA3, a degree of ligand selectivity reversal was obtained, without altering responsiveness to C3 (Schmidt et al. 2011). As well as providing useful information about other potential residues that might line the binding pocket, these studies noted the small and compact scale of this area and that the high ligand efficiency of the SCFAs and small carboxylic acids indicated that increased potency/affinity of synthetic ligands for these receptors would have to be generated by either targeting a different site or by extending outwith the core orthosteric agonist binding region.

Clearly some efforts have been made to do so. Ulven (2012) has reviewed and summarised key data contained within a series of patents from Euroscreen SA (Hoveyda et al. 2010, 2011a, b, c, d; Brantis et al. 2011). Within these reports, FFA2 agonist compounds have been reported to increase glucose uptake by both mouse 3T3-L1 adipocytes and isolated adipocytes as well as to enhance release of the incretin glucagon-like peptide 1 (GLP-1) from each of NCI-H716 cells and a rat intestinal cell preparation. A chemically distinct FFA2 agonist from a 5-aryl-2-acylpyrrollidinecarboxylic acid series has also been reported to inhibit isoprenaline-induced lipolysis in rat adipocytes and blood glucose load in the *ob*/*ob* mouse model of obesity and diabetes after chronic treatment for 4 weeks. Despite these important observations and reports, very few studies using synthetic FFA2 agonists have so far been subjected to peer review.

3 Orthosteric Ligands

3.1 Orthosteric Agonist Ligands

As highlighted by Schmidt et al. (2011), a substantial gain in potency over the endogenous agonist ligands is required to deliver synthetic FFA2/FFA3 agonists that might be useful to explore the biology of these receptors in an ex vivo or in vivo context. Despite recent studies suggesting that agonism of FFA2 might be a useful approach in therapeutic areas such as diabetes and obesity (Kimura et al. 2013; McNelis et al. 2015) (note that Tang et al. (2015) have instead suggested that FFA2 or FFA3 antagonists might be of interest here, whilst Forbes et al. (2015) argue that an FFA2 agonist might be useful in the treatment of obesity but not diabetes), there remains a serious lack of tool compounds available to test these hypotheses. Based on information in Hoveyda et al. (2010), Hudson et al. (2013) synthesised both 3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid (compound 1) (**1**) (Fig. 1 and Table 1) and (*R*)-3-(cyclopentylmethyl)-4-(cyclopropyl-(4-(2,6-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid (compound 2) (**2**) (Fig. 1 and Table 1) and assessed their activity as agonists of FFA2 in greater detail. At human FFA2 both these compounds acted as relatively potent agonists of signalling via both G_i- and $G_q/_{11}$-mediated pathways and were also able to promote interactions between the receptor and β-arrestin-2. Importantly, neither

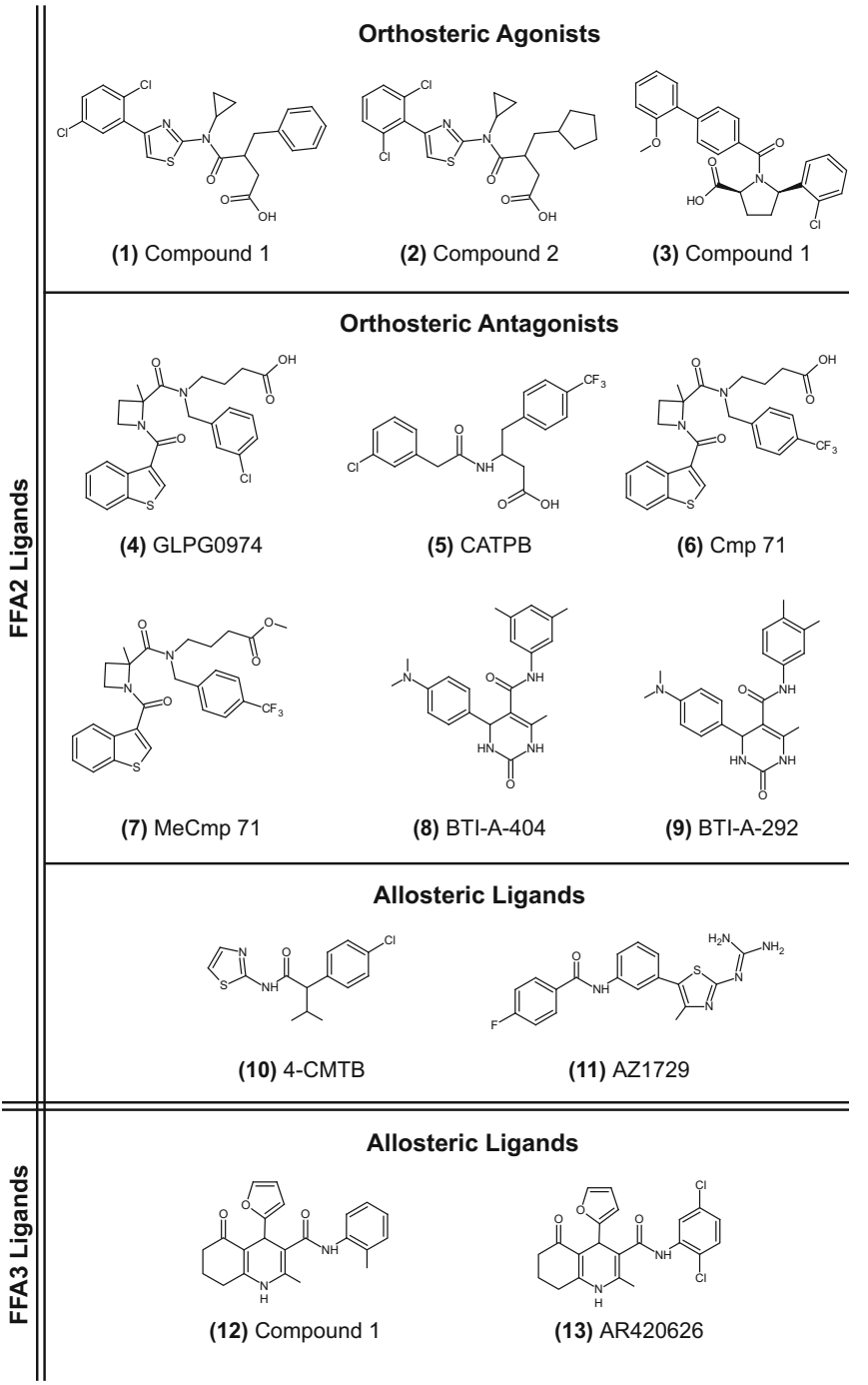

Fig. 1 FFA2 and FFA3 ligands. Structures of the FFA2/FFA3 ligands discussed in the text are shown

Table 1 FFA2 and FFA3 ligand pharmacology

#	Compound	Target	Pharmacology	pK_i
1	**Compound 1** (Hudson et al. 2013)	FFA2	Orthosteric agonist	6.91 ± 0.12
2	**Compound 2** (Hudson et al. 2013)	FFA2	Orthosteric agonist	
3	**Compound 1** (Forbes et al. 2015)	FFA2	Orthosteric agonist	6.32 ± 0.02
4	**GLPG0974** (Sergeev et al. 2016)	FFA2	Orthosteric antagonist	7.88 ± 0.08 (Sergeev et al. 2016)
5	**CATPB** (Brantis et al. 2011)	FFA2	Orthosteric antagonist/ inverse agonist	7.87 ± 0.08 (Sergeev et al. 2016)
6	**Cmp71** (Sergeev et al. 2016)	FFA2	Orthosteric antagonist	7.39 ± 0.04 (Sergeev et al. 2016)
7	**MeCmp71** (Sergeev et al. 2016)	FFA2	Orthosteric antagonist	6.22 ± 0.09 (Sergeev et al. 2016)
8	**BTI-A-404** (Park et al. 2016)	FFA2	Orthosteric antagonist/ inverse agonist	
9	**BTI-A-292** (Park et al. 2016)	FFA2	Orthosteric antagonist/ inverse agonist	
10	**4-CMTB** (Smith et al. 2011)	FFA2	Allosteric agonist	6.52 ± 0.17 (Bolognini et al. 2016b)
11	**AZ1729** (Bolognini et al. 2016b)	FFA2	Biased allosteric agonist	6.84 ± 0.11 (Bolognini et al. 2016b)
12	**Compound 1** (Hudson et al. 2014)	FFA3	Allosteric agonist	
13	**AR420626** (Hudson et al. 2014)	FFA3	Allosteric agonist	

The ligands shown in Fig. 1 are listed indicating their mode of action and including the designations used in publications reporting their activity. Where information is available in peer-reviewed publications, ligand affinity is also provided

compound displayed activity at FFA3, nor at the pair of longer-chain fatty acid receptors, FFA1 and FFA4 (Hudson et al. 2013). The carboxylate moiety of these compounds was integral to function because both methyl and *tert*-butyl esters of (**2**) lacked activity (Hudson et al. 2013) and, as for the SCFAs and small carboxylic acids, mutation of either Arg 5.39 or Arg 7.35 ablated responses to both (**1**) and (**2**), defining interaction within the orthosteric binding pocket (Hudson et al. 2013). More extensive mutagenesis, based around docking these compounds to a homology model of human FFA2, also identified a number of residues as being outwith the core binding pocket for SCFAs but which, when mutated, reduced the affinity/ potency of (**1**) and (**2**). These included the valine at residue position 5.38, located next to Arg 5.39, whilst in studies which replaced extracellular loop 2 from FFA2 with the equivalent sequence from FFA3, there was a substantial loss of potency of both (**1**) and (**2**) but only a small reduction in potency of the SCFA C3. In accord with this area being an important region for interaction of these synthetic ligands,

alteration of a glutamine within this loop to glutamic acid all but eliminated response to (**1**) and (**2**) with a much more limited, and in some assay end points, negligible, effect on C3 potency. Interestingly a marked difference in the effect of mutation of Ser 86 to Gly was observed for (**1**) versus (**2**). This alteration resulted in a marked reduction in potency of (**2**) in a number of assay end points but had no effect on (**1**) or the SCFA C3 (Hudson et al. 2013). This alteration was assessed because this residue varies between human (Ser) and both mouse and rat (Gly) FFA2 and it had been noted that (**2**) was substantially less potent at these rodent orthologs of FFA2 than at human, whilst for (**1**) this species effect was much less pronounced (Hudson et al. 2013). Likely because of this species-dependent difference, both (**1**) and (**2**) were effective in producing reduction of glycerol release from differentiated SW872 cells, a human adipocyte cell line, whilst in differentiated 3T3-L1 cells, a mouse-derived adipocyte-like system (**1**) but not (**2**) was able to demonstrate this to be an FFA2-mediated effect (Hudson et al. 2013). (**1**) was also able to mimic the ability and extent of the SCFA C3 to promote the release of the incretin GLP-1 from the murine enteroendocrine cell line STC-1, suggesting that although both FFA2 and FFA3 are expressed by these cells (Hudson et al. 2013), the effect of C3 is mediated largely if not exclusively by FFA2.

Recently, Forbes et al. (2015) employed a novel FFA2 agonist (2S, R5)-5-(2-chlorophenyl)-1-1(2′-methoxy-[1,1′-biphenyl]-4carbonyl)pyrrolidine-2-carboxylic acid (**3**) (Fig. 1 and Table 1), also derived from a Euroscreen SA patent (Hoveyda et al. 2011b), to explore functions and consequences of FFA2 in regulating release of both GLP-1 and neuropeptide YY in mouse models of diet-induced obesity. Although the mode of binding and recognition of (**3**) by the receptor was not explored, it is likely that the carboxylic acid moiety anchors the ligand via Arg 5.39 and Arg 7.35 in a similar way as for (**1**) and (**2**). (**3**) was reported not to interact with FFA1 or FFA3 and to have no significant effects at a broad range of other drug targets (Forbes et al. 2015). Moreover, in functional studies, much of the effect of (**3**) was absent in FFA2 knockout mice, indicating key 'on-target' pharmacology of the ligand. In these studies it was noted that (**3**) was highly potent ($EC_{50} = 81$ nM) in elevating [Ca^{2+}] levels in CHO-K1 cells transfected to express human FFA2 and was also potent in suppressing forskolin-induced cAMP elevation. Much lower potency was observed in GLP-1 release studies in primary colonic cultures from mice where 10 μM (**3**) was required to produce a statistically significant effect (Forbes et al. 2015). It remains unclear if the high potency recorded in the in vitro assays following heterologous expression of FFA2 is simply a reflection of high receptor reserve in the cell line employed, because no affinity values for (**3**) at FFA2 were provided. Similar variations in measures of potency have been noted for (**1**) in assays that move between heterologously and endogenously expressed FFA2 (Hudson et al. 2013). (**1**) has reported affinity ($pK_i = 6.91 \pm 0.12$) (Table 1) at human FFA2 compared to 2.96 ± 0.11 for the SCFA C3 (Sergeev et al. 2016), and it would be helpful to have equivalent information for (**3**). Overall the studies of Forbes et al. (2015) concluded that FFA2 plays a key role in controlling intestinal transit time and that by reduction of this, in a neuropeptide YY-dependent manner, it also can limit food intake.

A series of phenylthiazole-carboxamido acid derivatives has also been described recently as FFA2 agonists (Ma et al. 2016). However, the best compounds from this series were only slightly more potent than the SCFA C3 in a cAMP inhibition assay, and no information was provided about selectivity over FFA3 or other receptors.

3.2 Orthosteric Antagonist Ligands

Although studies in FFA2 knockout mice have provided considerable insights into key roles of FFA2, they have also generated a number of sets of conflicting data as to potential therapeutic utility (see Bolognini et al. 2016a; Milligan et al. 2016; Ang and Ding 2016, for review). To date, a single FFA2 antagonist has entered clinical trials for assessment of efficacy. GLPG0974 (4-[[1-(benzo[*b*]thiophene-3-carbonyl)-2-methylazetidine-2-carbonyl]-(3-chlorobenzyl)amino]butyric acid) (**4**) (Fig. 1 and Table 1) was developed by Galapagos NV (Saniere et al. 2012; Namour et al. 2016) as a potential treatment for ulcerative colitis (Pizzonero et al. 2014). SCFAs can promote chemotaxis of various white cells, including neutrophils via FFA2 (Vinolo et al. 2011). Based on a view that neutrophil chemotaxis into the lower gut might be associated with disrupted mucosal barrier function and the development of lower gut inflammatory conditions, (**4**) was employed in first-in-man trials following demonstration that it potently blocked acetate-induced human neutrophil chemotaxis and engaged FFA2 in situ as measured by a capacity to block acetate-induced expression of the neutrophil activation marker CD11bAE in human blood (Pizzonero et al. 2014). Sadly, a lack of improvement of clinical symptoms, after relatively short-term treatment, resulted in these trials being terminated. The studies of Pizzonero et al. (2014) explored the development of this compound series to improve potency and pharmacokinetic properties and indicated the ligand to both be highly selective for FFA2 over FFA3 and to lack activity at a number of other GPCRs, ion channels and transporters.

Subsequently Sergeev et al. (2016) explored the mode of binding to FFA2 of both (**4**) and some related ligands and, to do so, generated [^{3}H]GLPG0974. [^{3}H]GLPG0974 bound human FFA2 with high affinity ($K_d = 7.5 \pm 0.4$ nM) and was fully displaced by the SCFA C3, (**1**) and both non-radiolabelled (**4**) and the chemically distinct FFA2 antagonist CATPB ((*S*)-3-(2-(3-chlorophenyl) acetamido)-4-(4-(trifluoromethyl)phenyl)butanoic acid) (**5**) (Brantis et al. 2011) (Fig. 1 and Table 1). As both the SCFAs and (**1**) require their carboxylate moiety for function, and both lack function when either Arg 5.39 or Arg 7.35 of FFA2 is replaced by alanine, it was unsurprising that affinity of both SCFAs and (**1**) was essentially eliminated at either Arg 5.39 Ala FFA2 or Arg 7.35 Ala FFA2 mutants (Sergeev et al. 2016). However, unlike for the agonists, the binding affinity of [^{3}H]GLPG0974 at each of these two point mutants was only reduced slightly. Indeed, combined elimination of both Arg 5.39 and Arg 7.35 was required to abolish high-affinity binding of [^{3}H]GLPG0974. This led Sergeev et al. (2016) to assess whether the carboxylate was essential for recognition or high-affinity interaction with human FFA2. A carboxylate (**6**)/methyl ester (**7**) pair (Fig. 1 and Table 1) of an

azetidine closely related to (**4**) showed that the carboxylate was not required to either bind or to antagonise agonist function at human FFA2. However, in both situations, (**6**) displayed some 15–20 fold higher affinity than (**7**) (Sergeev et al. 2016) indicating the clear contribution of the carboxylate to affinity. These studies, in concert with docking of both the carboxylate-containing (**4**) and (**6**) into a homology model of FFA2 that was developed from the atomic level structure of FFA1 (Srivastava et al. 2014), and the observed parallel and dextral shifts in potency of the SCFA C3 and of (**1**) in the presence of increasing concentrations of (**4**), indicated that the azetidine-containing ligands act as orthosteric antagonists of human FFA2 (Sergeev et al. 2016).

Previous to these studies, Hudson et al. (2012) had demonstrated that (**5**) was also an orthosteric blocker of human FFA2 and, indeed, acted as an inverse agonist because this ligand was also able to suppress ligand-independent, constitutive activity of the receptor in a concentration-dependent fashion. However, these studies also first highlighted that (**5**) had no significant affinity for either the mouse or rat orthologs of FFA2, seriously limiting efforts to use preclinical rodent models of disease to predict human efficacy (Hudson et al. 2012). This is also true of (**4**) and chemically related molecules (Sergeev et al. 2016). Despite this, (**5**) has been used to effectively block the ability of (**1**) and (**2**) to reduce forskolin-enhanced glycerol release from human SW872 adipocytes (Hudson et al. 2013). Interestingly, however, in these cells the SCFA C3 surprisingly rather caused an increase in glycerol production. This was not blocked by (**5**), and as mRNA encoding FFA3 was not expressed in these cells at detectable levels (Hudson et al. 2013), this implies that this effect of C3 was produced by a non-FFA receptor mechanism. As with (**6**) and (**7**), the methyl ester of (**5**) acted as an antagonist of human FFA2 but with substantially lower potency/affinity (Sergeev et al. 2016). Chemically related to (**5**), *N*-(4-chlorobenzoyl)-L-tryptophan has also been described as an orthosteric antagonist selective for human FFA2, with no activity at rat FFA2 (Brown et al. 2015). This compound has been shown to effectively antagonise C3-mediated elevation of $[Ca^{2+}]$ in human neutrophils and to have a small, but clearly non-competitive, effect on the ability of the allosteric agonist 4-CMTB (see below) to generate this signal (Brown et al. 2015).

Most recently, two novel and closely related pyrimidinecarboxamide-based FFA2 antagonist/inverse agonists have been described (Park et al. 2016): BTI-A-404 ([4-[4-(dimethylamino)phenyl]-*N*-(3,5-dimethylphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxamide] (**8**) and BTI-A-292 [4-[4-(dimethylamino)phenyl]-*N*-(4,5-dimethylphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxamide] (**9**) (Fig. 1 and Table 1). As with the FFA2 antagonists described earlier, both of these ligands lack activity at FFA3 and fail to antagonise agonist function at mouse FFA2 (Park et al. 2016). Because direct comparison with the potency of (**5**) indicated each of (**8**) and (**9**) to be some 30-fold less effective in a range of assays, the broader utility of (**8**) and (**9**) is likely to be limited unless a structure-activity-based chemistry effort is able to improve their affinity for FFA2 substantially. However, the lack of a carboxylate or another fixed negative charge within (**8**) and (**9**) is interesting and does indicate that, as for methyl esters from

both the other two described FFA2 antagonist series (Sergeev et al. 2016), this is not a prerequisite for function. Although the limited data set presented is consistent with (**8**) and (**9**) acting as competitive orthosteric blockers of human FFA2 (Park et al. 2016) further, more detailed, studies will be required to define this fully.

4 Allosteric Regulators of FFA2 and FFA3

4.1 Allosteric Regulators of FFA2

The first reported synthetic activators of FFA2 were a pair of phenylacetamides, identified in a high-throughput screen conducted by Amgen and most widely exemplified by 4-chloro-α-(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide (**10**) (Fig. 1 and Table 1). In various reports, this ligand has been described as 'phenylacetamide 1' (Lee et al. 2008), '4-CMTB' (Smith et al. 2011), 'AMG7703' (Swaminath et al. 2011) and 'PAAT' (Lee et al. 2013). In the initial report, two closely related ligands were shown to be essentially equipotent, but in the region of 1,000-fold more potent than the SCFAs C2 and C3 at human FFA2 (Lee et al. 2008). They also appeared to display at least equivalent efficacy as the SCFAs (Lee et al. 2008). Despite clearly causing activation of FFA2, certain reports have indicated that (**10**) may not be equivalent to SCFAs in terms of efficacy, at least at certain end points (Smith et al. 2011), and, as such, results obtained with this and related ligands should be interpreted with care. Even in these initial studies, it was clear that (**10**) and the related 'phenylacetamide 2' were allosteric agonists of FFA2 because, as well as directly activating the receptor, co-addition of (**10**) with a SCFA increased the observed potency of the SCFA and vice versa (Lee et al. 2008). Importantly, (**10**) was also able to activate mouse FFA2 as this ligand inhibited lipolysis in a concentration-dependent manner in both primary mouse and 3T3-L1 adipocytes (Lee et al. 2008; Brown et al. 2015) and also promoted release of GLP-1 as effectively as C3 from mouse enteroendocrine STC-1 cells (Brown et al. 2015). Orthosteric activation of FFA2 is able to transduce signals via both G_i and G_q/G_{11} family G proteins, and (**10**) displayed both direct agonist and positive allosteric effects on the potency of SCFA in end points that reflect engagement with each G protein class (Lee et al. 2008). Both Wang et al. (2010) and Smith et al. (2011) reported substantive structure-activity studies around the parental compound but, although confirming the initial observations of Lee et al. (2008), were unable to improve on the potency of (**10**). However, 'phenylacetamide 2' (**11**) (Lee et al. 2008) (Fig. 1 and Table 1) displayed substantially improved pharmacokinetic properties and has been used to demonstrate an acute ability to reduce plasma nonesterified fatty acid levels in wild-type but not in FFA2 knockout mice (Wang et al. 2010). Although clearly non-orthosteric in that (**10**) retains function at both Arg 5.39 and Arg 7.35 mutants of FFA2 (Swaminath et al. 2011; Smith et al. 2011), the exact mode of interaction of these ligands with FFA2 remains uncertain. However, Smith et al. (2011) provided evidence for a key role of extracellular

loop 2, and specifically of Leu 173, in the allosteric communication between (**10**) and the SCFAs. However, interestingly, no allosteric interactions were noted between (**10**) and (**1**) (Hudson et al. 2013).

Although 4-CMTB clearly has characteristics of an allosteric agonist, recently Grundmann et al. (2016) have posited a more complex and potentially unique behaviour of this ligand. Based on studies following the kinetic responses of FFA2-expressing cells over extended time periods after addition of the ligand, 4-CMTB was identified to mediate responses at early times by binding to the orthosteric site of the receptor. However, kinetically later and pharmacologically separable responses were then produced by engagement with the allosteric site, resulting in the authors describing 4-CMTB as a sequentially activating ligand (Grundmann et al. 2016).

Although not stated explicitly, it is likely that the 'GPR43 agonist' used by Agus et al. (2016) was (**10**). Oral treatment of mice with 5 mg/kg/day over a one-week period was reported to reduce body weight, to reduce cytokine production from colonic mucosa and to lessen the susceptibility to develop symptoms in a dextran sulphate sodium-induced model of colitis (Agus et al. 2016). These results are potentially antithetical with the rationale for using (**4**) in clinical trials to treat ulcerative colitis (see also Ang and Ding 2016 in this context). (**10**) has also been reported to potentiate insulin secretion ex vivo in both mouse and human islets (McNelis et al. 2015). It should be noted that Priyadarshini et al. (2015) found that 4-CMTB inhibited glucose stimulated insulin secretion in both mouse and human pancreatic islets. However, this effect may not have been FFA2-mediated as 4-CMTB was able to induce a similar response in ffar2 -/- islets (Priyadarshini et al. 2015).

To consider whether certain FFA2 active ligands might span the orthosteric SCFA binding site and the site at which (**10**) binds, Brown et al. (2015) recently studied a series of ligands containing both N-thiazolylamide and carboxylate groups, which could potentially allow such a scenario. However, the results across the series were complex and could not be interpreted simply as these acting as 'bitopic' (Lane et al. 2013; Mohr et al. 2013), i.e. two binding site, ligands.

A further allosteric agonist at FFA2 is AZ1729 (**11**) (Bolognini et al. 2016b) (Fig. 1 and Table 1). Although an effective direct activator of G_i-mediated signals mediated by FFA2, and more than tenfold more potent than (**10**) when assessed in parallel, this ligand displays marked signal bias. Unlike both the SCFAs and (**10**), (**11**) appears unable to stabilise a conformation of FFA2 that interacts effectively with the G_q/G_{11} group of G proteins in that although both the SCFA C3 and (**10**) promote the generation of inositol phosphates in HEK293 cells transfected to express human FFA2, (**11**) was completely inactive in this assay (Bolognini et al. 2016b). Moreover, when assessing the mechanisms of phosphorylation and activation of the ERK1/2 MAP kinases in FFA2-expressing HEK293 cells, inhibitor studies employing both pertussis toxin to block G_i-mediated signalling and the selective G_q/G_{11} inhibitor FR900359 (Schrage et al. 2015) showed that both G protein classes contributed to the effect of the SCFA C3 (Bolognini et al. 2016b). However, AZ1729 was only a partial agonist at this end point and FR900359 had no

effect whilst pertussis toxin treatment fully attenuated the signal, consistent with this compound being a G_i-biased ligand. As a 'G_i'-biased allosteric agonist, (**11**) effectively inhibited isoprenaline-promoted release of glycerol from primary mouse adipocytes, but not in such cells pre-treated with Pertussis toxin. (**11**) displayed marginal ability to compete with [^{3}H]GLPG0974 to bind to human FFA2 but, when co-added with C3, acted as a positive allosteric modulator of the affinity of the SCFA, resulting in a large shift to higher affinity in the concentration dependence of C3 to compete with [^{3}H]GLPG0974. Finally, although acting as a positive allosteric regulator of SCFA G_i-mediated signalling of FFA2 (**11**) acted as a negative allosteric modulator and non-competitive antagonist of SCFA signalling through G_q/G_{11} (Bolognini et al. 2016b). Such complexity in functional pharmacology is fascinating from a mechanistic standpoint, but this may make interpretation of results challenging when using this ligand in cell type mixtures or in tissues (Kenakin 2015).

4.2 Allosteric Regulators of FFA3

Non-SCFA ligand pharmacology at FFA3 is exceedingly limited. Apart from the group of small carboxylic acids described by Schmidt et al. (2011) to be selective for FFA3 over FFA2, the only other potential orthosteric ligand reported to date is the ketone β-hydroxybutyrate, and this has been described as either a low-potency agonist (Won et al. 2013) or possibly antagonist (Kimura et al. 2011) of this receptor. This remains to be clarified. However as a potential ligand with very modest potency and one that is both an endogenous metabolite and for which levels can fluctuate markedly, it would be challenging to use this to explore the biology of FFA3 in detail.

In terms of synthetic ligands, a group of hexahydroquinolone-3-carboxamides, described initially in a patent from Arena Pharmaceuticals (Leonard et al. 2006), have been the only ligands characterised in any level of detail (Hudson et al. 2014). 4-(furan-2-yl)-2-methyl-5-oxo-*N*-(*o*-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**12**) (Fig. 1 and Table 1) was able to effectively activate human FFA3 as measured in both G_i activation and cAMP inhibition studies, but was inactive at FFA2. However, as anticipated from the structure, (**12**) clearly acted as an allosteric agonist in that mutation of either Arg 5.39 or Arg 7.35 had no discernible effect on the function of this ligand, although abolishing response to the SCFA C3 (Hudson et al. 2014). Moreover, as with allosteric agonists of FFA2, (**12**) is also a positive allosteric modulator of the potency of SCFAs, and although analysis predicted rather lower affinity and co-operativity with SCFAs of (**12**) at mouse and rat FFA3 compared to human, it was clearly active at each specific orthologue. Analogues of (**12**) displayed a range of pharmacological behaviours, but importantly, a further molecule from this series, *N*-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**13**) (Fig. 1 and Table 1), described as AR420626 and used to define a role for FFA3 in the regulation of ghrelin release (Engelstoft et al. 2013), was also shown to be an allosteric agonist at FFA3 (Hudson

et al. 2014). This molecule has also been used to demonstrate a role for FFA3 in GLP-1 release from murine colonic crypt cultures (Nohr et al. 2013). A further molecule from this series, detailed only as AR19, has been described as a FFA3 antagonist (Engelstoft et al. 2013). It is uncertain how this molecule relates structurally to other members of this series, but it did not affect FFA2/C3-mediated inhibition of cAMP levels (Engelstoft et al. 2013) and may be closely related to compounds described by Hudson et al. (2014) as negative allosteric modulators (and therefore functional antagonists) of SCFA function, but positive allosteric modulators of the affinity of the SCFAs.

5 Conclusions

Despite a range of FFA2- and FFA3-selective ligands being described in the patent literature, few of these have been employed to date in peer-reviewed publications. Moreover, in the case of FFA2, all the antagonist ligands described to date do not have significant affinity at rodent orthologues of the receptor. Despite many suggestions in the literature that either or both FFA2 and FFA3 might be interesting therapeutic targets in a range of diseases, the only ligand to progress into phase II clinical trials, the FFA2 antagonist GLPG0974, did not show clinical efficacy, resulting in termination of these trials. It is clear that a broader range of both FFA2 and FFA3 ligands, particularly those with an orthosteric binding mode and high affinity/potency at mouse and rat receptors, would be potentially of great use in unravelling the therapeutic potential of these receptors.

References

Agus A, Denizot J, Thévenot J, Martinez-Medina M, Massier S, Sauvanet P, Bernalier-Donadille-A, Denis S, Hofman P, Bonnet R, Billard E, Barnich N (2016) Western diet induces a shift in microbiota composition enhancing susceptibility to adherent-invasive E. coli infection and intestinal inflammation. Sci Rep 6:19032

Ang Z, Ding JL (2016) GPR41 and GPR43 in obesity and inflammation – protective or causative? Front Immunol 7:28

Ballesteros JA, Weinstein H (1995) Integrated methods for modeling G-protein coupled receptors. Methods Neurosci 25:366–428

Bolognini D, Tobin AB, Milligan G, Moss CE (2016a) The pharmacology and function of short chain fatty acid receptors. Mol Pharmacol 89:388–398

Bolognini D, Moss CE, Nilsson K, Petersson AU, Donnelly I, Sergeev E, König GM, Kostenis E, Kurowska-Stolarska M, Miller A, Dekker N, Tobin AB, Milligan G (2016b) A novel allosteric activator of free fatty acid 2 receptor displays unique Gi-functional bias. J Biol Chem 291:18915–18931

Brantis C, Ooms F, Bernard J (2011) Novel amino acid derivatives and their use as gpr43 receptor modulators. PCT Int Appl WO2011092284

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ

(2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319

Brown AJ, Tsoulou C, Ward E, Gower E, Bhudia N, Chowdhury F, Dean TW, Faucher N, Gangar A, Dowell SJ (2015) Pharmacological properties of acid N-thiazolylamide FFA2 agonists. Pharmacol Res Perspect 3, e00141

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2:376–392

Forbes S, Stafford S, Coope G, Heffron H, Real K, Newman R, Davenport R, Barnes M, Grosse J, Cox H (2015) Selective FFA2 agonism appears to act via intestinal PYY to reduce transit and food intake but does not improve glucose tolerance in mouse models. Diabetes 64:3763–3771

Grundmann M, Tikhonova IG, Hudson BD, Smith NJ, Mohr K, Ulven T, Milligan G, Kenakin T, Kostenis E (2016) A molecular mechanism for sequential activation of a G protein-coupled receptor. Cell Chem Biol 23:392–403

Hoveyda H, Brantis CE, Dutheuil G, Zoute L, Schils D, Bernard J (2010) Compounds, pharmaceutical composition and methods for use in treating metabolic disorders. PCT Int Appl WO2011076732

Hoveyda H, Brantis CE, Dutheuil G, Zoute L, Schils D, Bernard J (2011a) Compounds, pharmaceutical composition and methods for use in treating inflammatory diseases. PCT Int Appl WO2011076734

Hoveyda H, Brantis CE, Dutheuil G, Zoute L, Schils D, Fraser G (2011b) Compounds, pharmaceutical composition and methods for use in treating for use in the treatment of gastrointestinal disorders. PCT Int Appl WO2011076732

Hoveyda H, Schils D, Zoute L, Parcq J (2011c) Pyrrolidine or thiazolidine carboxylic acid derivatives, pharmaceutical compositions and methods for use as in treating metabolic disorders as agonists of G-protein coupled receptor 43 (GPR43). PCT Int Appl WO2011073376

Hoveyda H, Zoute L, Lenoir F (2011d) Azepanes, azocanes and related compounds as GPR43 modulators and their preparation and use for the treatment of inflammatory, gastrointestinal and metabolic disorders. PCT Int Appl WO2011151436

Hudson BD, Christiansen E, Murdoch H, Jenkins L, Hansen AH, Madsen O, Ulven T, Milligan G (2014) Complex pharmacology of novel allosteric free fatty acid 3 receptor ligands. Mol Pharmacol 86:200–210

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, Tikhonova IG, Ulven T, Milligan G (2013) Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288:17296–17312

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G (2012) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. J Biol Chem 287:41195–41209

Kenakin T (2015) The effective application of biased signaling to new drug discovery. Mol Pharmacol 88:1055–1061

Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, Tsujimoto G (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc Natl Acad Sci U S A 108:8030–8035

Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, Tani T, Takahashi T, Miyauchi S, Shioi G, Inoue H, Tsujimoto G (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829

Lane JR, Sexton PM, Christopoulos A (2013) Bridging the gap: bitopic ligands of G-protein-coupled receptors. Trends Pharmacol Sci 34:59–66

Lee SU, In HJ, Kwon MS, Park BO, Jo M, Kim MO, Cho S, Lee S, Lee HJ, Kwak YS, Kim S (2013) beta-Arrestin 2 mediates G protein-coupled receptor 43 signals to nuclear factor-kappaB. Biol Pharm Bull 36:1754–1759

Lee T, Schwandner R, Swaminath G, Weiszmann J, Cardozo M, Greenberg J, Jaeckel P, Ge H, Wang Y, Jiao X, Liu J, Kayser F, Tian H, Li Y (2008) Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. Mol Pharmacol 74:1599–1609

Leonard JN, Chu ZL, Bruce MA, Boatman PD (2006) GPR41 and modulators thereof for the treatment of insulin-related disorders. PCT Int Appl WO 2006052566

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Duprie V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278:25481–25489

Nilsson NE, Kotarsky K, Owman C, Olde B (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 303:1047–1052

McNelis JC, Lee YS, Mayoral R, van der Kant R, Johnson AM, Wollam J, Olefsky JM (2015) GPR43 potentiates β-cell function in obesity. Diabetes 64:3203–3217

Ma L, Wang T, Shi M, Fu P, Pei H, Ye H (2016) Synthesis, activity and docking study of novel phenylthiazole-carboxamido acid derivatives as FFA2 agonists. Chem Biol Drug Des 88:26–37

Milligan G, Shimpukade B, Ulven T, Hudson BD (2016) Complex pharmacology of the free fatty acid receptors. Chem Rev (in press)

Milligan G, Stoddart LA, Smith NJ (2009) Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3. Br J Pharmacol 158:146–153

Mohr K, Schmitz J, Schrage R, Tränkle C, Holzgrabe U (2013) Molecular alliance-from orthosteric and allosteric ligands to dualsteric/bitopic agonists at G protein coupled receptors. Angew Chem Int Ed Engl 52:508–516

Namour F, Galien R, Van Kaem T, Van der Aa A, Vanhoutte F, Beetens J, Van't Klooster G (2016) Safety, pharmacokinetics and pharmacodynamics of GLPG0974, a potent and selective FFA2 antagonist, in healthy male subjects. Br J Clin Pharmacol 82:139–148

Nohr MK, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, Han S, Jones RM, Offermanns S, Schwartz TW et al (2013) GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinology 154:3552–3564

Park BO, Kim SH, Kong GY, da Kim H, Kwon MS, Lee SU, Kim MO, Cho S, Lee S, Lee HJ, Han SB, Kwak YS, Lee SB, Kim S (2016) Selective novel inverse agonists for human GPR43 augment GLP-1 secretion. Eur J Pharmacol 771:1–9

Pizzonero M, Dupont S, Babel M, Beaumont S, Bienvenu N, Blanqué R, Cherel L, Christophe T, Crescenzi B, De Lemos E, Delerive P, Deprez P, De Vos S, Djata F, Fletcher S, Kopiejewski S, L'Ebraly C, Lefrançois JM, Lavazais S, Manioc M, Nelles L, Oste L, Polancec D, Quénéhen V, Soulas F, Triballeau N, van der Aar EM, Vandeghinste N, Wakselman E, Brys R, Saniere L (2014) Discovery and optimization of an azetidine chemical series as a free fatty acid receptor 2 (FFA2) antagonist: from hit to clinic. J Med Chem 57:10044–10057

Priyadarshini M, Villa SR, Fuller M, Wicksteed B, Mackay CR, Alquier T, Poitout V, Mancebo H, Mirmira RG, Gilchrist A, Layden BT (2015) An acetate-specific GPCR, FFAR2, regulates insulin secretion. Mol Endocrinol 29:1055–1066

Saniere LRM, Pizzonero MR, Triballeau N, Vandeghinste NER, De VSIJ, Brys RCX, Pourbaix-L'ebraly CD (2012) Preparation of azetidine derivatives as GPR43 antagonists useful in the treatment of metabolic and inflammatory diseases. PCT Int Appl WO2012098033

Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, Ward RJ, Drewke C, Milligan G, Kostenis E, Ulven T (2011) Selective orthosteric free fatty acid

receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. J Biol Chem 286:10628–10640

Schrage R, Schmitz AL, Gaffal E, Annala S, Kehraus S, Wenzel D, Büllesbach KM, Bald T, Inoue A, Shinjo Y, Galandrin S, Shridhar N, Hesse M, Grundmann M, Merten N, Charpentier TH, Martz M, Butcher AJ, Slodczyk T, Armando S, Effern M, Namkung Y, Jenkins L, Horn V, Stößel A, Dargatz H, Tietze D, Imhof D, Galés C, Drewke C, Müller CE, Hölzel M, Milligan G, Tobin AB, Gomeza J, Dohlman HG, Sondek J, Harden TK, Bouvier M, Laporte SA, Aoki J, Fleischmann BK, Mohr K, König GM, Tüting T, Kostenis E (2015) The experimental power of FR900359 to study Gq-regulated biological processes. Nat Commun 6:10156

Sergeev E, Hansen H, Pandey SK, MacKenzie AE, Hudson BD, Ulven T, Milligan G (2016) Non-equivalence of key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. J Biol Chem 291:303–317

Smith NJ, Stoddart LA, Devine NM, Jenkins L, Milligan G (2009) The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1. J Biol Chem 284:17527–31759

Smith NJ, Ward RJ, Stoddart LA, Hudson BD, Kostenis E, Ulven T, Morris JC, Tränkle C, Tikhonova IG, Adams DR, Milligan G (2011) Extracellular loop 2 of the free fatty acid receptor 2 mediates allosterism of a phenylacetamide ago-allosteric modulator. Mol Pharmacol 80:163–173

Srivastava A, Yano J, Hirozane Y, Kefala G, Gruswitz F, Snell G, Lane W, Ivetac A, Aertgeerts K, Nguyen J, Jennings A, Okada K (2014) High-resolution structure of the human GPR40 receptor bound to allosteric agonist TAK-875. Nature 513:124–127

Stoddart L, Smith NJ, Milligan G (2008a) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev 60:405–417

Stoddart LA, Smith NJ, Jenkins L, Brown AJ, Milligan G (2008b) Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. J Biol Chem 283:32913–32924

Sum CS, Tikhonova IG, Neumann S, Engel S, Raaka BM, Costanzi S, Gershengorn MC (2007) Identification of residues important for agonist recognition and activation in GPR40. J Biol Chem 282:29248–29255

Swaminath G, Jaeckel P, Guo Q, Cardozo M, Weiszmann J, Lindberg R, Wang Y, Schwandner R, Li Y (2011) Mutational analysis of G-protein coupled receptor--FFA2. Biochem Biophys Res Commun 405:122–127

Tang C, Ahmed K, Gille A, Lu S, Gröne HJ, Tunaru S, Offermanns S (2015) Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nat Med 21:173–177

Ulven T (2012) Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. Front Endocrinol (Lausanne) 3:111

Vinolo MA, Ferguson GJ, Kulkarni S, Damoulakis G, Anderson K, Bohlooly-Y M, Stephens L, Hawkins PT, Curi R (2011) SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. PLoS One 6, e21205

Wang Y, Jiao X, Kayser F, Liu J, Wang Z, Wanska M, Greenberg J, Weiszmann J, Ge H, Tian H, Wong S, Schwandner R, Lee T, Li Y (2010) The first synthetic agonists of FFA2: discovery and SAR of phenylacetamides as allosteric modulators. Bioorg Med Chem Lett 20:493–498

Won YJ, Lu VB, Puhl HL 3rd, Ikeda SR (2013) Beta-hydroxybutyrate modulates N-type calcium channels in rat sympathetic neurons by acting as an agonist for the G-protein-coupled receptor FFA3. J Neurosci 33:19314–19325

Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJ, Smith DM, Arch JR (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett 584:2381–2386

Pharmacological Tool Compounds for the Free Fatty Acid Receptor 4 (FFA4/GPR120)

Steffen V.F. Hansen and Trond Ulven

Contents

Abstract

The free fatty acid receptor 4 (FFA4), also known as GPR120, is a G protein-coupled receptor that is activated by long-chain fatty acids and that has been associated with regulation of appetite, release of insulin controlling hormones, insulin sensitization, anti-inflammatory and potentially anti-obesity activity, and is progressively appearing as an attractive potential target for the treatment of metabolic dysfunctions such as obesity, type 2 diabetes and inflammatory disorders. Ongoing investigations of the pharmacological functions of FFA4 and validation of its potential as a therapeutic target depend critically on the appropriateness and quality of the available pharmacological probes or tool compounds. After a brief summary of the pharmacological functions of FFA4 and some general considerations on desirable properties for these pharmacological tool compounds, the individual compounds that have been or are currently

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being used as tools for probing the function of FFA4 in various in vitro and in vivo settings will be discussed and evaluated.

Keywords
Chemical probes • FFA4 • GPR120 • Tool compound

1 Introduction

The G protein-coupled receptor (GPCR) GPR120 was deorphanized in 2005 when it was found to be activated by long-chain fatty acids (LCFAs) (Hirasawa et al. 2005). Despite a sequence only distantly related to the other free fatty acid receptors FFA1-3, GPR120 was later renamed free fatty acid receptor 4 (FFA4) (Davenport et al. 2013). FFA4 is expressed in the gastrointestinal tract, lungs, adipose tissue, macrophages, pancreas, bone-related cells, taste buds and in the brain and has since its deorphanization become subject of intensive interest (Milligan et al. 2016; Moniri 2016; Offermanns 2014; Oh da and Olefsky 2016; Ulven and Christiansen 2015). Several studies have convincingly suggested FFA4 to be a promising potential target for treatment of obesity, type 2 diabetes (T2D) and related inflammatory diseases. For example, FFA4 is reported to regulate the secretion of a range of hormones, such as glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), cholecystokinin (CCK), ghrelin, somatostatin, and glucagon, all implicated in regulation of glucose metabolism and appetite (Ulven and Christiansen 2015). Inflammation is playing an important role in the pathogenesis of T2D and obesity (Donath and Shoelson 2011; Talukdar et al. 2011). It has been shown in several studies that activation of FFA4 leads to anti-inflammatory and insulin sensitizing effects, and it is believed that part of the established anti-inflammatory effects of omega-3 polyunsaturated fatty acids (PUFAs) derive from interaction with FFA4 (Oh da and Olefsky 2016). Many of these studies depend on the use of synthetic ligands as tools to clarify the biological role of FFA4. This chapter seeks to give an overview of the different tool compounds that have been (and continue to be) used in studies of FFA4 pharmacology. However, it is instructive to begin the chapter with a discussion on what makes a good tool compound and how a tool compound is distinct from a drug.

2 What Is a Good Tool Compound?

A tool compound or chemical probe is a small molecule that can assist researchers in making specific inquiries about a biological target. To generate trustworthy answers, there are certain requirements that the chemical probes should fulfil (Table 1) (Arrowsmith et al. 2015; Bunnage et al. 2013; Frye 2010; Kodadek 2010; Workman and Collins 2010). As the purpose of a chemical probe is to study the biological role or function of a specific target, it is required to specifically

Table 1 Generally desirable properties of tool compounds

Potency	Sufficient to ensure full target engagement at concentrations that do not cause off-target effects. Typically EC_{50} or $IC_{50} < 100$ nM
Selectivity	Sufficient to ensure full target engagement at concentrations that do not cause off-target effects. Typically >100-fold selective over off-targets
Chemical stability	Stable under the assay conditions for the extent of the study. Devoid of reactive and/or promiscuous functional groups
Solubility	Sufficient for intended use (e.g. avoid DMSO concentrations that may interfere with the assay)
In vivo pharmacokinetics	Information on bioavailability and formulation necessary for selection of appropriate dose and method of administration. Distribution and clearance properties that ensure target exposure for the appropriate period of time
Availability	Commercially or synthetically available

engage the target in question without concurrent interactions with other targets that may cause effects that can interfere with the readout and confuse the interpretation. To achieve this, the probe must be able to reach its target (e.g. it should be sufficiently soluble and chemically stable for in vitro studies), it should have sufficient in vitro potency and selectivity to specifically engage its target without causing off-target effects, and its mode of action (e.g. agonistic vs. antagonistic, orthosteric vs. allosteric, signalling bias) must be well defined. It is prudent to avoid groups generally associated with reactivity and promiscuity, including pan-assay interfering compounds (PAINS) (Baell and Holloway 2010; Pouliot and Jeanmart 2016). For in vivo studies, the compound must have a distribution and clearance profile that ensures sufficient target exposure for a sufficient period of time, implying a certain metabolic stability. The compound should not give rise to biologically active metabolites. It is usually desirable to have good oral bioavailability to avoid administration routes that can be stressful to the animal, even if intravenous (iv) or intraperitoneal (ip) administration can be viable alternatives in case of insufficient oral bioavailability.

It is important to keep in mind that a drug and a chemical probe are two very different things with different purposes. The ultimate goal for a drug is not to answer questions about biology but to elicit an effect of therapeutic value. A drug might well have several modes of action – drugs can even be designed to engage multiple mechanisms against a disease (Anighoro et al. 2014). The usefulness of a drug does not depend on a well-characterized mode of action. In contrast, the role of a tool compound is to probe the function of one specific biological target and should therefore ideally only have one well-defined mode of action. Another set of rules applies for drugs; a drug needs to have good pharmacokinetic properties and to be safe for human consumption at the dose required for the biological activity. There has previously been a mentality in drug design of "one drug, one target", which is changing to "one drug, multiple targets" (Anighoro et al. 2014; Peters 2013; Reddy and Zhang 2013). The concept of polypharmacology is used to describe such ligands with activity on more than one biological target (Anighoro et al. 2014;

Peters 2013; Reddy and Zhang 2013). Many drugs currently on the market are of polypharmacological nature, for example the new antidepressant vortioxetine (Bang-Andersen et al. 2011). In contrast, multiple activities will in most cases be inappropriate for a tool compound, with the exception of well-characterized dual activities where the study aims to investigate concomitant modulation of both targets.

An ideal tool compound will reach and specifically bind to and either activate or inhibit a specific target without causing any effect that is not target related. This task is more challenging to fulfil for in vivo studies because of the more complex biological system with a larger variety of off-targets, natural barriers, efflux pumps and metabolic enzymes designed to eliminate xenobiotics. It is rare that all these properties can be found in a tool compound. Due to the sheer number of potential biological targets, it is impossible to guarantee that a probe does not have any unforeseen off-target effects. Use of the tool in combination with knockout or gene silencing techniques is one way to avoid the risk of confusing off-target effects and build confidence and robustness into the study. Like chemical probes, the variety of techniques available to disrupt normal gene expression can have off-target effects in addition to the desired on-target effects (Boettcher and McManus 2015), however, these will typically be different from off-target effects of chemical probes and the use of both methods in parallel will usually result in more robust studies and conclusions that can be drawn with greater confidence (Workman and Collins 2010).

Another approach to add confidence to the link between target engagement and the observed effects is to use a second probe belonging to a structurally different chemical class that therefore would be unlikely to share the same activity profile (referred to as a structurally orthogonal probe) (Bunnage et al. 2013). The complementary strategy is to include a structurally close analogue of the chemical probe that is likely to have a similar activity profile but is confirmed to be devoid of activity on the target of interest. Candidates for such inactive analogues can frequently be found in the original publications describing the discovery of the probe. These approaches can preferably be used in combination.

One important point that has often been overlooked is the variability of activity between species orthologues of the protein target. Since early target validation frequently involves rodent models and many immortalized cell lines used in research are derived from rodents, a tool compound's activity in rodents is typically of equal and frequently of higher interest than its activity in humans. High potency of a compound on the human receptor is, however, no guarantee of activity on the murine or other species orthologues. Examples of this are known from ligands of free fatty acid receptor 2 (FFA2/GPR43), where the Euroscreen compound CATPB and Galapagos' GLPG0974, a previous clinical candidate for inflammatory bowel disease, both are potent on the human orthologue but devoid of activity in several other species including mouse (Pizzonero et al. 2014; Sergeev et al. 2015). GLPG0974 is therefore unusual in progressing to clinical trials without having been through studies in rodent disease models. A potent and selective tool compound in humans can also have preserved potency on the target of interest but

Table 2 Checklist for design of experiments with chemical probes

Target activity
• The potency and selectivity of the probe should be adequate for the intended study • The applied concentration should be appropriate relative to the potency of the probe on the target • Evidence that the probe engages the intended target
Specific effect
• Implementation of structurally unrelated active compounds and/or structurally related inactive compounds used to support target specific effects • Use of knockout or gene silencing techniques
Chemical quality
• Known chemical structure and confirmed identity of the probe • Confirmed purity and stability
In vivo pharmacokinetics
• Sufficient target exposure – Appropriate formulation and route of administration (e.g. oral administration requires bioavailability) – Confirmed appropriate plasma concentration and half-life • Absence of interfering metabolites

increased activity on off-targets in other species of the study, rendering the compound unselective. The FFA4 agonist TUG-891 is an example of this, as will be detailed below. It is therefore essential to characterize the tool compound on the receptor for the relevant species. The issue of potency variability between species orthologues of the FFA receptors has been discussed in detail elsewhere (Hudson et al. 2013a). Inspired by Arrowsmith et al. (2015), Table 2 provides a list of points to consider in the design of experiments with chemical probes.

3 Controlling FFA1-Mediated Effects

FFA1 is another GPCR activated by LCFAs that is also established as a target for treatment of T2D and has been connected to regulation of insulin and secretion of incretin hormones (Defossa and Wagner 2014; Milligan et al. 2015). Some degree of co-activity on FFA1 is a property shared by all FFA4-activating LCFAs (Ulven and Christiansen 2015), and is also an issue for many of the synthetic tool compounds (see below). As a strategy to control confounding effects related to co-activation of FFA1 in studies with FFA4 agonists, it is common and relevant to include control experiments using a specific FFA1 antagonist. This has been particularly relevant in conjunction with the use of probes that are equipotent on FFA4 and FFA1 or even have up to two orders of magnitude higher potency on FFA1, such as LCFAs or GW9508, respectively (see below). Several FFA1 antagonists are known (Fig. 1). The first published antagonist was GW1100 with $pIC_{50} = 5.5–6.0$ against GW9508 and PUFAs at the human FFA1. The compound is also active on the mouse orthologue, as only 1 μM GW1100 completely blocked the

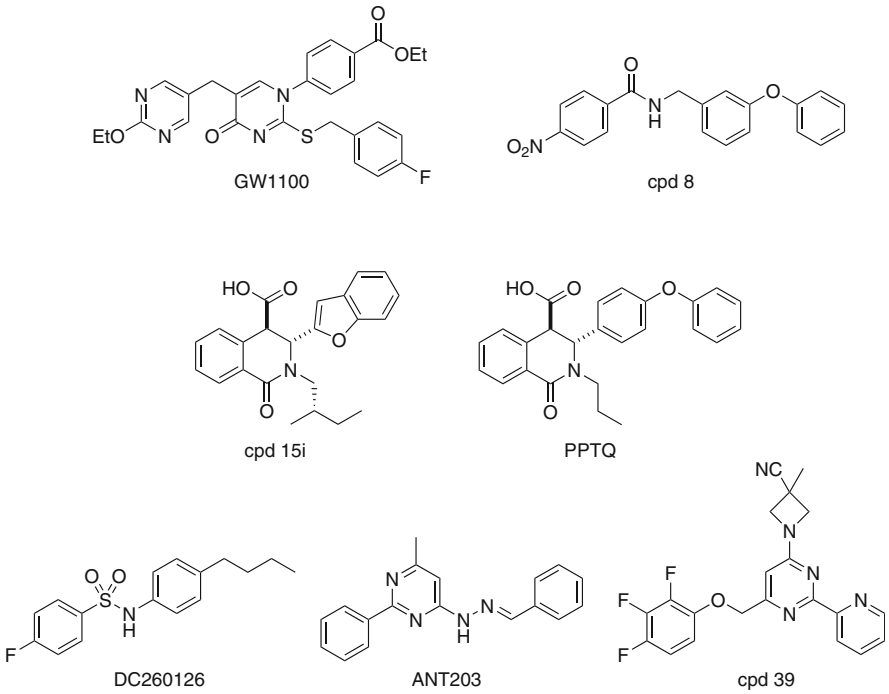

Fig. 1 FFA1 antagonists

enhancement of glucose-stimulated insulin secretion from the mouse-derived MIN6 β-cell line produced by up to 20 μM GW9508 (Briscoe et al. 2006). Importantly, GW1100 is confirmed not to affect the activity of agonists on FFA4 (Briscoe et al. 2006), however, the compound is otherwise incompletely characterized and may exert off-target activity on its own. GW1100 is not confirmed to be active in vivo and the metabolically labile ester functionality of the compound may represent a liability in this respect.

There are other FFA1 antagonists structurally orthogonal to GW1100 that are worthy of consideration. Another early antagonist is cpd 8, reported with $IC_{50} = 2.8$ μM against 100 nM GW9805 (Tikhonova et al. 2008). A series of 1,2,3,4-tetrahydroisoquinolin-1-ones (THIQs) were disclosed as FFA1 antagonists by Pfizer (Humphries et al. 2009). Optimization of pharmacokinetic properties led to identification of compound *15i*, which show satisfactory pharmacokinetic properties in rat. A representative from the series, PPTQ, has been used to specifically block FFA1 activity on mouse-derived β-cell lines and exhibited $pIC_{50} = 5.1–5.3$ in the inhibition of FFA1 agonists (Christiansen et al. 2011, 2012, 2013). These antagonists have, however, not been profiled on other receptors, including FFA4. The sulfonamide DC260126 was reported as a low potency FFA1 antagonist and has been used in vivo (Sun et al. 2013). However, as this compound recently was found to also behave as a β-arrestin-biased FFA4 agonist (Azevedo

et al. 2016), its use as an FFA1 antagonist within FFA4 studies cannot be recommended. The hydrazone ANT203 has been described as an FFA1 antagonist that at 2 μM concentration protected against palmitate-induced lipotoxicity (Kristinsson et al. 2013). However, as might have been expected from a hydrazone (Baell and Holloway 2010), the compound resulted in β-cell apoptosis above 5 μM, lacks bioavailability and is unsuitable for in vivo studies (Kristinsson et al. 2013). Waring and co-workers recently reported their discovery of compound 39, a potent FFA1 antagonist with $pIC_{50} = 6.8$ and 7.2 on the human and mouse orthologue, respectively, and good pharmacokinetic properties that makes it suitable for in vivo studies (Waring et al. 2015). Compound 39 is relatively well characterized as a chemical probe as it has been screened against 144 protein targets in the Cerep panel, unfortunately not including FFA4. Only seven targets showed measurable activity and only pig Na+/K+ ATPase had sub 1 μM activity. Compound 39 is likely to become the preferred FFA1 antagonist if lack of interference with FFA4 can be confirmed.

4 Chemical Probes for Studies of FFA4

FFA4 has seen a surge of interest in recent years, and the growing number of FFA4 ligands found in the patent literature has recently been the subject of reviews (Formicola et al. 2015; Halder et al. 2013). The following sections will discuss the compounds that have been used as tools to investigate FFA4 in vitro and in vivo and evaluate their properties as chemical probes. The current FFA4 probes (Fig. 2) can overall be classified as either carboxylic acid probes or sulfonamide probes.

4.1 Carboxylic Acid Probes

4.1.1 Long-Chain Fatty Acids

LCFAs are recognized to be the endogenous ligands for FFA4 (Davenport et al. 2013). The initial publications highlighted unsaturated fatty acids as functional endogenous FFA4 agonists and also recognized that saturated fatty acids of 14 carbon atoms or longer activate the receptor but without translation into GLP-1 secretion (Hirasawa et al. 2005). In particular omega-3 fatty acids have been associated with FFA4 and their anti-inflammatory effects have been linked to the receptor (Oh et al. 2010). A subsequent study on a broader selection of fatty acids found both saturated fatty acids with carbon chain length at least ten and unsaturated fatty acids to activate the receptor (Christiansen et al. 2015). Several studies have employed unsaturated LCFAs, typically docosahexaenoic acid (DHA) and α-linolenic acid (aLA) (Fig. 2), as tool compounds for study of FFA4 pharmacology. Initially, this was out of necessity, as no high-quality synthetic agonists were available. This is no longer the case and the use of LCFAs as exclusive pharmacological tool compounds cannot be recommended. Despite being the endogenous ligands for FFA4, LCFAs are not optimal as tool compounds owing to their low

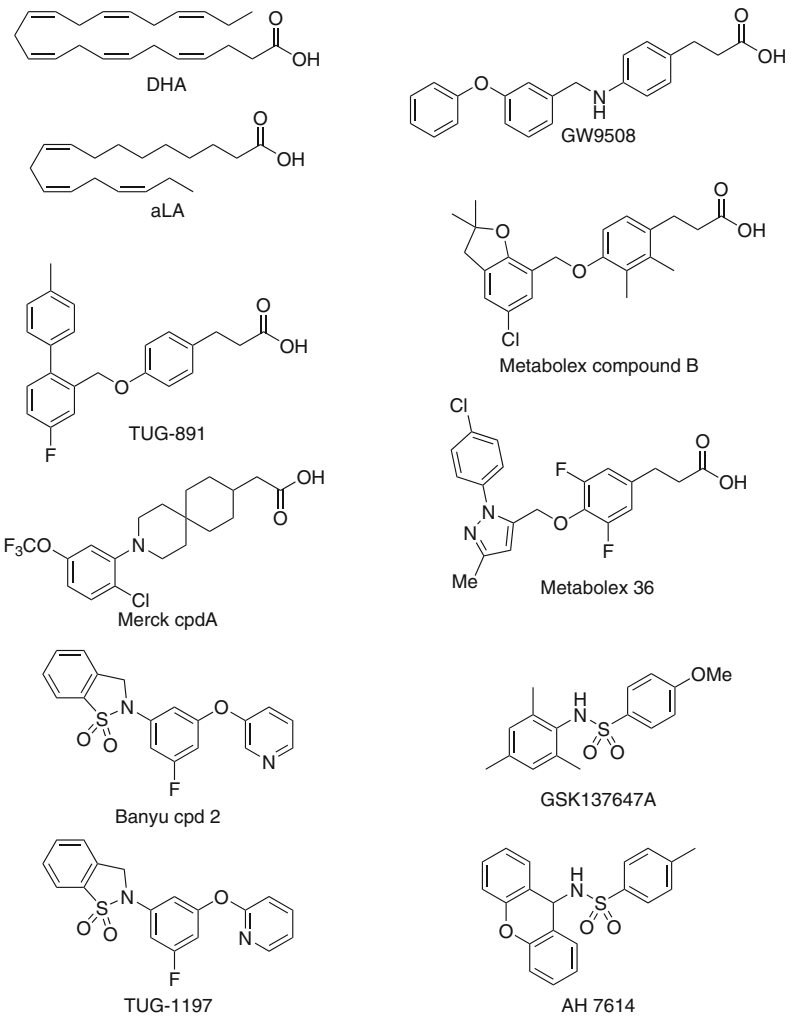

Fig. 2 FFA4 probes

potency and multiple other biological effects (Calder 2012). Due to their importance as fuel sources, the body is equipped with several proteins just for shuttling and metabolising LCFAs. Moreover, LCFAs are known to modulate several biological targets directly. For example, at the concentration needed to activate FFA4 [$EC_{50} = 4$ μM (Ulven and Christiansen 2015)], DHA also has significant activity on other targets e.g. FFA1 [$EC_{50} = 1–8$ μM (Ulven and Christiansen 2015)], retinoid X receptor α [$EC_{50} = 5–10$ μM, (Lengqvist et al. 2004)] and the peroxisome proliferator-activated receptors [$EC_{50} = 145$ nM on PPARα (Hostetler et al. 2006)]. In addition, PUFAs act as precursors for important messenger molecules with biological activity, such as the prostaglandins, leukotrienes,

lipoxins, protectins, marseins, and resolvins (Serhan 2014; Ulven and Kostenis 2006). The physicochemical properties of LCFAs, including poor solubility and the tendency to micelle formation in the relevant concentration range, also make them unsuitable as tool compounds. Nevertheless, studies with LCFAs for the elucidation of the ability of, for example, dietary-derived fatty acids to cause effects through these receptors are of high interest and relevance.

4.1.2 GW9508

The first synthetic ligand used in studies of FFA4 was GW9508 (Fig. 2), disclosed as a potent and selective FFA1 ligand a decade ago and also described in the same publication as a low potency agonist for FFA4 with approximately the same potency as the endogenous LCFAs (Briscoe et al. 2006). Despite only moderate potency on FFA4 and a 100-fold higher potency on FFA1, the compound has been frequently used as a tool compound for studies of FFA4, initially due to a paucity of better tools. GW9508 is well characterized as a chemical probe for FFA1 as it has been shown in either functional or binding assays to be at least 100-fold selective against 220 other GPCRs, 60 kinases, 63 proteases, 7 integrins and 20 nuclear receptors including PPARα, δ and γ (Briscoe et al. 2006). Unfortunately, since GW9508 is also approximately 100-fold selective for FFA1 over FFA4, little is known about the selectivity for FFA4 over off-targets. Notably, GW9508 was reported to have $pEC_{50} = 4.9$ on PPARγ (Briscoe et al. 2006), implying only four-fold selectivity for FFA4. Nevertheless, due to lack of better alternatives, GW9508 has frequently been used as a tool compound in studies of FFA4 and can with appropriate controls still generate meaningful data.

GW9508 was, for example, used to study the anti-osteoclastogenic role of FFA4 in RAW264.7 cells, where the expression levels of FFA1 were found to be 100-fold lower than of FFA4 (Cornish et al. 2008). The compound has also been used as a "functionally specific" FFA4 agonist to study FFA4 in macrophages and adipocytes since these do not express FFA1 (Oh et al. 2010), the compound was found to elicit anti-inflammatory effects that were ablated upon FFA4 knockdown.

GW9508 can be used to rule out that an effect is related to either FFA1 or FFA4: using GW9508 and GW1100 as tools, Garrel et al. showed that neither receptor is involved in fatty acid induced secretion of the gonadotropin hormone LH although both are expressed in LβT2 gonadotrope cells (Garrel et al. 2011). Murine RAW264.7 cells were used to study if FFA4 is involved in DHA-mediated cyclo-oxygenase-2 (COX-2) induction (Li et al. 2013a). Here, GW9508 and PPAR specific ligands were used in concert to demonstrate that DHA attenuates COX-2 expression in a PPAR-independent manner but, at least in part, through FFA4. At a concentration of 100 μM GW9508 was found to reverse homocysteine-induced inflammation in adipocytes (Li et al. 2013b). Since human colorectal carcinoma cells have been shown to not express FFA1, GW9508 was used as a "functionally specific" FFA4 agonist on these cells (Wu et al. 2013). Backed up by knockdown of FFA4, this study showed activation of FFA4 to be promoting angiogenesis in vitro and in vivo. Yan et al. used GW9508 and knockdown of FFA1, FFA4 or both in

their study of the NLRP3 inflammasome in human THP-1 derived macrophages that express both receptors (Yan et al. 2013).

In a study on ghrelin producing SG-1 cells, GW9508 and knockdown approaches were used to show that activation of FFA4 and not of FFA1 leads to reduced secretion of this hunger-promoting hormone (Gong et al. 2014). GW9508 and DHA were used to further study the mechanism of FFA4 signalling through phospholipase A_2 in murine RAW264.7 cells (Liu et al. 2014). FFA4 was found to be expressed on Kupffer cells, hepatic stellate macrophages, and activation of FFA4 on these cells with GW9508 promoted an anti-inflammatory state which appeared to be beneficial against hepatic ischemia reperfusion injury and possibly other liver injuries (Raptis et al. 2014). The neuronal cell line rHypoE-7, derived from rat hypothalamus, expresses FFA4 at abundant levels but does not express FFA1. GW9508 has therefore been used as a "functionally specific" FFA4 tool in this cell model. Both GW9508 and DHA were found to produce anti-inflammatory effects mediated through FFA4, results that were supported by siRNA-mediated FFA4 knockdown (Wellhauser and Belsham 2014).

PANC-1 is a pancreatic cancer cell line that expresses both FFA1 and FFA4. GW9508 and a combination of GW1100 and FFA1 and FFA4 knockdown were used to study the role of the receptors in these cells (Fukushima et al. 2015). The authors found that FFA4 stimulates and FFA1 inhibits motility, invasion and tumorigenicity of pancreatic cancer cells. Using the same tools, the group has also studied the effects of FFA4 and FFA1 on ethionine induced liver epithelial cell motility (WB-F344 cells) and the role of the receptors in several lung cancer cell lines (Ishii et al. 2015a, b; Kita et al. 2016). Kim et al. reported that FFA4 negatively regulates osteoclast differentiation, survival, and function, by using GW9508 as an FFA4 probe despite expression of FFA1 at a very low level, and provided support for the conclusion through the use of shRNA-mediated FFA4 knockdown (Kim et al. 2015).

Tsukahara and co-workers found both FFA4 and FFA1 to be overexpressed in enteroendocrine L cells of the inflamed ileum of Crohn's disease patients. The combination of GW9508 and GW1100 helped them to elucidate that FFA1 had stimulatory, and FFA4 inhibitory, effects on the production of the anti-inflammatory glucagon-like peptide-2 (GLP-2) from L-cells (Tsukahara et al. 2015). Using GW9508 and GW1100 as tools, Konno et al. investigated the expression and functions of FFA4 on human eosinophils, where 100 μM GW9508 induced the release of IL-4 and inhibited apoptosis, effects that were not affected by pre-treatment with 10 μM GW1100, thus leading the authors to conclude that the effects were mediated by FFA4 and not FFA1 (Konno et al. 2015).

As testified by the studies mentioned above, GW9508 is probably the most extensively used tool compound in studies of FFA4. It is commercially available and thus easily accessible. However, its modest potency on FFA4, similar potency on PPARγ and 100-fold higher potency on FFA1 render GW9508 a suboptimal tool compound and unsuitable for such studies when used alone. The high concentration necessary to ensure full activation of FFA4 implies that off-target activity beyond FFA1 cannot be excluded. It is therefore recommended that any use of this

compound as a probe for FFA4 also includes receptor silencing and/or an FFA4-specific antagonist such as AH 7416 (see below).

4.1.3 TUG-891

Screening of a series of FFA1 agonists and optimization of a hit that exhibited selectivity for FFA4 led to the identification of TUG-891 (Fig. 2) as the first potent and selective FFA4 agonist (Shimpukade et al. 2012). Depending on the type of assay, TUG-891 is 1.7–3.2 orders of magnitude selective for hFFA4 over hFFA1 and has no detectable activity on the two short-chain fatty acid receptors FFA2 and FFA3. Unfortunately, whereas the compound is even more potent on the murine orthologue of FFA4, its selectivity over FFA1 in this species is only 3-fold and 50-fold selective in a calcium mobilization and β-arrestin-2 recruitment assay, respectively (Hudson et al. 2013b). TUG-891 has also been used for investigation of the porcine variant of FFA4, where on the wild-type receptor it has pEC_{50} of 5.7–5.9 in the nuclear factor of activated T-cell response element (NFAT) and serum response element (SER) based reporter systems (Song et al. 2015). Despite the less pronounced selectivity between FFA4 and FFA1 at the mouse orthologues, the compound still represents a useful tool compound for both in vitro and in vivo studies of the biology of FFA4.

Following its discovery, TUG-891 was confirmed to promote Ca^{2+} mobilization, β-arrestin recruitment and ERK1/2 phosphorylation via FFA4, but with a clear bias for signalling through Ca^{2+} and β-arrestin (Hudson et al. 2013b). As also observed for LCFAs and GW9508 (Hirasawa et al. 2005; Watson et al. 2012), activation of FFA4 by TUG-891 was followed by rapid receptor internalization and desensitization, effects that were equally rapidly reversed after washout. TUG-891 was also tested together with the selective FFA1 agonist TUG-905 on the enteroendocrine cell lines STC-1 and GLUTag that express both receptors, and it was found that GLP-1 release could be ascribed predominantly to FFA4 activation (Hudson et al. 2013b). In the same study TUG-891 was reported to be as efficacious as aLA in causing inhibition of lipopolysaccharide-induced TNF-α release from RAW264.7 macrophages (Hudson et al. 2013b). TUG-891 was later used in a mass spectrometry-based proteomics study of FFA4 in an effort to map the sites of phosphorylation and residues interacting with arrestin-3, it was found that activation of the receptor with this agonist leads to phosphorylation of Thr^{347}, Thr^{349}, Ser^{350} Ser^{357} and Ser^{360} (Butcher et al. 2014). Mutagenesis and modelling studies have confirmed that TUG-891 is an orthosteric ligand that binds to essentially the same pocket as aLA (Hudson et al. 2014).

A hallmark of osteoporosis is an imbalance in the osteoblast/adipocyte ratio. TUG-891 has recently been used as a tool to investigate the role of FFA4 in relation to differentiation of bone marrow mesenchymal stem cells (Gao et al. 2015). It was found that low concentrations (0.1–1 μM) of the FFA4 agonist led to increased adipogenesis whereas high concentrations (30–100 μM) led to increased osteogenesis. Furthermore, local injection of high doses of TUG-891 to the proximal femur of ovariectomized mice rescued the animals from oestrogen-deficient bone loss. In light of the activity of TUG-891 on mFFA1 and the expression of also this receptor

in bone-related cells (Wauquier et al. 2013), inclusion of either a specific antagonist or gene silencing would have been interesting to confirm and clarify the roles of the receptors.

TUG-891 and GW9508 were used as selective FFA4 and FFA1 agonists, respectively, in a study of the function of the two receptors in human and guinea pig airway smooth muscle, where it was concluded that, of these two, FFA1 is the sole receptor responsible for ligand-induced airway smooth muscle contraction (Mizuta et al. 2015). The paper claims that TUG-891 is a highly selective FFA4 agonist without providing support for this. The claim is true for the human but not for the murine orthologue, whilst data on the guinea pig orthologue is lacking. The compounds are therefore more appropriately used as selective agonists on human than murine cells and tissue. The same two agonists have been used as tool compounds in a study of human prostate cancer cell lines, where it was found that TUG-891 acted as a potent inhibitor of proliferation induced by lysophosphatidic acid or serum (Liu et al. 2015).

Obstructive sleep apnoea (OSA) is a common health problem that is associated with the presence of insulin resistance and altered lipid homeostasis. Chronic sleep fragmentation can be used to mimic the disrupted sleep patterns of OSA and it has been shown to induce deregulation of appetite and promote the emergence of obesity and insulin resistance as well as adipose tissue inflammation in mice being fed normal diets (Hakim et al. 2015a, b). A recent study employing TUG-891 found that the compound attenuated these effects when dosed to mice subjected to chronic sleep fragmentation, suggesting that FFA4 agonists may in the future become valuable to combat some of the side effects of OSA, although it was correctly noted that further examination of causal pathways is justified (Gozal et al. 2016).

As part of their effort to clarify the mechanism for FFA4-induced adipogenesis of 3T3-L1 adipocytes Song et al. employed TUG-891 as well as the PUFAs DHA, aLA and linoleic acid (Song et al. 2016). Addition of 1, 10 or 100 μM TUG-891 led to dose-dependent increase of triglyceride accumulation and PPARγ expression in differentiated 3T3-L1, effects that disappeared upon FFA4 knockdown. Using a reporter assay, the authors were able to conclude that TUG-891 has no direct effect on PPARγ.

Because TUG-891 is commercially available and a relatively potent and selective FFA4 agonist it has become a frequently employed tool compound in both in vitro and in vivo studies. The activity and potency of TUG-891 has been well characterized on both arrestin and G protein-dependent signalling pathways, which is noteworthy since both pathways have been implicated in important functions of the receptor. The compound should, however, be used with care in mice and murine-derived cells, since only a three-fold selectivity has been observed over FFA1 in a calcium mobilization assay (Hudson et al. 2013b). Furthermore, a broader characterization of the selectivity profile of the compound, including on PPARs, is still lacking.

4.1.4 Metabolex Compounds

The company Metabolex (now Cymabay Therapeutics) has developed and patented FFA4 agonists (Ma et al. 2010; Shi et al. 2010). Two of these agonists, referred to as Metabolex 36 and compound B (example 209 in the patent), respectively, have been used as tool compounds in peer reviewed studies (Fig. 2) (Egerod et al. 2015; Engelstoft et al. 2013; Stone et al. 2014).

Metabolex 36 (as well as two proprietary FFA4 agonists from AstraZeneca, AZ-423 and AZ-670 with pEC_{50} 5.8–6.4 on mFFA4, that have undisclosed structures and thereby violate an obvious requirement for tool compounds) was used by Stone et al. to study the function of FFA4 in pancreatic delta cells (Stone et al. 2014). The compound was found to have pEC_{50} 6.0 (DMR) and 5.9 (FLIPR) on mFFA4 and the pEC_{50} on mFFA1 was found to be <4.0 (FLIPR) (Stone et al. 2014). Metabolex 36 lowered both basal and glucose induced somatostatin secretion from murine pancreatic islets with maximum inhibition reached at 30 μM. The agonist was found to be without effect on somatostatin levels when tested on islets isolated from FFA4 knockout mice. The authors did not observe any change in insulin secretion under either basal or elevated glucose conditions after dosing of Metabolex 36. Interestingly DHA was found in the same study to be without influence on somatostatin secretion, whereas this endogenous agonist did influence the insulin levels in a dose-dependent manner in islets from wild-type mice but not from FFA4-knockout mice.

Compound B was used by Engelstoft et al. in their study of the role of FFA4 in ghrelin secreting cells (Engelstoft et al. 2013). The compound was found to have $EC_{50} = 15$ nM on FFA4 and >1,000-fold selectivity over FFA1 in an inositol trisphosphate assay in transfected COS7 cells. Compound B dose-dependently inhibited ghrelin secretion in isolated primary gastric mucosal cells, an effect that was absent in cells isolated from FFA4 knockout mice. Oral dosing of compound B to fasting wild-type mice also significantly and dose-dependently decreased ghrelin levels (Engelstoft et al. 2013). The same group subsequently used compound B in their investigation of primary gastric mucosal cells where they found significant and dose-dependent inhibition of somatostatin release from cells isolated from wild-type but not from FFA4 knockout mice (Egerod et al. 2015).

Whereas Metabolex 36 appears to be only moderately potent, compound B may be the most potent compound used as an FFA4 probe. However, the compound was tested in a different assay than the others. To reliably compare potency, the compounds should be tested head-to-head in the same assay. Furthermore, only data from a G protein-dependent assay is currently available. Potency in an arrestin-dependent assay would also be relevant since the anti-inflammatory effects of FFA4 are believed to be mediated by this signalling pathway (Talukdar et al. 2011). Furthermore, the data is only from one unspecified receptor orthologue. The selectivity of compound B over FFA1 appears to be excellent but no further counter-screening appears to have been performed. Data disclosed in the patent, where oral dose of 30 mg/kg of compound B led to 43.6% reduction of area under the curve (AUC) in an intraperitoneal glucose tolerance test (Shi et al. 2010), indicates that compound B also is suitable for in vivo studies. Neither Metabolex 36 nor compound B is commercially available and both compounds require multistep synthesis.

4.1.5 Merck cpdA

This selective FFA4 agonist (Fig. 2) entered the public domain in early 2014 in a patent from Merck, in which it was reported to have a $pEC_{50} = 6.1$ on hFFA4 in FLIPR Ca^{2+} assays (Chelliah et al. 2014). Shortly thereafter, it was published in the peer-reviewed literature and reported with $pEC_{50} = 7.6$ on hFFA4 and negligible activity on hFFA1 up to 1 μM in FLIPR assays (Oh da et al. 2014). The compound was also tested in a β-arrestin-2 recruitment assays on human and murine FFA4 with $pEC_{50} \approx 6.5$ in both assays. In this report, cpdA and DHA were used to investigate FFA4 mediated anti-inflammatory properties in vitro. Both compounds exerted potent anti-inflammatory effects on macrophages from wild-type mice but not from FFA4-knockout mice. CpdA was then tested in both wild-type and FFA4-knockout high-fat diet (HFD) fed mice at a dose of 30 mg/kg, resulting in markedly improved glucose and insulin tolerance, decreased insulin secretion in the wild-type mice, but not in the FFA4-knockout mice. Furthermore, cpdA was shown to improve systemic and hepatic insulin sensitivity and beneficial effects were also seen on hepatic lipid metabolism (decrease in hepatic steatosis, liver triglyceride and diacylglycerol levels and fewer saturated free fatty acids). No effects on GLP-1 levels were found upon treatment of HFD-fed mice with cpdA (Oh da et al. 2014).

CpdA is an FFA4 probe with high selectivity over FFA1 that is proven to be useful in both in vitro and in vivo assays. As for the other tool compounds above, data from more extensive profiling is still lacking and its use therefore requires compensating control studies such as with FFA4-knockout mice as used by Oh et al. The compound is commercially available.

4.2 Sulfonamide Probes

4.2.1 GSK137647A

A medium throughput screening campaign at GlaxoSmithKline yielded a diarylsulfonamide that after optimization led to the discovery of GSK137647A (Fig. 2), an FFA4 agonist with comparable potency across species [$pEC_{50} = 6.3$ (human), 6.2 (mouse) and 6.1 (rat) in FLIPR assays] (Sparks et al. 2014). The compound was shown to be quite selective for FFA4, having pEC_{50} of less than 4.5 on both on human and rodent orthologues of FFA1, FFA2 and FFA3. The agonist was also screened and found to have minimal activity on 58 other targets, including PPAR α, δ and γ. The compound thus represents a good and selective probe for in vitro studies and is the currently best profiled FFA4 probe. Unfortunately, the compound suffers from low solubility in simulated intestinal fluid (FASSIF) (2.9 μg/mL), which limits its usefulness for in vivo studies (Sparks et al. 2014). The agonist was tested on MIN6 cells and found to produce a dose-dependent increase in glucose-stimulated insulin secretion. GSK137647A was also shown to cause a modest increase in GLP-1 release from a human intestinal cell line (NCI-H716) (Sparks et al. 2014). Before the discovery of GSK137647A was published, the compound was used as a tool compound to investigate FFA4 as a lipid sensor in mouse taste buds (Martin et al. 2012). At a concentration of 50 μM,

the agonist led to a small but significant increase in GLP-1 release from freshly isolated mouse circumvallate papillae. To date, this compound has only been used in these two studies, but as it is commercially available and easily synthesized, this may change. The compound is suitable as a structurally orthogonal probe in combination with any of the carboxylic acid probes above.

4.2.2 Cyclic Sulfonamides, Banyu cpd 2 and TUG-1197

A patent from Banyu Pharmaceutical disclosed a series of cyclic sulfonamide FFA4 agonists where cpd 2 (Fig. 2) appeared as the most potent ($pEC_{50} = 6.7$ in a FLIPR assay) of two compounds with activity data (Arakawa et al. 2010). The compound was found to be unbiased in β-arrestin recruitment and a Ca^{2+} mobilization assays with $pEC_{50} = 6.4$ and 6.5, respectively (Azevedo et al. 2016). Interestingly, and in contrast to all other known FFA4 agonists (including GSK137647A above, which has a weakly acidic function), the scaffold in this series contains no acidic group and cannot be negatively charged under physiological conditions. In light of its distinct structure, it is also interesting that cpd 2 also binds to the orthosteric site of FFA4 (Azevedo et al. 2016).

Structure-activity explorations starting with cpd 2 led Azevedo et al. to identify the closely related TUG-1197 (Fig. 2), a compound with $pEC_{50} = 6.9$ and 6.6 in the human FFA4 in a β-arrestin recruitment and a Ca^{2+} mobilization assay, respectively, as well as $pEC_{50} = 6.3$ and 6.8 in the same assays on the murine FFA4. Both TUG-1197 and cpd 2 were found active in an acute oral tolerance test in normal mice after oral dosing at 10 mg/kg. TUG-1197 was investigated further in diet-induced obese (DIO) mice, where TUG-1197 again significantly lowered the glucose level in wild-type but not in FFA4-knockout mice. The compound was investigated further in a chronic study in DIO mice where it caused significant insulin sensitization and a moderate but significant bodyweight loss, effects that were not observed in FFA4-knockout mice (Azevedo et al. 2016).

Cpd 2 and especially TUG-1197 are reasonably potent compared to the other FFA4 agonists and none of these compounds show activity on FFA1 at up to 100 μM concentration. TUG-1197 is also confirmed to lack activity on FFA2 and FFA3 (Azevedo et al. 2016). A more comprehensive profiling is still lacking. The compounds are non-acidic and structurally distinctly different from the carboxylic acid containing FFA4 agonists (see above), and would therefore be expected to have different activity profiles (preliminarily confirmed by lack of activity on FFA1). They would therefore be suitable as orthogonal FFA4 probes in conjunction with carboxylic acid probes (Bunnage et al. 2013). The absence of a charge causes low aqueous solubility but not to a degree that it interfered with the in vivo studies (Azevedo et al. 2016). Neither cpd 2 nor TUG-1197 is currently commercially available, but they are both synthetically easily accessible by a three-step sequence from saccharin (Azevedo et al. 2016).

4.3 Antagonists

4.3.1 AH 7614

During their work towards GSK137647A, Sparks et al. also identified the sulfonamide AH 7614 (compound 39) as a potent FFA4 antagonist with pIC_{50} of 7.1, 8.1 and 8.1 on the human, mouse and rat orthologue, respectively (Fig. 2) (Sparks et al. 2014). The compound was also confirmed to lack agonist activity on hFFA4 and either agonist or antagonist activity on hFFA1. The antagonist counteracted effects of GSK137647A on insulin and GLP-1 secretion in MIN6 and NCI-H716 cells, respectively. The exact mode of antagonism for AH 7614 is yet to be clarified. However, the compound lacks properties appropriate for utilization in an in vivo setting (Sparks et al. 2014), presumably due to low solubility. As AH 7614 is the only FFA4 antagonist published to date, it is nevertheless an important tool compound with confirmed high potency across human and rodent FFA4, and it is commercially available. The antagonist was recently used in a study on the phosphorylation patterns of mFFA4, where the compound was reported to effectively block FFA4 activation of TUG-891 (Prihandoko et al. 2016).

5 Outlook

FFA4 has in recent years appeared as a hot target for new potential drugs aimed at the treatment of metabolic and inflammatory disorders such as T2D, obesity and potentially non-alcoholic steatohepatitis. Both selective FFA4 agonists and polypharmacological activities, such as dual FFA1/FFA4 agonists, are of interest. There is, however, still many unanswered questions regarding the physiology and pharmacology of FFA4 and its interplay with other receptors. Tool compounds with appropriate properties are therefore required for further investigations. As outlined above, several reasonably potent FFA4 agonists are now available, thus, there is no longer any need to use low potency and non-selective agonists like the LCFAs or GW9508 in future studies where specific FFA4 activation is desired. We are now also in a position where structurally orthogonal FFA4 agonists that may be presumed to have non-overlapping activity profiles are available (even if this remains to be supported by broader profiling). Thus, inclusion of a carboxylic acid based agonist such as TUG-891, a Metabolex compound or Merck's cpdA, may be used together with sulfonamide based agonists such as GSK137647A or a Banyu-derived compound to provide strong pharmacological evidence for specific engagement of FFA4. Furthermore, the first antagonist for FFA4 was recently disclosed and has already been employed as an in vitro tool in the study of FFA4.

Despite this, there is still a need for better profiling of all current FFA4 probes, as well as a need for further improved probes. The properties of the current FFA4 probes are summarized in Table 3. Since FFA4 is linked to effects mediated by both G protein and β-arrestin pathways, it is critical that tools are characterized on both of these. A more extensive profiling is currently only available for GSK137647A. Properties such as solubility, chemical stability and the more detailed

Table 3 Properties of currently used FFA4 tool compounds

	FFA4 potency	Off-target activity (*if known*)	Solubility	In vivo PK	Commercial availability (06/2016)
Agonists	*pEC$_{50}$*	*pEC$_{50}$*			
LCFAs	<6[1]	<6[1]	Generally poor[1]	Rapidly metabolized[1]	
GW9508	h: 5.5[a,2] m: 6.5[b,4]	hFFA1: 7.3[a,2] PPARγ: 4.9[2]	ND	ND Used in several mouse studies	✔
TUG-891	h: 7.4–7.0[a,3], 7.2–6.8[b,3,4], 5.8[c,4], 7.3[d,4] m: 7.8[a,3,4], 7.0[b,4]	hFFA1: 4.2[b,3] mFFA1: 5.9[b,4] FFA2, FFA3: <4[3]	ND	ND Used orally in mice (20 mg/kg)[5]	✔
Metabolex compound B	m: 7.8 (IP)[6]	mFFA1: <5 (IP)[6]	ND	ND Used in mice (30 mg/kg)[6]	✘
Metabolex 36	m: 6.0[e,7], 5.9[d,7]	mFFA1: <4[d,7]	ND	ND	✘
Merck cpdA	h: 7.6[d,8], 6.1[d,9], 6.5[b,8] m: 6.5[b,8]	hFFA1: <5[d,8]	ND	ND Used in mice[8]	✔
GSK137647A	h: 6.3[d,10] m: 6.2[d,10] r: 6.1[d,10]	h/m/rFFA1/2/3: <4.5[d,10]	<10 μm (FASSIF)[10]	ND Deemed unsuitable for in vivo studies[10]	✔
Banyu cpd 2	h: 6.5[a], 6.4[b]	hFFA1: <4[a]	11 μm (PBS)[11]	ND Used in mice[11]	✘
TUG-1197	h: 6.6[a], 6.9[b] m: 6.3[b]	hFFA1: <4[a]	14 μm (FASSIF), 1.3 μm (PBS)[11]	ND Used in mice[11]	✘

(continued)

Table 3 (continued)

	FFA4 potency	Off-target activity (*if known*)	Solubility	In vivo PK	Commercial availability (06/2016)
Antagonists	pIC_{50}				
AH 7614	h: 7.1[d,10] m: 8.1[d,10] r: 8.1[d,10]	hFFA1: $pEC_{50} < 4.3$[d,10] $pIC_{50} < 4.6$[d,10]	ND	ND Deemed unsuitable for in vivo studies[10]	✔

Source: [1]Ulven and Christiansen (2015); [2]Briscoe et al. (2006); [3]Shimpukade et al. (2012); [4]Hudson et al. (2013b); [5]Gozal et al. (2016); [6]Engelstoft et al. (2013); [7]Stone et al. (2014); [8]Oh da et al. (2014); [9]Chelliah et al. (2014); [10]Sparks et al. (2014); [11]Azevedo et al. (2016)
h human, *m* mouse, *r* rat, *ND* no data available
[a]Calcium mobilization assay
[b]β-Arrestin recruitment assay
[c]pERK
[d]Internalization
[e]Dynamic mass redistribution assay and FLIPR Ca^{2+} assay

pharmacokinetic properties, including oral bioavailability, metabolic stability and propensity to enter the CNS, are still mostly unknown for all known probes, even if several have been successfully used in vivo. It is clear that although TUG-891, Metabolex compound B and Merck cpdA are of reasonable potency, agonists with even higher potency, and thereby increased chance of appropriate target exposure and reduced risk of off-target effects and pharmacokinetic issues, are still desirable.

Likewise, the FFA4 antagonist AH 7614 has appreciable potency at human and especially at rodent receptors, and represents a significant advance since the compound enables studies with pharmaceutical ablation of FFA4 and an alternative method for controlling specific activity of FFA4 agonists. However, further characterization is required to establish specificity, chemical stability, and pharmacological mode of action. It seems clear that the compound has certain suboptimal properties, including poor solubility, that interfere with its use in vivo (Sparks et al. 2014). Additional FFA4 antagonists with complementary properties and improved solubility are therefore still desirable.

None of the current FFA4 probes are sufficiently characterized to make them optimal for in vivo studies, even though several of the compounds have been used in such studies. For in vivo studies in general, information on bioavailability, preferred formulations, expected plasma concentrations following specific doses, clearance, major metabolites and their activity profiles, is useful but rarely available information. The widespread expression and multiple functions of FFA4 make this a particularly challenging case. It is difficult to ensure complete and concomitant target engagement in all organs relevant for regulation of the metabolic state, including intestines (where exposure from the vascular or luminal side will not necessarily result in the same effect), pancreas, adipose tissue and the CNS, and imbalanced exposure may affect the response. The role of FFA4 in the brain is not as well studied as in other tissues, but studies in which intracerebroventricular (icv) injection of LCFAs has been performed indicate that the receptor is mediating anti-inflammatory effects (Cintra et al. 2012). Probes with well-characterized CNS penetration are therefore desirable for in vivo studies, where the preferred compound would either be completely excluded from the CNS or have good penetration that could engage FFA4 in the CNS and enable studies of their effects without the need for icv injection.

In conclusion, although none of the FFA4 probes described hitherto are completely characterized or have ideal properties, tool compounds are available that are of sufficient quality for exploration of FFA4 provided that appropriate precautions are taken in the experimental design. Strategies to minimize the risk of confounding readouts include concomitant use of structurally orthogonal probes, for example both a carboxylic acid and a sulfonamide, FFA4 antagonists, of which only one currently is available, and gene knockout or knockdown techniques. Meanwhile, the high current activity in the field is likely to result in new probes with further improved properties that will contribute to the further elucidation of the function of FFA4 and its potential as a drug target.

References

Anighoro A, Bajorath J, Rastelli G (2014) Polypharmacology: challenges and opportunities in drug discovery. J Med Chem 57:7874–7887

Arakawa K, Nishimura T, Sugimoto Y, Takahashi H, Shimamura T (2010) Preparation of heteroaryloxyphenyldihydrobenzisothiazoledioxide derivatives and analogs for use as GPR120 receptor modulators. Banyu Pharmaceutical, Tokyo. WO2010104195A1

Arrowsmith CH, Audia JE, Austin C, Baell J, Bennett J, Blagg J et al (2015) The promise and peril of chemical probes. Nat Chem Biol 11:536–541

Azevedo CM, Watterson KR, Wargent ET, Hansen SVF, Hudson BD, Kepczynska M et al (2016) A non-acidic free fatty acid receptor 4 agonists with antidiabetic activity. J Med Chem 59:8868–8878. doi:10.1021/acs.jmedchem.6b00685

Baell JB, Holloway GA (2010) New substructure filters for removal of Pan Assay Interference Compounds (PAINS) from screening libraries and for their exclusion in bioassays. J Med Chem 53:2719–2740

Bang-Andersen B, Ruhland T, Jorgensen M, Smith G, Frederiksen K, Jensen KG et al (2011) Discovery of 1-[2-(2,4-dimethylphenylsulfanyl)phenyl]piperazine (Lu AA21004): a novel multimodal compound for the treatment of major depressive disorder. J Med Chem 54:3206–3221

Boettcher M, McManus MT (2015) Choosing the right tool for the job: RNAi, TALEN, or CRISPR. Mol Cell 58:575–585

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR et al (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 148:619–628

Bunnage ME, Chekler EL, Jones LH (2013) Target validation using chemical probes. Nat Chem Biol 9:195–199

Butcher AJ, Hudson BD, Shimpukade B, Alvarez-Curto E, Prihandoko R, Ulven T et al (2014) Concomitant action of structural elements and receptor phosphorylation determines arrestin-3 interaction with the free fatty acid receptor FFA4. J Biol Chem 289:18451–18465

Calder PC (2012) Mechanisms of action of (n-3) fatty acids. J Nutr 142:592s–599s

Chelliah M, Chu HD, Cox JM, Debenham JS, Eagen K, Lan P et al (2014) Preparation of substituted spiropiperidinyl compounds useful as GPR120 agonists. Merck Sharp & Dohme, Kenilworth, NJ. WO2014059232A2

Christiansen E, Urban C, Grundmann M, Due-Hansen ME, Hagesaether E, Schmidt J et al (2011) Identification of a potent and selective free fatty acid receptor 1 (FFA1/GPR40) agonist with favorable physicochemical and in vitro ADME properties. J Med Chem 54:6691–6703

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schroder R, Hudson BD et al (2012) Free fatty acid receptor 1 (FFA1/GPR40) agonists: mesylpropoxy appendage lowers lipophilicity and improves ADME properties. J Med Chem 55:6624–6628

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schmidt J, Hansen SVF et al (2013) Discovery of a potent and selective free fatty acid receptor 1 agonist with low lipophilicity and high oral bioavailability. J Med Chem 56:982–992

Christiansen E, Watterson KR, Stocker CJ, Sokol E, Jenkins L, Simon K et al (2015) Activity of dietary fatty acids on FFA1 and FFA4 and characterization of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. Br J Nutr 113:1677–1688

Cintra DE, Ropelle ER, Moraes JC, Pauli JR, Morari J, Souza CT et al (2012) Unsaturated fatty acids revert diet-induced hypothalamic inflammation in obesity. PLoS One 7, e30571

Cornish J, MacGibbon A, Lin JM, Watson M, Callon KE, Tong PC et al (2008) Modulation of osteoclastogenesis by fatty acids. Endocrinology 149:5688–5695

Davenport AP, Alexander SPH, Sharman JL, Pawson AJ, Benson HE, Monaghan AE et al (2013) International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. Pharmacol Rev 65:967–986

Defossa E, Wagner M (2014) Recent developments in the discovery of FFA1 receptor agonists as novel oral treatment for type 2 diabetes mellitus. Bioorg Med Chem Lett 24:2991–3000

Donath MY, Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11:98–107

Egerod KL, Engelstoft MS, Lund ML, Grunddal KV, Zhao M, Barir-Jensen D et al (2015) Transcriptional and functional characterization of the G protein-coupled receptor repertoire of gastric somatostatin cells. Endocrinology 156:3909–3923

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S et al (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2:376–392

Formicola R, Pevarello P, Kuhn C, Liberati C, Piscitelli F, Sodano M (2015) FFA4/GPR120 agonists: a survey of the recent patent literature. Pharm Pat Anal 4:443–451

Frye SV (2010) The art of the chemical probe. Nat Chem Biol 6:159–161

Fukushima K, Yamasaki E, Ishii S, Tomimatsu A, Takahashi K, Hirane M et al (2015) Different roles of GPR120 and GPR40 in the acquisition of malignant properties in pancreatic cancer cells. Biochem Biophys Res Commun 465:512–515

Gao B, Huang Q, Jie Q, Lu W-G, Wang L, Li X-J et al (2015) GPR120: a bi-potential mediator to modulate the osteogenic and adipogenic differentiation of BMMSCs. Sci Rep 5:14080

Garrel G, Simon V, Denoyelle C, Cruciani-Guglielmacci C, Migrenne S, Counis R et al (2011) Unsaturated fatty acids stimulate LH secretion via novel PKCepsilon and -theta in gonadotrope cells and inhibit GnRH-induced LH release. Endocrinology 152:3905–3916

Gong Z, Yoshimura M, Aizawa S, Kurotani R, Zigman JM, Sakai T et al (2014) G protein-coupled receptor 120 signaling regulates ghrelin secretion in vivo and in vitro. Am J Physiol Endocrinol Metab 306:E28–E35

Gozal D, Qiao Z, Almendros I, Zheng J, Khalyfa A, Shimpukade B et al (2016) Treatment with TUG891, a free fatty acid receptor 4 agonist, restores adipose tissue metabolic dysfunction following chronic sleep fragmentation in mice. Int J Obes 40:1143–1149

Hakim F, Kheirandish-Gozal L, Gozal D (2015a) Obesity and altered sleep: a pathway to metabolic derangements in children? Semin Pediatr Neurol 22:77–85

Hakim F, Wang Y, Carreras A, Hirotsu C, Zhang J, Peris E et al (2015b) Chronic sleep fragmentation during the sleep period induces hypothalamic endoplasmic reticulum stress and PTP1b-mediated leptin resistance in male mice. Sleep 38:31–40

Halder S, Kumar S, Sharma R (2013) The therapeutic potential of GPR120: a patent review. Expert Opin Ther Pat 23:1581–1590

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M et al (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94

Hostetler HA, Kier AB, Schroeder F (2006) Very-long-chain and branched-chain fatty acyl-CoAs are high affinity ligands for the peroxisome proliferator-activated receptor α (PPARα). Biochemistry 45:7669–7681

Hudson BD, Murdoch H, Milligan G (2013a) Minireview: the effects of species ortholog and SNP variation on receptors for free fatty acids. Mol Endocrinol 27:1177–1187

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E et al (2013b) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 84:710–725

Hudson BD, Shimpukade B, Milligan G, Ulven T (2014) The molecular basis of ligand interaction at free fatty acid receptor 4 (FFA4/GPR120). J Biol Chem 289:20345–20358

Humphries PS, Benbow JW, Bonin PD, Boyer D, Doran SD, Frisbie RK et al (2009) Synthesis and SAR of 1,2,3,4-tetrahydroisoquinolin-1-ones as novel G-protein-coupled receptor 40 (GPR40) antagonists. Bioorg Med Chem Lett 19:2400–2403

Ishii S, Hirane M, Kato S, Fukushima N, Tsujiuchi T (2015a) Opposite effects of GPR120 and GPR40 on cell motile activity induced by ethionine in liver epithelial cells. Biochem Biophys Res Commun 456:135–138

Ishii S, Hirane M, Kitamura Y, Mori S, Fukushima N, Honoki K et al (2015b) Role of GPR120 in cell motile activity induced by 12-O-tetradecanoylphorbol-13-acetate in liver epithelial WB-F344 cells. Mol Cell Biochem 400:145–151

Kim HJ, Yoon HJ, Kim BK, Kang WY, Seong SJ, Lim MS et al (2015) G protein-coupled receptor 120 signaling negatively regulates osteoclast differentiation, survival, and function. J Cell Physiol 231:844–851

Kita T, Kadochi Y, Takahashi K, Fukushima K, Yamasaki E, Uemoto T et al (2016) Diverse effects of G-protein-coupled free fatty acid receptors on the regulation of cellular functions in lung cancer cells. Exp Cell Res 342:193–199

Kodadek T (2010) Rethinking screening. Nat Chem Biol 6:162–165

Konno Y, Ueki S, Takeda M, Kobayashi Y, Tamaki M, Moritoki Y et al (2015) Functional analysis of free fatty acid receptor GPR120 in human eosinophils: implications in metabolic homeostasis. PLoS One 10, e0120386

Kristinsson H, Smith DM, Bergsten P, Sargsyan E (2013) FFAR1 is involved in both the acute and chronic effects of palmitate on insulin secretion. Endocrinology 154:4078–4088

Lengqvist J, Mata De Urquiza A, Bergman AC, Willson TM, Sjovall J, Perlmann T et al (2004) Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. Mol Cell Proteomics 3:692–703

Li X, Yu Y, Funk CD (2013a) Cyclooxygenase-2 induction in macrophages is modulated by docosahexaenoic acid via interactions with free fatty acid receptor 4 (FFA4). FASEB J 27:4987–4997

Li Y, Zhang H, Jiang C, Xu M, Pang Y, Feng J et al (2013b) Hyperhomocysteinemia promotes insulin resistance by inducing endoplasmic reticulum stress in adipose tissue. J Biol Chem 288:9583–9592

Liu Y, Chen LY, Sokolowska M, Eberlein M, Alsaaty S, Martinez-Anton A et al (2014) The fish oil ingredient, docosahexaenoic acid, activates cytosolic phospholipase A(2) via GPR120 receptor to produce prostaglandin E(2) and plays an anti-inflammatory role in macrophages. Immunology 143:81–95

Liu Z, Hopkins MM, Zhang Z, Quisenberry CB, Fix LC, Galvan BM et al (2015) Omega-3 fatty acids and other FFA4 agonists inhibit growth factor signaling in human prostate cancer cells. J Pharmacol Exp Ther 352:380–394

Ma J, Novack A, Nashashibi I, Pham P, Rabbat CJ, Song J et al (2010) [(Heterocyclylmethoxy) aryl]alkanoic acid derivatives as GPR120 receptor agonists and preparation and uses thereof. Metabolex, Hayward, CA. WO2010048207A2

Martin C, Passilly-Degrace P, Chevrot M, Ancel D, Sparks SM, Drucker DJ et al (2012) Lipid-mediated release of GLP-1 by mouse taste buds from circumvallate papillae: putative involvement of GPR120 and impact on taste sensitivity. J Lipid Res 53:2256–2265

Milligan G, Alvarez-Curto E, Watterson KR, Ulven T, Hudson BD (2015) Characterising pharmacological ligands to study the long chain fatty acid receptors GPR40/FFA1 and GPR120/FFA4. Br J Pharmacol 172:3254–3265

Milligan G, Shimpukade B, Ulven T, Hudson BD (2016) Complex pharmacology of free fatty acid receptors. Chem Rev. doi:10.1021/acs.chemrev.6b00056

Mizuta K, Zhang Y, Mizuta F, Hoshijima H, Shiga T, Masaki E et al (2015) Novel identification of the free fatty acid receptor FFAR1 that promotes contraction in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 309:L970–L982

Moniri NH (2016) Free-fatty acid receptor-4 (GPR120): cellular and molecular function and its role in metabolic disorders. Biochem Pharmacol 110–111:1–15

Offermanns S (2014) Free fatty acid (FFA) and hydroxy carboxylic acid (HCA) receptors. Annu Rev Pharmacol Toxicol 54:407–434

Oh da Y, Olefsky JM (2016) G protein-coupled receptors as targets for anti-diabetic therapeutics. Nat Rev Drug Discov 15:161–172

Oh da Y, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR et al (2014) A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med 20:942–947

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan WQ et al (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698

Peters JU (2013) Polypharmacology – foe or friend? J Med Chem 56:8955–8971

Pizzonero M, Dupont S, Babel M, Beaumont S, Bienvenu N, Blanque R et al (2014) Discovery and optimization of an azetidine chemical series as a free fatty acid receptor 2 (FFA2) antagonist: from hit to clinic. J Med Chem 57:10044–10057

Pouliot M, Jeanmart S (2016) Pan Assay Interference Compounds (PAINS) and other promiscuous compounds in antifungal research. J Med Chem 59:497–503

Prihandoko R, Alvarez-Curto E, Hudson BD, Butcher AJ, Ulven T, Miller AM et al (2016) Distinct phosphorylation clusters determine the signaling outcome of free fatty acid receptor 4/G protein-coupled receptor 120. Mol Pharmacol 89:505–520

Raptis DA, Limani P, Jang JH, Ungethum U, Tschuor C, Graf R et al (2014) GPR120 on Kupffer cells mediates hepatoprotective effects of omega3-fatty acids. J Hepatol 60:625–632

Reddy AS, Zhang S (2013) Polypharmacology: drug discovery for the future. Expert Rev Clin Pharmacol 6:41–47

Sergeev E, Hojgaard Hansen A, Pandey SK, Mackenzie AE, Hudson BD, Ulven T et al (2015) Non-equivalence of key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. J Biol Chem 291:303–317

Serhan CN (2014) Pro-resolving lipid mediators are leads for resolution physiology. Nature 510:92–101

Shi DF, Song J, Ma J, Novack A, Pham P, Nashashibi I et al (2010) Preparation of benzofuranyl substituted phenylalkylcarboxylic acids as Gpr120 receptor agonists. Metabolex, Hayward, CA. WO2010080537A1

Shimpukade B, Hudson BD, Hovgaard CK, Milligan G, Ulven T (2012) Discovery of a potent and selective GPR120 agonist. J Med Chem 55:4511–4515

Song T, Peng J, Ren J, Wei HK, Peng J (2015) Cloning and characterization of spliced variants of the porcine G protein coupled receptor 120. Biomed Res Int 2015:813816

Song T, Zhou Y, Peng J, Tao YX, Yang Y, Xu T et al (2016) GPR120 promotes adipogenesis through intracellular calcium and extracellular signal-regulated kinase 1/2 signal pathway. Mol Cell Endocrinol 434:1–13

Sparks SM, Chen G, Collins JL, Danger D, Dock ST, Jayawickreme C et al (2014) Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). Bioorg Med Chem Lett 24:3100–3103

Stone VM, Dhayal S, Brocklehurst KJ, Lenaghan C, Sorhede Winzell M, Hammar M et al (2014) GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans. Diabetologia 57:1182–1191

Sun P, Wang T, Zhou Y, Liu H, Jiang H, Zhu W et al (2013) DC260126: a small-molecule antagonist of GPR40 that protects against pancreatic beta-Cells dysfunction in db/db mice. PLoS One 8, e66744

Talukdar S, Olefsky JM, Osborn O (2011) Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. Trends Pharmacol Sci 32:543–550

Tikhonova IG, Sum CS, Neumann S, Engel S, Raaka BM, Costanzi S et al (2008) Discovery of novel agonists and antagonists of the free fatty acid receptor 1 (FFAR1) using virtual screening. J Med Chem 51:625–633

Tsukahara T, Watanabe K, Watanabe T, Yamagami H, Sogawa M, Tanigawa T et al (2015) Tumor necrosis factor alpha decreases glucagon-like peptide-2 expression by up-regulating G-protein-coupled receptor 120 in Crohn disease. Am J Pathol 185:185–196

Ulven T, Christiansen E (2015) Dietary influences on regulation of the long-chain fatty acid receptor FFA4/GPR120. Annu Rev Nutr 35:239–263

Ulven T, Kostenis E (2006) Targeting the prostaglandin D_2 receptors DP and CRTH2 for treatment of inflammation. Curr Top Med Chem 6:1427–1444

Waring MJ, Baker DJ, Bennett SNL, Dossetter AG, Fenwick M, Garcia R et al (2015) Discovery of a series of 2-(pyridinyl)pyrimidines as potent antagonists of GPR40. Med Chem Commun 6:1024–1029

Watson SJ, Brown AJ, Holliday ND (2012) Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 81:631–642

Wauquier F, Philippe C, Leotoing L, Mercier S, Davicco MJ, Lebecque P et al (2013) The free fatty acid receptor G protein-coupled receptor 40 (GPR40) protects from bone loss through inhibition of osteoclast differentiation. J Biol Chem 288:6542–6551

Wellhauser L, Belsham DD (2014) Activation of the omega-3 fatty acid receptor GPR120 mediates anti-inflammatory actions in immortalized hypothalamic neurons. J Neuroinflammation 11:60

Workman P, Collins I (2010) Probing the probes: fitness factors for small molecule tools. Chem Biol 17:561–577

Wu Q, Wang H, Zhao X, Shi Y, Jin M, Wan B et al (2013) Identification of G-protein-coupled receptor 120 as a tumor-promoting receptor that induces angiogenesis and migration in human colorectal carcinoma. Oncogene 32:5541–5550

Yan Y, Jiang W, Spinetti T, Tardivel A, Castillo R, Bourquin C et al (2013) Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation. Immunity 38:1154–1163

Application of GPCR Structures for Modelling of Free Fatty Acid Receptors

Irina G. Tikhonova

Contents

Abstract

Five G protein-coupled receptors (GPCRs) have been identified to be activated by free fatty acids (FFA). Among them, FFA1 (GPR40) and FFA4 (GPR120) bind long-chain fatty acids, FFA2 (GPR43) and FFA3 (GPR41) bind short-chain fatty acids and GPR84 binds medium-chain fatty acids. Free fatty acid receptors have now emerged as potential targets for the treatment of diabetes, obesity and immune diseases. The recent progress in crystallography of GPCRs has now enabled the elucidation of the structure of FFA1 and provided reliable templates for homology modelling of other FFA receptors. Analysis of the crystal structure and improved homology models, along with mutagenesis data and structure activity, highlighted an unusual arginine charge-pairing interaction in FFA1–3 for receptor modulation, distinct structural features for ligand binding to FFA1

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and FFA4 and an arginine of the second extracellular loop as a possible anchoring point for FFA at GPR84. Structural data will be helpful for searching novel small-molecule modulators at the FFA receptors.

Keywords
Allosteric site • FFA1 • FFA2 • FFA3 • FFA4 • Free fatty acid receptors • G protein-coupled receptors • GPR84 • Molecular modelling • Mutagenesis • Orthosteric site • SAR

1 Introduction

The free fatty acid receptors are members of the rhodopsin family of G protein-coupled receptors (GPCRs) and relay binding of endogenous free fatty acids at the cell surface into intracellular activation of a heterotrimeric G protein. Currently, five GPCRs have been identified to bind free fatty acids with a different level of specificity. The free fatty acid receptor 1 (FFA1), previously known as GPR40, binds preferably long- and medium-chain fatty acids with more than 12 carbon atoms (Briscoe et al. 2006; Itoh and Hinuma 2005). The free fatty acid receptor 2 (FFA2), previously known as GPR43, and the free fatty acid receptor 3 (FFA3), known as GPR41, respond to short-chain fatty acids with less than 5 carbon atoms (Brown et al. 2003). FFA1–3 have more than 30% of sequence identity and belong to the branch of nucleotide receptors in the phylogenetic tree of the rhodopsin family of GPCRs (Fig. 1). The free fatty acid receptor 4 (FFA4), also known as GPR120, has a preference to bind long-chain fatty acids (Hirasawa et al. 2005), like FFA1, but has a substantially low sequence identity with FFA1 (<19%) and other members of the family. Phylogenetically, FFA4 is clustered with several orphan receptors. The last receptor, GPR84, is activated by medium-chain fatty acids with 7–12 carbon atoms (Wang et al. 2006), has low similarity with FFA1–4 (<21%) and is branched with the prostanoid receptors. Since free fatty acid signalling is a molecular messenger system regulating energy storage, metabolism and inflammation, these receptors are thought to be involved in numerous metabolic and inflammatory conditions such as obesity, type 2 diabetes, atherosclerosis, cardiovascular diseases, ulcerative colitis, Crohn's disease and irritable bowel disease (Ulven 2012; Dranse et al. 2013; Milligan et al. 2014; Bindels et al. 2013).

Knowledge of the receptor three-dimensional structure is crucial for an understanding of the molecular mechanisms underlying diseases as well as for structure-based design of small-molecule modulators. The FFA receptors share the overall topological structure typical of GPCRs: seven alpha-helices that cross the cell membrane and are connected by three extracellular and intracellular loops, the N terminus in the extracellular side and the C terminus in the intracellular side of the receptors. The recent breakthrough in structural biology of GPCRs enabled crystallographic structures for 27 receptors of the rhodopsin family of GPCRs, including the FFA1 receptor. A FFA1 structure is solved in the complex with the

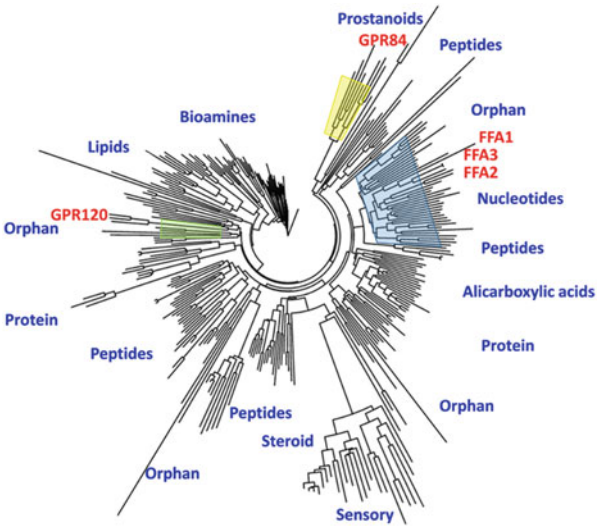

Fig. 1 A phylogenetic tree of the rhodopsin family of GPCRs. The phylogenetic tree was constructed using the sequence of the transmembrane helices by the online GPCRDB server (www.gpcrdb.org). The circular tree type, neighbour-joining distance calculation and no bootstrapping options were used. Clusters to which the free fatty acid receptors belong are highlighted

ago-allosteric ligand, TAK-875 (Srivastava et al. 2014), and provides a crucial point to study ligand recognition at FFA1. However, FFA2, FFA3, GPR120 and GPR84 have not been yet crystallised. The crystallisation of GPCRs remains a challenging and long process, and it will take years to get the structure of other free fatty acid receptors. Solving GPCR structures in the complex with various ligands is also labour-intensive and time-consuming. Therefore, computational techniques such as homology modelling and ligand docking will play an important role in mapping ligand-receptor contacts and providing working hypotheses for site-directed mutagenesis of the receptors.

Homology modelling in combination with mutagenesis data enables indirect structural information and has been proven to be insightful in an understanding of ligand-protein interactions in many GPCRs. In homology modelling, the structure of the protein is constructed from its amino acid sequence and an experimental structure of a related homologous protein. A detailed procedure of GPCR homology modelling is provided in the recent article by Costanzi (Costanzi 2012). Crystal structures as well as homology models of GPCRs serve as a basis to explore ligand binding through various molecular docking protocols. Ligand docking provides a rapid solution as to how and where a ligand binds in a receptor and predicts key interactions important for ligand recognition. Besides scoring functions, the obtained ligand docking pose is evaluated based on its agreement with mutagenesis data and structure-activity relationships (SARs) of the ligands. GPCR docking has been recently reviewed in several studies (Costanzi 2013; Beuming et al. 2015; Weiss et al. 2016; Levit et al. 2012). An ability to explain experimental data and accurately predict the results of new experiments defines the quality of receptor modelling.

In this chapter, we will focus on molecular modelling of the FFA structures and ligand binding using available structural information, mutagenesis data and ligand

SARs. We will analyse the recently published FFA1 crystal structure and assess past and future modelling of ligand interactions at FFA1. We then examine the application of available structural templates in modelling of structures and prediction of ligand-receptor interactions for other FFA receptors. In the text, we will provide the Ballesteros-Weinstein index (Ballesteros and Weinstein 1995) for residues in the transmembrane helices as a superscript. This index represents X.Y, where X is the helix number and Y is the residue number relative to position 50, which is assigned to the most conserved residue in a given helix.

2 Free Fatty Acid Receptor 1

2.1 X-Ray Crystallography of FFA1

In 2014, an X-ray crystallographic structure of the human FFA1 in complex with the ago-allosteric ligand, TAK-875, provided unprecedented three-dimensional insight into molecular recognition at FFA1 (Srivastava et al. 2014). The crystal structure revealed that TAK-875 binds to the site involving transmembrane helices 3–5 and the second extracellular loop (EL2). In the binding site, the oxygen atom of the carboxyl group of the ligand forms hydrogen bonds with two arginines, $R183^{5.39}$ and $R258^{7.53}$, whereas the other one is in hydrogen bonding with two tyrosines, $Y240^{6.51}$ and $Y91^{3.37}$; the dihydrobenzofuran ring of TAK-875 is stabilised via π-π stacking with $W174^{EL2}$ and $F142^{EL2}$; the biphenyl scaffold protrudes into the interhelical gap between helices 3 and 4; and the methylsulfonyl linker is pointed to the extracellular side (Fig. 2a, b).

Interestingly, the guanidinium side chains of $R183^{5.39}$ and $R258^{7.53}$ are in an unusual charge-pairing interaction. Despite being both positively charged residues, these arginines do not repulse but are in planar stacking with the distance between the C_ζ-C_ζ atom pairs of 3.6 Å. From the detailed analysis of the crystal structure, it becomes clear that the electrostatic repulsion between positively charged guanidinium groups is balanced by the surrounding environment. The counterion from the aspartate acid residue $E172^{EL2}$ forms an ionic bridge with $R258^{7.53}$, interacting with two hydrogens of the guanidinium (Fig. 2c). Two other guanidinium donors of $R258^{7.53}$ are involved in a direct hydrogen bond with $Y240^{6.51}$ and through a water-mediated hydrogen bond with $N244^{6.55}$. In the case of $R183^{5.39}$, two guanidinium donors of $R183^{5.39}$ form a direct hydrogen bond with the backbone of $L171^{EL2}$ and a water-mediated hydrogen bond with the backbone of $W174^{EL2}$ and $E172^{EL2}$. Considering that the guanidinium group can be involved in five hydrogen bonds, $R258^{7.53}$ and $R183^{5.39}$ have one and three guanidinium donors, respectively, that are free from interactions and could coordinate the carboxyl group of a ligand (Fig. 2). Indeed the carboxyl group of TAK-875 forms one hydrogen bond with $R258^{7.53}$ and two hydrogen bonds with $R183^{5.39}$, thus fully balancing the arginine pairing.

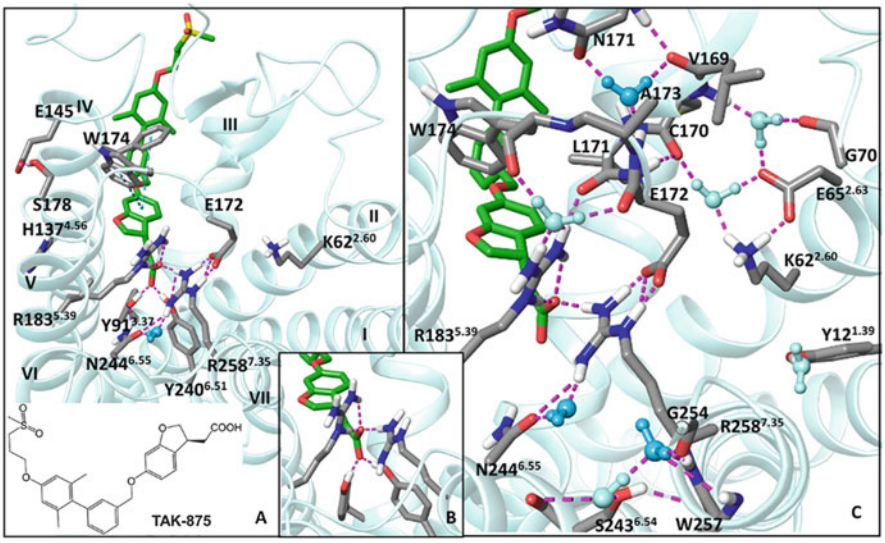

Fig. 2 The binding mode of TAK-875 in the crystal structure of FFA1. (**a**) The overall view of the binding site. (**b**) The zoomed view of the hydrogen bonding between the carboxyl group of TAK-875 and four residues of the binding site. (**c**) The binding site of TAK-875 in the presence of water molecules. Hydrogen bonds and π-π stacking are in *pink* and *blue*, respectively. The crystal water molecules are shown in *light cyan* and the water molecules with a low B factor are shown in *dark cyan*. Residues involved in ligand binding, water-mediated interactions and coordination of the arginine pairing, as well as used in site-directed mutagenesis, are shown in *sticklike* representation. Numbering of receptor helices in *Roman numerals*

Although this is the first arginine pairing observed in GPCRs, arginine-arginine short-range interactions are found in many other proteins, where these residues are involved in the binding of nucleotides, organic acids and other negatively charged bioactive molecules. From analysis of 67,520 crystal structures containing clusters of positively charged arginines, Neves and colleagues (Neves et al. 2012) demonstrated that guanidinium groups tend to sit in polar pockets or are exposed to solvent. In a much earlier study, computer simulations of Magalhaes and colleagues (Magalhaes et al. 1994) have predicted the importance of solvent environment for arginine pairing. Similar to other proteins, the two pairing arginines in FFA1 are solvent exposed and have interactions with hydrophilic residues. It is believed that controlling a countercharge in arginine-arginine interactions represents a biological regulatory mechanism. I propose here that stabilising the arginine-arginine pairing might be crucial for activation of FFA1.

In addition to water molecules involved in the arginine pairing, several other water molecules in the binding site are observed in the crystal structure of 2.33 Å resolution (Fig. 2c). Although water molecules do not interact with the ligand directly, they are engaged in an hydrogen bonding network with the residues of the binding site. There are three water molecules with a low B factor that are engaged in several hydrogen bonds with the receptor (Fig. 2c). One water molecule

links R258$^{7.53}$ via a water-mediated interaction with N244$^{6.55}$, the second water molecule is within the extracellular tip of helix 7 and the third water molecule is trapped in EL2. With a recognised importance of water molecules in GPCRs (Mason et al. 2012), it is likely that ordered water molecules found in the FFA1 binding site could also play a critical role in ligand binding and receptor modulation.

Our recent examination of the crystal structure has revealed K62$^{2.60}$ as a third positively charged residue in the binding cavity of FFA1 (Tikhonova and Poerio 2015). This residue is in the binding site due to a proline kink causing unwinding of helix 2. Notably, the residue in the same position in the peptide (PAR1, opioid and CXCR4), lysosphingolipid (S1P1) and nucleotide (P2Y12) receptor crystal structures contributes to ligand recognition.

The intracellular side of helix 6, which is known to move away from the helical bundle during receptor activation (Rasmussen et al. 2011), is in the inwards position, suggesting that the FFA1 receptor in the crystal structure is in the inactive state.

Four point mutations, A88F, L42A, G103A and Y202F, were made to improve expression and thermal stability of FFA1 for crystallisation. The alanine mutation at position 3.34 is located close to TAK-875 (5 Å). Our docking study indicates that phenylalanine of the wild type at this position slightly changes a docking position of the biphenyl moiety of TAK-875 (Tikhonova and Poerio 2015).

2.2 Molecular Modelling of FFA1

The FFA1 X-ray structure follows upon a nearly decade long progression of knowledge of the binding site at FFA1 and other structural features based on homology modelling and mutagenesis. The first structural model of FFA1 was obtained through an iterative approach that combined rhodopsin-based homology modelling and receptor mutagenesis (Tikhonova et al. 2007). Rhodopsin, a light-activated receptor found in retinal rod cells was a single template available in 2007 to model GPCRs before breakthroughs in GPCR crystallography. Although the sequence identity in the transmembrane helices with FFA1 is only 16%, the first model of FFA1 helped to identify the putative binding cavity within helices 3, 4 and 5 and the positively charged residues R183$^{5.39}$ and R258$^{7.35}$ anchoring the negative carboxylic group of agonists. The homology model in conjunction with mutagenesis also predicted Y91$^{3.37}$, Y240$^{6.51}$, N244$^{6.55}$, H137$^{4.56}$ and L186$^{5.42}$ to form interactions with an agonist. From the available FFA1 crystal structure bound to TAK-875, it is clear that Y91$^{3.37}$ and Y240$^{6.51}$ are in hydrogen bonding with the carboxyl group of the ligand and not in aromatic and hydrophobic contacts, as it was predicted in homology modelling, and H137$^{4.56}$, L186$^{5.42}$ and N244$^{6.55}$ do not form a direct contact with the ligand. Overall, the hydrophobic tail of the ligand is pointed to the gap between helices 3 and 4 and not situated inside the helical bundle as was previously predicted.

Because EL2 is buried into the helical bundle in rhodopsin and prevents ligand interactions with the anchoring residues, the first FFA1 model was built without involvement of EL2 (Tikhonova et al. 2007). The next model of FFA1 was constructed using a template based on the β_2-adrenergic receptor, the first GPCR with a diffusible ligand crystallised (Sum et al. 2009). Although there is also a low sequence conservation in the transmembrane region (18%) and no homology in EL2, the β_2-adrenergic-based model allowed mapping the solvent-accessible orientation of EL2 and made predictions of ionic interactions between two glutamates, E145 and E172, of EL2 and two arginines, $R183^{5.39}$ and $R258^{7.35}$, of transmembrane helices. Molecular dynamics (MD) simulations of the receptor and mutagenesis of $E145^{EL2}$ and $E172^{EL2}$ suggested that these ionic interactions play roles of ionic locks keeping the receptor in an inactive state and breaking upon receptor activation. The FFA1 crystal structure has confirmed the existence of the ionic interaction between $E172^{EL2}$ and $R258^{7.35}$ (Fig. 2). However, it remains unclear whether this ionic interaction breaks upon activation, as the available crystal structure is an inactive conformation of the receptor. $E145^{EL2}$ is outside of the binding cavity and in hydrogen bonding with $S178^{EL2}$ in the experimental structure (Fig. 2a).

FFA1 homology modelling based on the templates with 16–18% sequence identity helped to map the ligand-binding area and anchoring residues but were not precise in predicting the type of interactions and the ligand-binding mode (Tikhonova and Poerio 2015). This conclusion fits to the results of community-wide GPCR homology modelling assessments conducted by the Abagyan and Stevens labs (Michino et al. 2008; Kufareva et al. 2011, 2014). According to GPCR homology modelling assessments, templates with a sequence identity of at least 30–35% allow building accurate homology models (Michino et al. 2008; Kufareva et al. 2011, 2014).

2.3 Docking to the FFA1 Crystal Structure

The crystal structure of FFA1 provides a good starting point for ligand docking. In our recent work, we have used different docking protocols including standard docking, induced fit docking and docking to conformations obtained from conformational search of the binding site residues to explore docking of linoleate, GW9508, TUG-770, AMG837, AM1638 and AM8182 in the wild-type receptor (Tikhonova and Poerio 2015). The carboxyl group of all the compounds is coordinated by two arginines and two tyrosines in all docking studies, similar to TAK-875. However, there is a difference in position of the hydrophobic tail of the ligands. While a standard docking protocol with a rigid receptor enables a TAK-875-like binding mode for most of the agonists, in which the hydrophobic tail is in the interhelical gap between helices 3 and 4, docking to the flexible receptor predicts, in addition, a new binding mode, where the hydrophobic tail of the agonists is pointed to the gap between helices 4 and 5. Whether it is an artefact of docking or this mode truly exists could be further validated by receptor

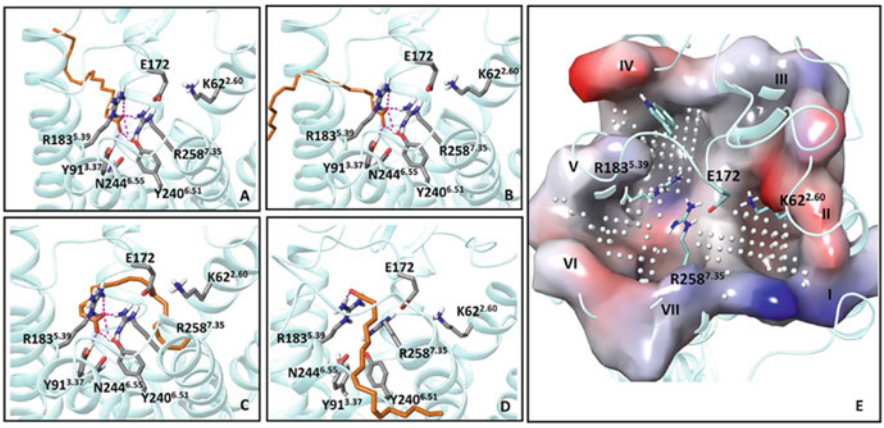

Fig. 3 Putative binding sites in FFA1. (**a**–**d**) Docking solutions for linoleate binding at FFA1. (**e**) The surface-based representation of the FFA1 extracellular cavity with contiguous dummy atoms that define the putative binding sites. Prediction of the binding sites was made with the site map (Schrodinger, LLC, New York, NY, USA 2014b SiteMap 3.3). The surface is coloured based on the electrostatic potential. Hydrogen bonds are in *pink*. Numbering of receptor helices in *Roman numerals*

mutagenesis. For example, residues facing one of the interhelical gaps could be mutated to bulky ones so that they provide hindrance in binding to one gap and validate binding to another gap.

Figure 3 shows different binding modes on the example of linoleate. For linoleate, in addition to two modes of binding in the gaps between helices (Fig. 3a, b), there are two other possible binding modes (Fig. 3c, d). In one of the modes, the carboxyl group has the same interactions with four residues, but the hydrophobic tail is within the helical bundle, around helices 1, 2 and 7. In another mode, the carboxyl group forms hydrogen bonding with arginines from the extracellular side, and the rest of the molecule occupies the sites of water molecules protruding between helices 6 and 7. Although docking of flexible linoleate is the least reliable, it helps to generate hypotheses as to how agonistic activity of linoleate could be amplified by synthetic agonists, like TAK-875, at a structural level. For example, targeting arginines from the extracellular side by substituting existing water-mediated contacts could still leave a possibility of targeting the arginines from the side involved in interactions with tyrosines.

Interestingly, docking of bulky AM1638 does not allow to place the ligand between helices in either way and gives a solution where the ligand is located within the extracellular side (Tikhonova and Poerio 2015). Since mutagenesis studies suggests that AM1638 does not bind to the arginines (Lin et al. 2012; Luo et al. 2012), the carboxyl group of AM1638 might be coordinated by other charged or hydrophilic residues within the extracellular binding cavity. We have previously shown that $K62^{2.60}$ is the third positively charged residues in the extracellular side and potentially could interact with AM1638 (Tikhonova and Poerio 2015).

The fact that the ligands could have different binding modes and occupy different binding sites at FFA1 can be also seen from a site map search (Schrodinger, LLC, New York, NY, USA 2014b) (Fig. 3). This computational technique is able to add dummy atoms and cluster contiguous dummy atoms into a putative site in several areas of the FFA1 extracellular binding cavity: sites around the arginine pairing and a site within helix 2 and 7, involving $K62^{2.60}$.

The use of X-ray structural data is now able to provide greater insight into ligand recognition than earlier approaches. Given that four residues are involved in coordination of the carboxyl group of an agonist, we could hypothesise that there could be more variation allowed in distances between ligand coordinating atoms of the residues than if only one residue, an arginine, chelates the carboxyl group (see the example with GPR120). Therefore, substitution of the carboxyl group to nonclassical bioisosteric groups could likely be more tolerated, providing a new route to further modify agonists and improve ligand selectivity.

Examination of available structures of antagonists (Holliday et al. 2012) shows that antagonists either do not have a carboxyl group in the structure or have a carboxyl group that is less approachable. This suggests that antagonists likely do not have interactions with the four anchoring residues or have interactions with only a few of them.

In summary, new mutagenesis efforts will be instrumental in mapping the precise binding mode of orthosteric and allosteric agonists and antagonists. The recent development of a fluorescent agonist could further assist in distinguishing between orthosteric and allosteric sites at FFA1 (Christiansen et al. 2016). The crystal structure is also helpful in initiation of MD simulation studies to probe flexibility of the receptor and explore the dynamics of the arginine pairing and the surrounding hydrogen bonding network in relation to ligand binding and receptor activation. In addition, simulations of the empty form of the receptor could help to clarify the position of helix 4 and therefore the dynamics of the interhelical gaps, further clarifying the possibility of different ligand-binding modes.

3 Free Fatty Acid Receptor 2

The initial structural model of FFA2 was built based on the crystal structure of the β_2-adrenergic receptor with sequence identity of 18%. This model, in conjunction with mutagenesis, was used for an understanding of the structural basis of selectivity at FFA2/FFA3 (Schmidt et al. 2011) and FFA2 species orthologues (Hudson et al. 2012a) for short carboxylic acids; elucidation of molecular determinants in binding of potent synthetic agonists, cmp 1 and cmp 2, in human and rodent orthologues of FFA2 (Hudson et al. 2013); and identification of possible ionic locks of activation (Hudson et al. 2012a, b), similar to FFA1.

The recently published crystal structure of FFA1 provides a more suitable template for the modelling of FFA2. FFA1 and FFA2 share 32% sequence identity and have conserved anchoring residues for the carboxylate of the ligand. It should be noted that, however, as receptor subtypes they have relatively low sequence

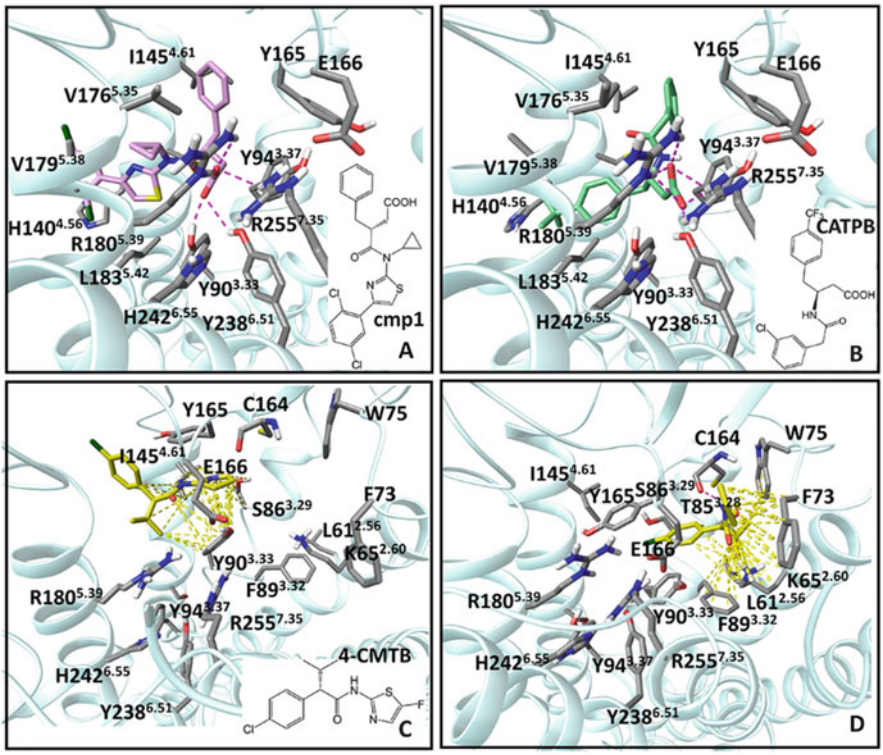

Fig. 4 Ligand binding at FFA2. (**a**) The agonist binding mode using the example of cmp 1 (Hudson et al. 2013). (**b**) Antagonists binding mode using the example of CATPB. (**c**–**d**) The binding mode of 4-CMTB in the orthosteric and allosteric sites. Hydrogen bonds and hydrophobic interactions are in *pink* and *yellow*, respectively

identity in comparison with the adrenergic, muscarinic and opioid receptor subtypes, which have over 60% sequence identity. This could suggest more structural divergence in the ligand-binding site between FFA1 and FFA2. Indeed, our recent modelling study suggests that the FFA2 binding cavity is notably smaller than in FFA1 as a result of a more intensive hydrogen bonding and aromatic network, created by nonconserved residues (Tikhonova and Poerio 2015). In terms of physicochemical properties, the FFA2 binding site is more aromatic compared to FFA1 and FFA3.

Redocking of selective carboxylic acids to the new FFA2 model and comparison with the FFA1-based FFA3 model suggest that $Y90^{3.33}$, $I145^{4.61}$ and $E166^{EL2}$ in FFA2 ($F96^{3.33}$, $Y151^{4.61}$ and $L171^{EL2}$ in FFA3) are likely responsible for ligand selectivity at FFA2/FFA3 (Tikhonova and Poerio 2015).

We compare docking of cmp 1, an agonist, and CATPB, an antagonist, in the new FFA1-based homology model in Fig. 4. Similar to FFA1, the carboxylate of the agonist can form interactions with two arginines and two tyrosines (Fig. 4). In contrast, docking of CATPB predicts that the carboxylate could form interactions

with only the arginines. More importantly, a recent mutagenesis study suggests that CATPB preferably binds to R255$^{7.35}$, whereas GLPG0974, another antagonist, binds to R180$^{5.39}$ (Sergeev et al. 2016). It appears that the carboxylate of the agonists and antagonists differently coordinate the arginine network of interactions. While agonists are able to be in hydrogen bonding with two arginines and two tyrosines, antagonists are unable to form interactions with all the residues. Like in FFA1, I suggest that modulation of the arginine pairing is critical in triggering receptor activation. The arginine pairing in FFA2 is also stabilised by countercharged E166$_{EL2}$ similar to FFA1.

A different hydrogen bonding network of the carboxylate in agonists and antagonists is likely due to variation in the hydrophobic and aromatic moieties. Docking predicts the position of the phenylthiazole moiety of cmp1 pointing towards helices 4 and 5, and the remaining group is directed towards helices 3 and 4 (Fig. 4a). Two aromatic moieties of CATPB are pointed in similar directions (Fig. 4b). Unlike FFA1, it appears that the interhelical space of helices 3 and 4 is less accessible. Although it seems that the conformationally restricted phenylthiazole moiety by an *ortho* substituent in the phenyl ring stabilises the position of the carboxylate in the agonist to be able to form hydrogen bonding with four polar residues, the precise position of this moiety needs to be further validated by mutagenesis.

Finally, the FFA1-based homology model of FFA2 was helpful in understanding a two-step activation process of 4-CMTB (also known as AMG7703) (Grundmann et al. 2016) at a molecular level. Innovative label-free biosensors and functional assays have shown that 4-CMTB briefly activates the receptor through the orthosteric site and subsequently induces prolonged activation through the allosteric site. We used the new FFA2 homology model to identify interactions of 4-CMTB with the orthosteric and allosteric sites and to characterise a precise scenario of dynamic binding that highlighted key interactions with the receptor, using steered MD simulations. In particular, the new model has helped to establish four residues S86$^{3.29}$, Y90$^{3.33}$, I145$^{4.61}$ and E166^{EL2} in the orthosteric site and K65$^{2.60}$ in the allosteric site (Fig. 4c, d). Similar to FFA1, K65$^{2.60}$ is within the binding cavity and not outside as it was predicted in earlier models based on the β_2-adrenergic receptor. Simulations of 4-CMTB binding and unbinding suggested the ligand enters the receptor through the extracellular tips of helices 4 and 5; moves to the orthosteric site composed of S68$^{3.29}$, Y90$^{3.33}$, I145$^{4.61}$, H242$^{6.55}$ and R180$^{5.39}$; and, subsequently, with the help of the inter-site translator residues Y165^{EL2} and Y90$^{3.33}$ occupies the allosteric site by interacting with K65$^{2.60}$. The obtained allosteric binding pose of 4-CMTB is consistent with the SAR studies (Smith et al. 2011; Wang et al. 2010). In the binding site, the carbonyl group of the amide group of 4-CMTB forms hydrogen bonding with K65$^{2.60}$, while the hydrogen of the amide group forms an hydrogen bond with the backbone of C164^{EL2}. The importance of the amide group to act as a hydrogen bond acceptor and donor in binding of 4-CMTB to the receptor is confirmed by the absence of activity for analogues containing amino or methylamide groups (Smith et al. 2011; Wang et al. 2010). The isopropyl moiety of 4-CMTB sticks to helices 2 and 3 forming hydrophobic interactions with L61$^{2.56}$, W75^{EL1}, T85$^{3.28}$, F89$^{3.32}$ and the hydrophobic tail of K65$^{2.60}$. The small hydrophobic pocket does not allow growing the

isopropyl group. Indeed, replacement of the isopropyl group for bulkier groups led to reduction of potency (Smith et al. 2011). The polar thiazole ring of 4-CMTB is pointed to the extracellular side and in π-π stacking with $F73^{EL1}$. The contribution of the aromatic interaction to the potency of the ligand is demonstrated by the absence of activity for analogues with acetylaminoethyl and cyclooctyl groups (Smith et al. 2011). The remaining phenyl group of 4-CMTB is pointed towards the region covered by EL2 and is in hydrophobic and aromatic interactions with $Y165^{EL2}$ and $Y90^{3.33}$.

Overall, the homology model built based on a template with sequence identity of 32% together with mutagenesis data enabled the characterisation of binding of orthosteric agonists and antagonists as well as a first sequential activator. Unequal binding to two arginines suggests that planar pairing of arginines is differently stabilised in the presence of agonists and antagonists and could be crucial for receptor activation.

4 Free Fatty Acid Receptor 3

FFA3 has higher sequence identity in the transmembrane domains with FFA2 (49%) than with FFA1 (33%). The first model of FFA3 was constructed based on the β_2-adrenergic receptor and used for an understanding of a preference in binding of short carboxylic acids with sp3 over sp2 hybridised alpha-carbons (Hudson et al. 2012a, b).

The recently available FFA1 crystal structure provides a better quality template for FFA3 modelling. Like in FFA1 and FFA2, this receptor has two conserved arginines and two tyrosines for coordination of the carboxyl group, suggesting a similar network of interactions, involving the arginine pairing (Fig. 5). Interestingly, FFA3 has $L171^{EL2}$ instead of glutamate of FFA1 and FFA2, indicating that this residue cannot be around the arginines to stabilise the planar pairing. It is likely that the conformation of EL2 in FFA3 should be notably distinct from FFA1 and FFA2, bringing available hydrophilic or negatively charged residues of EL2 close to the arginine pairing to balance this interaction. Indeed, there is low sequence homology in EL2 with FFA1. In the binding site of FFA3, there is a third arginine at position 2.60, contrasting with a lysine residue at this position in FFA1 and FFA2. The role of this residue in receptor function is unclear; however it likely contributes to ligand recognition, similar to a lysine in FFA2, and is clustered with other two arginines in planar pairing, due to close spatial proximity, thus potentially modulating receptor activation.

The medicinal chemistry of FFA3 is the least developed of the two subtypes, with only a series of allosteric modulators with modest activity available to date. Pharmacological studies have shown that mutation of $R185^{5.39}$ and $R258^{7.35}$ to alanine does not change the binding of these compounds (Hudson et al. 2014a, b). To further assess the recognition of these aromatic compounds by FFA3 residues, $F96^{3.33}$, $Y100^{3.37}$, $Y151^{4.61}$, $Y170^{EL2}$ and $F173^{EL2}$ forming a putative binding site could be mutated.

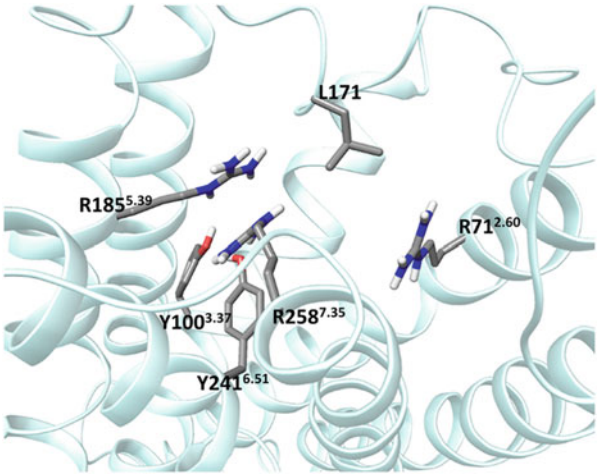

Fig. 5 The extracellular binding cavity of FFA3. The residues anchoring the carboxyl group of free fatty acids, L171, of the second extracellular loop and, R71 $^{2.60}$, the third positively charged residue in the extracellular binding cavity are visualised

With availability of a reasonable homology model of FFA3 progress in the development of ligands could be accelerated in the years to come. Indeed, the structural data allows a move from traditional high-throughput screening methods to cheaper and efficient virtual screening approaches for the identification of novel ligands. In the recent study, Huang and colleagues have used homology models of the poor-characterised receptors, GPR68 and GPR65, for virtual screening and identified potent ligands (Huang et al. 2015). A similar approach could be applied for FFA3 to find potent modulators for future pharmacological and modelling studies of the receptor.

5 Free Fatty Acid Receptor 4 (GPR120)

Although GPR120 binds long- and medium-chain fatty acids like FFA1 as well as some synthetic FFA1 agonists, the anchoring site for the carboxylate and the overall location of the binding site are distinct. While two arginines and two tyrosines of FFA1 are involved in coordinating one of the oxygen atoms of the carboxylate, these residues are absent in GPR120. Instead, R99$^{2.64}$ chelates the carboxylic group of the ligand by forming two hydrogen bonds as predicted initially by the rhodopsin-based homology model of GPR120 (Negoro et al. 2010; Takeuchi et al. 2013). The binding site is located in the centre of a helical bundle involving helices 2, 3, 5, 6 and 7 (Hudson et al. 2014a, b) (Fig. 6a). This is different from FFA1, where the TAK-875 binding site is on the side of the extracellular cavity within the helical bundle and is created by helices 3, 4, 5 and 6 (Fig. 3e).

Recently, docking of several agonists to the GPR120 homology model built based on the β_2-adrenergic receptor active state (24% sequence identity) and supported by residue mutagenesis has been reported by Hudson and colleagues (Hudson et al. 2014a, b). The authors suggested that besides R99$^{2.64}$, W104^{EL1} is in

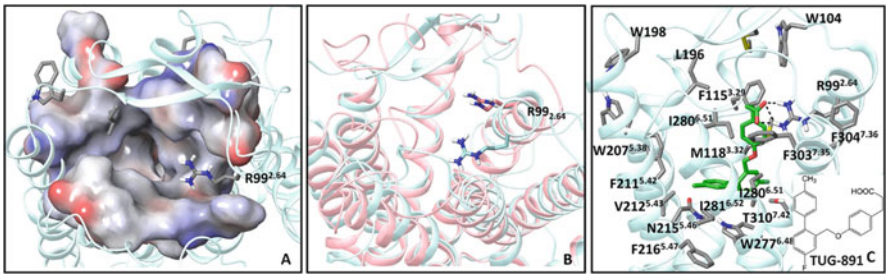

Fig. 6 The binding site of FFA4. (**a**) The surface-based representation of the FFA4 extracellular cavity. (**b**) The superimposition of the β_2-adrenergic and OX2-based homology models based on the backbone of the helices. The models are in *pink* and *cyan* ribbons, respectively. (**c**) The binding mode of TUG891. $R99^{2.64}$ coordinates the carboxylate of TUG891. The surface is coloured based on the electrostatic potential

hydrogen bonding with the caboxylate, while hydrophobic residues $F88^{2.53}$, $F115^{3.29}$, $W207^{5.38}$, $F211^{5.42}$, $I280^{6.51}$, $W277^{6.48}$, $F303^{7.35}$ and $F304^{7.36}$ create a favourable environment for the hydrophobic tail of linoleate, TUG891, TUG-670 and GW9508 (Hudson et al. 2014a, b). This study predicts that $V212^{5.43}$ and $I281^{6.52}$ form a hydrophobic pocket for the methyl group of TUG-891 (Hudson et al. 2014a, b). Unlike other ligands, GW9508 interacts with $F311^{7.43}$ and not $F88^{2.53}$, suggesting difference in the binding pose. The importance of hydrophilic residues $T119^{3.33}$, $T310^{7.42}$ and $N215^{5.46}$ in ligand binding is also demonstrated.

Recent advances in structural biology of GPCRs have provided alternative templates for homology modelling of GPR120. Among currently available templates, the orexin receptors (OX1 and OX2) have recently published crystal structures (Yin et al. 2015, 2016) and the highest sequence conservation, 30% in the transmembrane region. Moreover, EL2 of the OX2 receptor has a similar length and some similarity (16%), especially in the region after the disulphide bridge, making OX crystal structures more suitable templates than other available GPCR crystal structures. We have built the improved GPR120 model using these templates to evaluate the ligand-binding site. Unlike the β_2-adrenergic-based model of GPR120 with only the side chain of $R99^{2.64}$ clearly pointing to the centre of the extracellular cavity, both the backbone and side chain of $R99^{2.64}$ are orientated towards the centre of the helical bundle (Fig. 6b). This somewhat affects ligand docking. Figure 6c shows the docked pose of TUG891, in which the carboxyl group only interacts with $R99^{2.64}$. Other contacts are relatively similar to the β_2-adrenergic-based model of GPR120 apart from $W207^{5.38}$, $F303^{7.35}$ and $F304^{7.36}$, which are likely having a nondirect effect. The biphenyl moiety of TUG891 is predicted to be placed perpendicular to the helix and firmly locked in the pocket between helices 2–3 and 4–5. This pose is consistent with SAR data and provides steric reasons why large substitutions in the *para* position of the terminal ring are unfavourable as well as explaining the preference of *ortho* position of the terminal ring over *meta* or *para* positions. Comparison of the binding cavities of GPR120 with FFA1 shows that the GPR120 cavity is large and likely more exposed to the extracellular side,

contrasting with a narrow cavity of FFA1 that is covered by EL2 and extends to the interhelical space between helices 3 and 4 (Figs. 3 and 6). In GPR120 the ligand needs to adopt a 'V' shape and should be relatively short but bulky, while in FFA1 the ligand is longer but needs to be narrower to squeeze between helices to adopt a 'U' shape. The new model predicts the side chain of $L196^{EL2}$ and $W198^{EL2}$ facing the binding cavity. $W198^{EL2}$ is conserved with the OX receptors, suggesting a similar position in GPR120. In the GPR120 model, $W198^{EL2}$ is in π-π stacking with $W207^{5.38}$, a critical residue for ligand binding (Hudson et al. 2014a, b).

The known microswitches of activation and inactivation for the rhodopsin family of GPCRs are conserved in GPR120, contrasting to other free fatty acid receptors. Thus, W6.48 of the conserved 'CWxP' motif, the so-called toggle switch of GPCR activation, is conserved in GPR120 and predicted to be in close proximity to the ligand. In addition, GPR120 has conserved residues at the D[E]RY motif and aspartate at position 6.30, thus forming a salt bridge, known as an intracellular ionic lock that holds an inactive state of the receptor. The tyrosine of 'NPxxY' motif of GPR120 is predicted to interact with the tyrosine at position 7.53 in the active state, similar to rhodopsin and the β_2-adrenergic receptor. Therefore, GPR120 likely has a typical mechanism of activation observed in many GPCRs of the rhodopsin family.

The validated GPR120 binding site, together with the improved homology model, could be further used for establishing the binding properties of a recently identified antagonist (Sparks et al. 2014) and biased agonist (Li et al. 2015). For instance, by introducing modifications in the cholecystokinin 2 (CCK2) receptor as well as in its biased antagonist suggested by modelling, Magnan and colleagues (Magnan et al. 2013) were able to identify the key moiety in the ligand responsible for biased signalling and a microswitch of activation, involving M3.32 and Y7.43 that stabilises the β-arrestin active state of the CCK2 receptor. Such a bidirectional study could be explored in GPR120.

6 GPR84

The first attempt to delineate the binding site of GPR84 has been made recently by Nikaido and colleagues (Nikaido et al. 2015). They used a GPR84 homology model based on a β_2-adrenergic template with 20% sequence conservation to predict and then validate by mutagenesis the putative residues interacting with decanoic acid, a native agonist, and diindolylmethane, a synthetic agonist. They found that mutations of $L100^{3.32}$, $F101^{3.33}$ and $N104^{3.36}$ dramatic altered potency of decanoate. However, these residues are not important for binding of diindolylmethane. The authors suggested that $N104^{3.36}$ plays an anchoring role for the carboxylate of the ligand at GPR84.

To further explore the properties of this receptor, we modelled its structure using the recently published crystal structure of the OX1 receptor (Yin et al. 2016), the template with highest sequence identity, 31%. Importantly, the sequence analysis of EL2 among the receptors with available crystal structures shows that EL2 of GPR84 has 35% identity and a similar length with rhodopsin (Fig. 7a). This is

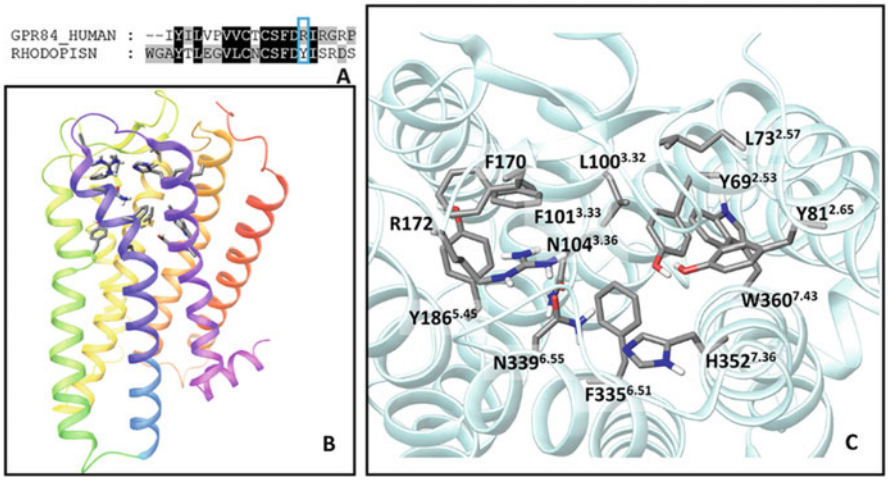

Fig. 7 A GPR84 homology model. (**a**) The sequence alignment of the second extracellular loop 2 (EL2) for GPR84 and rhodopsin. The putative anchoring $R172^{EL2}$ and the corresponding residue in rhodopsin are highlighted. (**b**) A homology model based on a hybrid template involving the OX1 receptor and EL2 of rhodopsin. (**c**) The putative ligand-binding cavity of GPR84. Only potentially interesting residues for ligand binding are visualised

interesting, as generally EL2 is quite divergent, even within the same receptor family. From such relatively high similarity, we could suggest that EL2 of GPR84 has a similar β-sheet conformation and covers the cavity within the helical bundle like in rhodopsin. We modelled the GPR84 structure using a hybrid template, where EL2 of rhodopsin was attached to the OX1 crystal structure. The obtained model was subjected to a short optimisation using molecular mechanics and dynamics tools of MacroModel (Schrodinger, LLC, New York, NY, USA 2014a). The resulting model is shown in Fig. 7b, c. The model predicts that R172 of EL2 is pointing into the binding cavity within the helical bundle and might play a coordinating role for the carboxylate of the ligand. This residue could not have been predicted with the β_2-adrenergic receptor template used by Nikaido and colleagues (Nikaido et al. 2015) due to the absence of any similarity in EL2. In addition, there are several residues with hydrogen bonding capability such as $Y81^{2.65}$, $Y69^{2.53}$, $N104^{3.36}$, $Y186^{5.45}$, $N339^{6.55}$ and $H352^{7.36}$ in the putative ligand-binding site. Interestingly, asparagine in position 6.55 is conserved with the OX1 receptor in which it forms hydrogen bonding with the ligand. Overall, the putative binding site is located at the centre of the binding cavity within the helical bundle and is created by aromatic residues, $F170^{EL2}$, $F101^{3.33}$, $F335^{6.51}$ and $W360^{7.43}$, and aliphatic residues, $L73^{2.57}$ and $L100^{3.32}$. Unlike FFA1–4, GPR84 has a few aliphatic residues in the binding cavity.

From sequence analysis and homology modelling, it is evident that the ligand-binding site of GPR84 is distinct from FFA1–4. GPR84 does not contain positively charged residues at positions 5.39 and 7.35 like in FFA1–3 or at position 2.64 like in

FFA4. Moreover no charged residues are found in the transmembrane bundle facing the putative binding cavity. The improved homology model based on the hybrid template predicts that EL2 could play an important role in anchoring a free fatty acid at GPR84 by means of a positively charged arginine. Given that phylogenetically GPR84 belongs to the prostanoid receptor subfamily, for which the positively charged residue is predicted to coordinate the negatively charged ligands, as exemplified by the prostacyclin receptor (Stitham et al. 2003), it is suggested that GPR84 also could attract the anionic part of the ligand via a countercharged residue.

7 Summary and Future Directions

The recent progress in GPCR crystallography has enabled the elucidation of the structure of FFA1 and provided reliable templates (>30% sequence conservation) for homology modelling of other free fatty acid receptors. The atomic models of the receptors could now be used for an understanding of ligand recognition and, subsequently, in structure-based ligand design. In particular, it will be important to explore the binding modes of ligands with different chemotypes as well as a different pharmacological profile (orthosteric agonists and antagonists, allosteric modulators and biased ligands) for FFA1, FFA2 and GPR120 using molecular docking in combination with mutagenesis and ligand SARs. This knowledge should pave the way for the discovery of small-molecule drugs with improved properties with a high chance of success.

For FFA3 and GPR84 receptors with a limited number of ligands available to date, it will be interesting to probe homology models for virtual screening with a hope to identify new binders and therefore to increase the arsenal of ligands to study receptor pharmacology and physiology. Structure-based virtual screening of chemical libraries using an X-ray structure or even a homology model is now an accepted method of discovering new chemotypes in GPCRs (Ngo et al. 2016). Indeed, prior to FFA1 crystallisation, the virtual screening based on the FFA1 homology model led to the identification of 15 compounds acting as agonists, partial agonists and antagonists (Tikhonova et al. 2008).

FFA1–3 represent the first GPCRs with an unusual arginine pairing interaction. It appears that the regulation of this interaction is crucial for defining the pharmacological property of ligands. Further studies are required to explore charge pairing of arginines in receptor modulation.

References

Ballesteros JA, Weinstein H (1995) Modeling transmembrane helix contacts in GPCR. Biophys J 68:A446

Beuming T, Lenselink B, Pala D, McRobb F, Repasky M, Sherman W (2015) Docking and virtual screening strategies for GPCR drug discovery. Methods Mol Biol 1335:251–276. doi:10.1007/978-1-4939-2914-6_17

Bindels LB, Dewulf EM, Delzenne NM (2013) GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci 34:226–232. doi:10.1016/j.tips.2013.02.002

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 148:619–628

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319. doi:10.1074/jbc.M211609200

Christiansen E, Hudson BD, Hansen AH, Milligan G, Ulven T (2016) Development and characterization of a potent free fatty acid receptor 1 (FFA1) fluorescent tracer. J Med Chem. doi:10.1021/acs.jmedchem.6b00202

Costanzi S (2012) Homology modeling of class a G protein-coupled receptors. Methods Mol Biol 857:259–279. doi:10.1007/978-1-61779-588-6_11

Costanzi S (2013) Modeling G protein-coupled receptors and their interactions with ligands. Curr Opin Struct Biol 23:185–190. doi:10.1016/j.sbi.2013.01.008

Dranse HJ, Kelly ME, Hudson BD (2013) Drugs or diet?--Developing novel therapeutic strategies targeting the free fatty acid family of GPCRs. Br J Pharmacol 170:696–711. doi:10.1111/bph.12327

Grundmann M, Tikhonova IG, Hudson BD, Smith NJ, Mohr K, Ulven T, Milligan G, Kenakin T, Kostenis E (2016) A molecular mechanism for sequential activation of a G protein-coupled receptor. Cell Chem Biol 23:392–403. doi:10.1016/j.chembiol.2016.02.014

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94

Holliday ND, Watson SJ, Brown AJ (2012) Drug discovery opportunities and challenges at g protein coupled receptors for long chain free fatty acids. Front Endocrinol (Lausanne) 2:112. doi:10.3389/fendo.2011.00112

Huang XP, Karpiak J, Kroeze WK, Zhu H, Chen X, Moy SS, Saddoris KA, Nikolova VD, Farrell MS, Wang S, Mangano TJ, Deshpande DA, Jiang A, Penn RB, Jin J, Koller BH, Kenakin T, Shoichet BK, Roth BL (2015) Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65. Nature 527:477–483. doi:10.1038/nature15699

Hudson BD, Christiansen E, Tikhonova IG, Grundmann M, Kostenis E, Adams DR, Ulven T, Milligan G (2012) Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. FASEB J 26:4951–4965. doi:10.1096/fj.12-213314

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G (2012) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. J Biol Chem 287:41195–41209. doi:10.1074/jbc.M112.396259

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, Tikhonova IG, Ulven T, Milligan G (2013) Defining the molecular

basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288:17296–17312. doi:10.1074/jbc.M113.455337

Hudson BD, Christiansen E, Murdoch H, Jenkins L, Hojgaard Hansen A, Madsen OB, Ulven T, Milligan G (2014) Complex pharmacology of novel allosteric free fatty acid 3 receptor ligands. Mol Pharmacol 86:200–210

Hudson BD, Shimpukade B, Milligan G, Ulven T (2014) The molecular basis of ligand interaction at free fatty acid receptor 4 (FFA4/GPR120). J Biol Chem 289:20345–20358

Itoh Y, Hinuma S (2005) GPR40, a free fatty acid receptor on pancreatic beta cells, regulates insulin secretion. Hepatol Res 33:171–173. pii: S1386-6346(05)00315-3

Kufareva I, Rueda M, Katritch V, Stevens RC, Abagyan R, GPCR Dock 2010 participants (2011) Status of GPCR modeling and docking as reflected by community-wide GPCR Dock 2010 assessment. Structure 19:1108–1126. doi:10.1016/j.str.2011.05.012

Kufareva I, Katritch V, Participants of GPCR Dock 2013, Stevens RC, Abagyan R (2014) Advances in GPCR modeling evaluated by the GPCR Dock 2013 assessment: meeting new challenges. Structure 22:1120–1139. doi:10.1016/j.str.2014.06.012

Levit A, Barak D, Behrens M, Meyerhof W, Niv MY (2012) Homology model-assisted elucidation of binding sites in GPCRs. Methods Mol Biol 914:179–205. doi:10.1007/978-1-62703-023-6_11

Li A, Yang D, Zhu M, Tsai KC, Xiao KH, Yu X, Sun J, Du L (2015) Discovery of novel FFA4 (GPR120) receptor agonists with beta-arrestin2-biased characteristics. Future Med Chem 7:2429–2437. doi:10.4155/fmc.15.160

Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J, Vimolratana M, Jiao XY, Wang Y, Birdsall NJ, Swaminath G (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859. doi:10.1124/mol.112.079640

Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, Vimolratana M, Houze JB, Wong S, Toteva M, Shan B, Li F, Zhuang R, Lin DC (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7:e46300. doi:10.1371/journal.pone.0046300

Magalhaes A, Maigret B, Hoflack J, Gomes JN, Scheraga HA (1994) Contribution of unusual arginine-arginine short-range interactions to stabilization and recognition in proteins. J Protein Chem 13:195–215

Magnan R, Escrieut C, Gigoux V, DE K, Clerc P, Niu F, Azema J, Masri B, Cordomi A, Baltas M, Tikhonova IG, Fourmy D (2013) Distinct CCK-2 receptor conformations associated with beta-arrestin-2 recruitment or phospholipase-C activation revealed by a biased antagonist. J Am Chem Soc 135:2560–2573. doi:10.1021/ja308784w

Mason JS, Bortolato A, Congreve M, Marshall FH (2012) New insights from structural biology into the druggability of G protein-coupled receptors. Trends Pharmacol Sci 33:249–260. doi:10.1016/j.tips.2012.02.005

Michino M, Abola E, GPCR Dock 2008 participants, Brooks CL, 3rd, Dixon JS, Moult J, Stevens RC (2009) Community-wide assessment of GPCR structure modelling and ligand docking: GPCR Dock 2008. Nat Rev Drug Discov 8:455–463. doi:10.1038/nrd2877

Milligan G, Ulven T, Murdoch H, Hudson BD (2014) G-protein-coupled receptors for free fatty acids: nutritional and therapeutic targets. Br J Nutr 111:S3–S7. pii: S0007114513002249

Negoro N, Sasaki S, Mikami S, Ito M, Suzuki M, Tsujihata Y, Ito R, Harada A, Takeuchi K, Suzuki N, Miyazaki J, Santou T, Odani T, Kanzaki N, Funami M, Tanaka T, Kogame A, Matsunaga S, Yasuma T, Momose Y (2010) Discovery of TAK-875: a potent, selective, and orally bioavailable GPR40 agonist. ACS Med Chem Lett 1:290–294. doi:10.1021/ml1000855

Neves MA, Yeager M, Abagyan R (2012) Unusual arginine formations in protein function and assembly: rings, strings, and stacks. J Phys Chem B 116:7006–7013. doi:10.1021/jp3009699

Ngo T, Kufareva I, Coleman JL, Graham RM, Abagyan R, Smith NJ (2016) Identifying ligands at orphan GPCRs: current status using structure-based approaches. Br J Pharmacol. doi:10.1111/bph.13452

Nikaido Y, Koyama Y, Yoshikawa Y, Furuya T, Takeda S (2015) Mutation analysis and molecular modeling for the investigation of ligand-binding modes of GPR84. J Biochem 157:311–320. doi:10.1093/jb/mvu075

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. Nature 477:549–555. doi:10.1038/nature10361

Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, Ward RJ, Drewke C, Milligan G, Kostenis E, Ulven T (2011) Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. J Biol Chem 286:10628–10640. doi:10.1074/jbc.M110.210872

Schrodinger, LLC, New York, NY, USA (2014a) MacroModel 10.6

Schrodinger, LLC, New York, NY, USA (2014b) SiteMap 3.3

Sergeev E, Hansen AH, Pandey SK, MacKenzie AE, Hudson BD, Ulven T, Milligan G (2016) Non-equivalence of key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. J Biol Chem 291:303–317. doi:10.1074/jbc.M115.687939

Smith NJ, Ward RJ, Stoddart LA, Hudson BD, Kostenis E, Ulven T, Morris JC, Trankle C, Tikhonova IG, Adams DR, Milligan G (2011) Extracellular loop 2 of the free fatty acid receptor 2 mediates allosterism of a phenylacetamide ago-allosteric modulator. Mol Pharmacol 80:163–173. doi:10.1124/mol.110.070789

Sparks SM, Chen G, Collins JL, Danger D, Dock ST, Jayawickreme C, Jenkinson S, Laudeman C, Leesnitzer MA, Liang X, Maloney P, McCoy DC, Moncol D, Rash V, Rimele T, Vulimiri P, Way JM, Ross S (2014) Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). Bioorg Med Chem Lett 24:3100–3103. doi:10.1016/j.bmcl.2014.05.012

Srivastava A, Yano J, Hirozane Y, Kefala G, Gruswitz F, Snell G, Lane W, Ivetac A, Aertgeerts K, Nguyen J, Jennings A, Okada K (2014) High-resolution structure of the human GPR40 receptor bound to allosteric agonist TAK-875. Nature 513:124–127. doi:10.1038/nature13494

Stitham J, Stojanovic A, Merenick BL, O'Hara KA, Hwa J (2003) The unique ligand-binding pocket for the human prostacyclin receptor. Site-directed mutagenesis and molecular modeling. J Biol Chem 278:4250–4257. doi:10.1074/jbc.M207420200

Sum CS, Tikhonova IG, Costanzi S, Gershengorn MC (2009) Two arginine-glutamate ionic locks near the extracellular surface of FFAR1 gate receptor activation. J Biol Chem 284:3529–3536. doi:10.1074/jbc.M806987200ER

Takeuchi M, Hirasawa A, Hara T, Kimura I, Hirano T, Suzuki T, Miyata N, Awaji T, Ishiguro M, Tsujimoto G (2013) FFA1-selective agonistic activity based on docking simulation using FFA1 and GPR120 homology models. Br J Pharmacol 168:1570–1583. doi:10.1111/j.1476-5381.2012.02052.x

Tikhonova IG, Poerio E (2015) Free fatty acid receptors: structural models and elucidation of ligand binding interactions. BMC Struct Biol 15:16-015-0044-2. doi:10.1186/s12900-015-0044-2

Tikhonova IG, Sum CS, Neumann S, Thomas CJ, Raaka BM, Costanzi S, Gershengorn MC (2007) Bidirectional, iterative approach to the structural delineation of the functional "Chemoprint" in GPR40 for agonist recognition. J Med Chem 50:2981–2989. doi:10.1021/jm0614782ER

Tikhonova IG, Sum CS, Neumann S, Engel S, Raaka BM, Costanzi S, Gershengorn MC (2008) Discovery of novel agonists and antagonists of the free fatty acid receptor 1 (FFAR1) using virtual screening. J Med Chem 51:625–633. doi:10.1021/jm7012425ER

Ulven T (2012) Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. Front Endocrinol (Lausanne) 3:111. doi:10.3389/fendo.2012.00111

Wang J, Wu X, Simonavicius N, Tian H, Ling L (2006) Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem 281:34457–34464

Wang Y, Jiao X, Kayser F, Liu J, Wang Z, Wanska M, Greenberg J, Weiszmann J, Ge H, Tian H, Wong S, Schwandner R, Lee T, Li Y (2010) The first synthetic agonists of FFA2: Discovery and SAR of phenylacetamides as allosteric modulators. Bioorg Med Chem Lett 20:493–498. doi:10.1016/j.bmcl.2009.11.112

Weiss DR, Bortolato A, Tehan B, Mason JS (2016) GPCR-bench: a benchmarking set and practitioners' guide for G protein-coupled receptor docking. J Chem Inf Model. doi:10.1021/acs.jcim.5b00660

Yin J, Mobarec JC, Kolb P, Rosenbaum DM (2015) Crystal structure of the human OX2 orexin receptor bound to the insomnia drug suvorexant. Nature 519:247–250. doi:10.1038/nature14035

Yin J, Babaoglu K, Brautigam CA, Clark L, Shao Z, Scheuermann TH, Harrell CM, Gotter AL, Roecker AJ, Winrow CJ, Renger JJ, Coleman PJ, Rosenbaum DM (2016) Structure and ligand-binding mechanism of the human OX1 and OX2 orexin receptors. Nat Struct Mol Biol 23:293–299. doi:10.1038/nsmb.3183

Using Biosensors to Study Free Fatty Acid Receptor Pharmacology and Function

Brian D. Hudson

Contents

Abstract

The free fatty acid (FFA) family of G protein coupled receptors (GPCRs) has generated significant interest for exploiting its members as potential drug targets. However, unravelling the complex pharmacology of this family of receptors has proven challenging. In recent years the use of biosensor technologies capable of assessing biological functions in living cells, and in real time, has greatly enhanced our ability to study GPCR pharmacology and function. These include genetically encoded sensors that change the intensity or wavelength of light emitted from a bioluminescent or fluorescent protein in response to a stimulus, as well as non-genetically encoded sensors able to measure more global cellular changes, such as mass redistribution within a cell. This chapter will examine

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how these sensors can be used to study GPCRs, and in particular how they are helping uncover the pharmacology of the FFA family of receptors.

Keywords
Bioluminescence resonance energy transfer (BRET) • Biosensor • Fluorescence resonance energy transfer (FRET) • Free fatty acid • G protein coupled receptor (GPCR) • Label-free biosensor

1 Introduction

Fatty acids are biomolecules that mediate many important biological processes. Fatty acids are defined chemically as a carboxylic acid head group attached to an aliphatic tail, and thus are structurally diverse, based on the composition of their aliphatic tail. The fatty acid aliphatic tail varies by carbon chain length, where examples shorter than 6 carbon are deemed short chain fatty acids (SCFAs), those between 7 and 12 carbon are medium chain fatty acids (MCFAs) and those longer than 12 are long chain fatty acids (LCFAs). In addition to chain length, the MCFAs and LCFAs are also defined by the number, position and conformation of unsaturations in their aliphatic tail. Together, the fatty acids constitute a complex group of biomolecules associated with diverse biological effects.

Historically fatty acids were believed to produce their biological effects primarily as building blocks for the phospholipids, or as a source of energy. However, it has become clear that fatty acids are also signalling molecules. Of particular interest, a family of four G protein coupled receptors (GPCRs) is now recognized as the free fatty acid (FFA) receptor family. This currently includes four members: FFA1 (previously GPR40), FFA2 (previously GPR43), FFA3 (previously GPR41) and FFA4 (previously GPR120) (Davenport et al. 2013; Stoddart et al. 2008). Of these FFA1 and FFA4 respond to MCFAs and LCFAs, while FFA2 and FFA3 respond to the SCFAs. Fatty acid signalling through each receptor regulates key aspects of metabolism and/or inflammation and, therefore, it is not surprising that each FFA receptor has received interest as a possible target for metabolic and/or inflammatory disease. However, before the full therapeutic potential of these receptors can be realized there is a need to better understand the complex pharmacology of this family. In this chapter I will examine how biosensor technologies are being used to study GPCRs and, specifically, uncover the detailed pharmacology and function of the FFA family.

2 GPCR Pharmacology and Function

The GPCR superfamily is the single largest family of membrane proteins, comprised of more than 800 members (Fredriksson et al. 2003). GPCRs are classically defined by their conserved seven transmembrane structures, as well as

by their ability to interact with and transduce signals through guanine nucleotide binding G proteins. These features allow GPCRs to activate intracellular signalling pathways in response to diverse extracellular stimuli, which has made these receptors the most historically successful drug targets. In recent times, however, it has become apparent that the signal transduction, pharmacology and function of GPCRs are far more complex than previously believed and, indeed, that there is a clear need to develop new approaches to study the complex actions of these receptors.

2.1 GPCR Signal Transduction

The classic GPCR signal transaction pathway involves in order: (1) agonist ligand binding to the GPCR; (2) the GPCR adopting an active conformation; (3) the active GPCR interacting with a heterotrimeric ($\alpha\beta\gamma$) G protein; (4) exchange of GTP for GDP on the G protein α subunit causing activation and dissociation of the α and $\beta\gamma$ subunits and (5) the Gα and G$\beta\gamma$ subunits regulating downstream effectors. The specific signalling pathways activated are largely defined by the particular Gα involved where: $G\alpha_s$ activates adenylyl cyclase to increase cAMP; $G\alpha_{i/o}$ inhibits adenylyl cyclase to decrease cAMP; $G\alpha_{q/11}$ activates phospholipase C leading ultimately to an increase in intracellular Ca^{2+} concentration and $G\alpha_{12/13}$, which typically activates a small GTPase RhoA-mediated signalling pathway. Traditionally, a given receptor was associated with one specific Gα, thus, for example, the β_2-adrenoceptor was commonly associated with $G\alpha_s$, therefore activation of this receptor would be primarily viewed to result in an increase in intracellular cAMP.

In addition to the classic GPCR activation pathway, there is also a well-established pathway for the inactivation of GPCR signalling. This involves: (1) phosphorylation of serine/threonine residues in the third intracellular loop or carboxy-terminal of the GPCR by a G protein receptor kinase (GRK); (2) recruitment of an arrestin to the phosphorylated receptor; (3) internalization of the arrestin-bound receptor via clathrin-mediated endocytosis and (4) dissociation of the arrestin and dephosphorylation of the receptor allowing for the receptor to either recycle back to the cell surface or be degraded.

2.2 Expanding Complexity in GPCR Signalling

Although these GPCR activation and deactivation pathways are well established, in recent times there has been an appreciation that GPCR signalling is far more complex. Indeed, it is now clear that individual GPCRs may couple to multiple G proteins and that the specific G protein activated by a receptor may depend on the cellular context, other GPCRs or adapter proteins present, or by changes that occur to the receptor during the kinetics of activation (Baillie et al. 2003; Rashid et al. 2007; Steen et al. 2014). Further, while arrestins were traditionally viewed as adaptor molecules responsible primarily for turning off GPCR signalling, it is

now recognized that arrestins are signalling molecule in their own right. Indeed, GPCRs are capable of arrestin-dependent, but G protein-independent, signalling (Shukla et al. 2011). Further, it is apparent that different ligands for the same receptor are able to generate unique responses, either through differences in their binding/activation kinetics, or by stabilizing distinct receptor active conformations, a concept often now referred to as 'biased agonism' (Steen et al. 2014). Many GPCRs are also recognized to possess allosteric binding sites, distinct from the orthosteric site of the receptor's endogenous ligand, and ligands binding to allosteric sites often generate distinct signalling responses (May et al. 2007). Adding a final level of complexity, there is substantial evidence that GPCRs form hetero-oligomers with other GPCRs, and that these interactions alter nearly every aspect of receptor function (Milligan 2013). To accommodate our increased understanding of GPCR complexity, there has been a need to develop new tools and technologies to study GPCR function. One area in particular that has evolved to meet this need is that of biosensor technologies capable of assessing nearly every aspect of GPCR function in living cells and in real time.

3 Biosensor Technologies to Study GPCRs

A biosensor is an analytical approach that uses a biological element in order to measure a specific function, protein conformation or analyte concentration. Most commonly in the field of GPCR research, biosensors take the form of genetically encoded sensor proteins that incorporate intrinsically fluorescent or bioluminescent proteins to measure specific functions of these receptors. However, there are also examples of non-genetically encoded sensors, most notably the "label-free" technologies, including those that assess changes in dynamic mass redistribution (DMR) or cellular impedance as global unbiased measures of cellular function. Biosensor technologies have now been developed to assess nearly every aspect of GPCR function and, indeed, these have been critical in defining the pharmacology of the FFA receptor family.

3.1 Genetically Encoded Biosensor Technologies

Genetically encoded biosensors typically incorporate fluorescent or bioluminescent proteins designed to alter either their intensity or spectral properties in response to the function to be measured. There are three common approaches that can be utilized to achieve this (Fig. 1), including: (1) intrinsically context-sensitive biosensors; (2) resonance energy transfer (RET) approaches and (3) protein fragment complementation approaches (Hattori and Ozawa 2014; Wehr and Rossner 2016). While the first of these techniques requires only a single fluorescent/bioluminescent tag, the other two require two separate tags that may be incorporated into either an intramolecular or intermolecular biosensor (Fig. 2). In either case, the

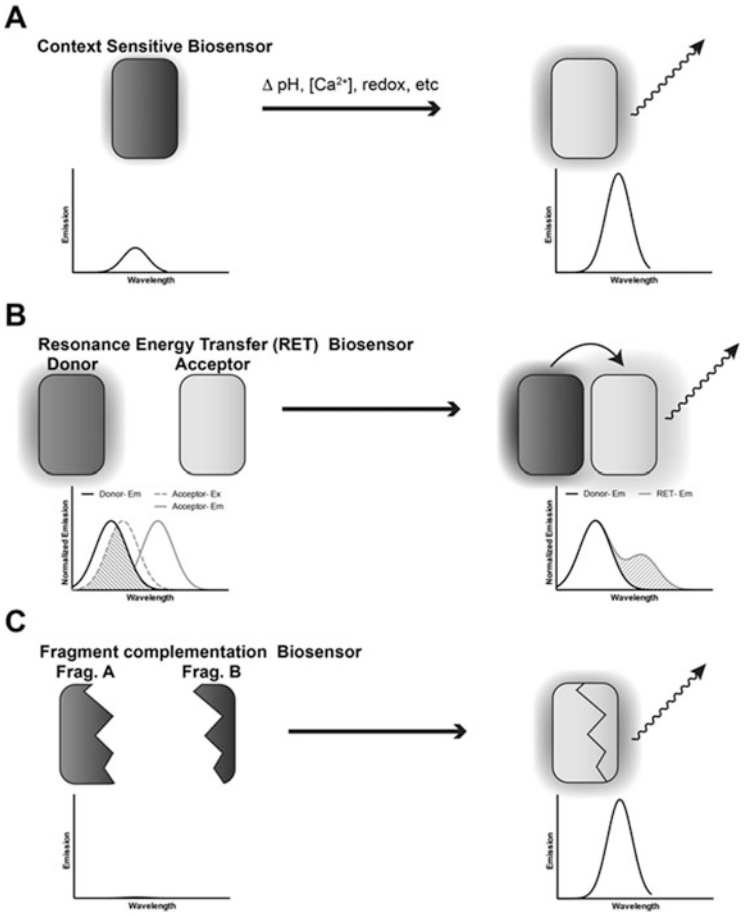

Fig. 1 Three common approaches utilizing genetically encoded bioluminescent and fluorescent biosensors. (**a**) In an intrinsically context-sensitive sensor the bioluminescent or fluorescent protein will alter its properties based on a change to the environment. This could result in either a change in intensity (shown) or the wavelength of emission. (**b**) Resonance energy transfer biosensors require a donor and an acceptor, where the emission spectrum of the donor overlaps the excitation spectrum of the acceptor (*left panel diagonal line fill*). When brought into close proximity, resonance energy transfer will occur, resulting in emission of the acceptor (*right panel diagonal line fill*). (**c**) Fragment complementation sensors split the bioluminescent or fluorescent protein into two non-functional halves, which become functional to emit fluorescence or bioluminescence when brought together

biosensor ultimately will measure changes in the distance between the two tags in order to assess various biological processes.

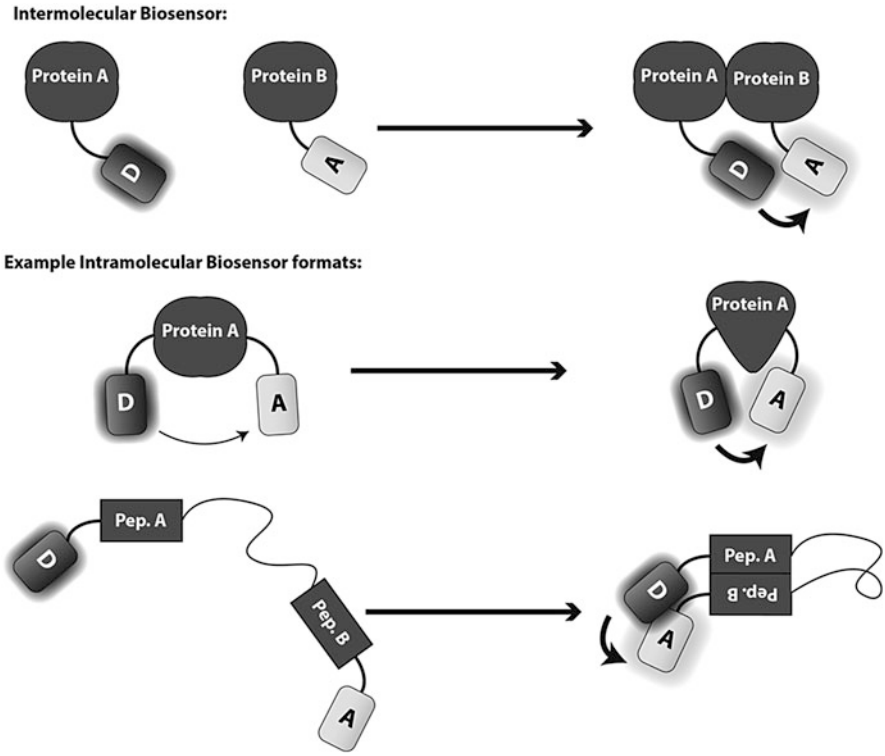

Fig. 2 Resonance energy transfer (RET) biosensor formats. General approaches to developing RET biosensors include intermolecular and intramolecular formats. In an intermolecular sensor, protein A is tagged with a donor (D), while protein B is tagged with the acceptor (A). If the two proteins come into close proximity RET will occur between donor and acceptor. There are several ways to generate intramolecular sensors, with two specific examples shown. In the first, protein A is tagged with both donor and acceptor in distinct locations, allowing for a basal RET. After the protein undergoes a conformational change, the relative distance of donor and acceptor decreases, resulting in increased RET. In the second example the sensor is constructed with the donor linked to peptide A, and the acceptor linked to peptide B. These are artificially separated with a linker, resulting in minimal basal RET. When a stimulus causes the two peptides to interact, it also brings the donor and acceptor into close proximity, increasing RET. Although this figure depicts RET biosensors, similar approaches are often suitable for fragment complementation sensors

3.1.1 Context-Sensitive Biosensors

It has long been known that both fluorescent proteins, such as green fluorescent protein (GFP) (Kneen et al. 1998), as well as bioluminescent luciferase proteins (Kitayama et al. 2003), are often sensitive to pH. Thus a number of context-sensitive biosensors have been developed utilizing GFP variants to assess changes in pH. For GPCRs, this may be of particular use since the luminal pH of secretory and endocytic compartments is acidic, and thus, pH sensors incorporated into the N-terminal of a GPCR may be used to assess cell surface delivery and internalization (Ashby et al. 2004). This has, for example, been useful in demonstrating the

dependence on receptor-activity-modifying proteins in delivering the calcium-sensing receptor to the cell surface (Bouschet et al. 2005). More recently, Robers et al. demonstrated that incorporating a pH sensitive NanoLuciferase (NLUC) (Hall et al. 2012), bioluminescent tag at the N-terminal of a GPCR allowed them to measure agonist induced internalization of several GPCRs (Robers et al. 2015). While these approaches are suitable to assess GPCR trafficking, they provide few advantages over tagging the receptor with a fluorescent protein at its intracellular C-terminal and using this to track subcellular location via either microscopy or high content imaging platforms (Zhang and Xie 2012). In addition to pH, context-sensitive biosensors have been described to measure the concentration of various other ions as well as redox state (Hochreiter et al. 2015). Most notably for GPCR studies, this includes sensors capable of assessing intracellular Ca^{2+} levels (Nakai et al. 2001), which may be useful as a readout of $G_{q/11}$ signalling.

3.1.2 RET-Based Biosensors

Fluorescent and bioluminescent RET biosensors have been by far the most widely employed technologies to study GPCR function. Fluorescent RET biosensors are based on the Förster resonance energy transfer (FRET) phenomenon dictating that when two fluorophores with overlapping emission and excitation spectra are in close proximity (<100 Å), excitation of the shorter wavelength donor will lead to a non-radiative transfer of energy to the acceptor, resulting in emission from the acceptor. The efficiency of energy transferred varies with the inverse of the distance between the donor and acceptor to the 6th power (Nakai et al. 2001), thus even very small changes in the distance between donor and acceptor, for example, those occurring within an intramolecular biosensor, will produce a measurable change in FRET. For bioluminescent RET sensors, bioluminescent resonance energy transfer (BRET) is employed, in which the fluorescent donor is replaced with a bioluminescent protein, but otherwise the same general principles apply (Pfleger and Eidne 2006). The scope for RET biosensors to study GPCRs expands to nearly every aspect of their function, with RET biosensors described to measure each of: ligand binding, receptor conformational changes, receptor/G protein interactions, G protein activation, intracellular levels of second messengers such as cAMP and Ca^{2+}, activation of downstream kinases, arrestin recruitment and receptor oligomerization, to name but a few (Lohse et al. 2012; Salahpour et al. 2012; Stoddart et al. 2015; van Unen et al. 2015). Considering this versatility, it is not surprising that RET-based biosensors have become an important tool in assessing the evolving complexities of GPCR pharmacology and function.

3.1.3 Fragment Complementation Biosensors

Protein fragment complementation-based sensors involve a fluorescent or bioluminescent protein split into two non-functional segments, able to recombine to form a functional protein only if brought into close proximity to each other. Therefore, complementation sensors function effectively as binary ‘on/off’ sensors and thus, are not well suited for measuring small changes in relative distance. This is

particularly true for the split fluorescent protein approach, bi-molecular fluorescent complementation (BiFC), where the process of combining the two non-functional halves is irreversible (Rose et al. 2010). While split luciferase sensors are reversible, and indeed have been developed to study many cellular processes and functions (Azad et al. 2014; Hattori and Ozawa 2014; Wehr and Rossner 2016); at least within the GPCR field, RET-based approaches remain far more widely used.

3.1.4 Fluorescent vs Bioluminescent-Based Technologies

The general principles are similar for fluorescent and bioluminescent-based biosensors, thus it is important to consider the advantages and disadvantages of each technology. Most notably, fluorescence yields a greater intensity of light emitted, and thus has historically been the preferred option for imaging-based applications (Boute et al. 2002; Lohse et al. 2012). Although BRET-based microscopy was first described some time ago (De and Gambhir 2005; Xu et al. 2007), the low amount of light emitted from *Renilla* luciferase (RLUC), the most commonly used luciferase in BRET biosensors, has greatly limited its utility. However, the development of brighter luciferases, for example, NLUC (Hall et al. 2012), is beginning to change this and NLUC/BRET imaging yielding subcellular resolution is now being described (Machleidt et al. 2015).

Although BRET has lagged behind FRET for imaging, it does have advantages when employed in plate-reader-based applications. The use of FRET biosensors is complicated by photobleaching, phototoxicity, autofluorescence and direct excitation of the FRET acceptor, none of which will affect BRET-based sensors (Boute et al. 2002). Many of the limitations of FRET can also be avoided if a long fluorescent lifetime donor is employed, allowing for time resolved (TR)-FRET. Although there are no known fluorescent proteins with suitable lifetimes for TR-FRET, the ability to incorporate long fluorescent lifetime lanthanide fluorophores into a protein of interest using reagents such as the SNAP, CLIP and HALO tags is now well established, and indeed has been used extensively in sensors assessing GPCR oligomerization and ligand binding (Emami-Nemini et al. 2013; Marsango et al. 2015; Maurel et al. 2008). Despite the possibilities for TR-FRET, and the recent advances in BRET imaging, FRET continues to be the primary imaging approach, while BRET remains more widely used in high throughput plate-based assays.

3.2 Non-genetically Encoded Biosensors

The advances in genetically encoded biosensor technologies have in many ways revolutionized the way we are able to assess the function of GPCRs in real time in living cells. However, these biosensors are not without limitation. Most generally, the need to express an exogenous biosensor in the cells under study. Non-genetically encoded biosensor technologies avoid this limitation, allowing for the assessment of GPCR function in unmodified cells. This is most commonly

achieved through the so-called label-free detection platforms and sensors utilizing antibodies.

3.2.1 Label-Free Biosensors

Many of the underlying concepts and technologies used in label-free biosensors originated in the 1990s and at the time were primarily focused on developing methods to characterize ligand-receptor interactions without the need for an exogenous label on either the ligand or the receptor. These technologies were either optically based: measuring, for example, DMR (Fang 2006; Fang et al. 2006); or electrically based: commonly measuring changes in cellular impedance (Cooper 2006). It quickly became clear that the signals these sensors generated were far more data rich than initially anticipated, and in a sense, that label-free biosensors provide an unbiased measure of the global changes occurring within a cell (Fang et al. 2006; Grundmann and Kostenis 2015; Scott and Peters 2010). This makes label-free techniques particularly powerful to identify ligand bias (Fang 2013). However, as the signal measured represents a composite of all pathways being activated within the cell, it is often difficult to deconvolute meaning. Despite this challenge, it has now been shown that pharmacological inhibitors can be used to deconvolute label-free signals arising from GPCR activation, at least to the level of identifying signals arising from specific G protein families (Schroder et al. 2010).

3.2.2 Antibodies as Biosensors

With the inherent ability to bind nearly any target molecule, antibodies provide numerous opportunities for use as biosensors. In particular, as antibodies specific for post-translationally modified proteins can be generated, this creates the opportunity to use antibodies recognizing phosphorylated GPCRs as biosensors of receptor activation. Recently, this approach elegantly yielded a biosensor antibody that demonstrated the location of muscarinic M_1 receptor activation in mouse brain during learning and memory (Butcher et al. 2016). Beyond post-translational modifications, antibodies against a specific active conformation of a target protein can also be developed. This has, for example, been used to generate antibodies capable of selectively identifying active G proteins (Lane et al. 2008), which could also be viewed as biosensors to assess GPCR activation of these G proteins.

While antibodies are incredibly useful tools, they are limited by their inability to cross the cell membrane, and thus cannot generally be used as biosensors to study function in living cells or in real time. To overcome this limitation, technologies such as nanobodies, derived from camelid single domain antibodies, are now making it possible to develop genetically encodable antibody-like biosensors. Of particular note, Irannejad et al. employed a nanobody, originally developed to mimic $G\alpha_s$ interaction and facilitate crystallization of the active state β_2 adrenoceptor (β_2AR) (Rasmussen et al. 2011), to build a biosensor able to show the subcellular localization of activated β_2ARs (Irannejad et al. 2013). This work demonstrated that the β_2AR remained in an active state even after internalization, providing critical evidence for endosomal signalling of GPCRs. Currently the full potential of antibodies, nanobodies and similar technologies as biosensors

to study GPCRs is only beginning to be realized, and it will be exciting to see how these may be employed in the future.

4 Biosensors to Study the FFA Receptors

Since being identified as receptors for FFAs between 2003 and 2005, uncovering the pharmacology of members of the FFA family of GPCRs has proven challenging (Hudson et al. 2011). This has been influenced by several factors, including a lack of pharmacological tools with selectivity for individual family members, low potency of the endogenous fatty acid ligands for the receptors, complex allosteric modes of binding for several receptors and the potential to signal through multiple G protein-dependent and independent pathways (Milligan et al. 2016). Biosensor-based approaches have proven to be valuable tools to address some of these complex aspects of FFA receptor pharmacology and the following sections will discuss how both genetically encoded and non-genetically encoded biosensors have improved our understanding of this important family of GPCR.

4.1 Studying FFA Receptors with Genetically Encoded Biosensors

Genetically encoded biosensors have been used to characterize many aspects of FFA receptor pharmacology. This has largely focused on using BRET-based biosensors to study the pharmacology and function of heterologously expressed receptors. In particular, such approaches have proven useful in interrogating ligand binding, G protein activation and arrestin interactions of these receptors.

4.1.1 Ligand Binding Sensors

Radioligand binding assays have long been the standard approach to directly assess ligand/GPCR interactions. However, such assays require a suitable radio-labelled tracer ligand with both high affinity and low non-specific binding, which typically are not available for recently deorphanized receptors, such as the FFA family members. Thus, directly measuring ligand binding to the FFA receptors has been challenging (Hudson et al. 2011). Although in recent years radio tracers have been reported for both FFA1 (Lin et al. 2012) and FFA2 (Sergeev et al. 2016), this approach remains challenging for the FFA receptors, in large part due to high lipophilicity and resultantly high non-specific binding of most ligands. One alternative approach to assess ligand binding gaining significant traction within the GPCR field is the use of fluorescent tracers (Briddon et al. 2011). Indeed, several studies have now described fluorescent tracer agonists for the FFA1 receptor (Bertrand et al. 2016; Christiansen et al. 2016; Hara et al. 2009; Ren et al. 2016). Although these have proven useful, the high lipophilicity of FFA1 ligands has made it difficult to directly assess the binding of these ligands to the receptor. To

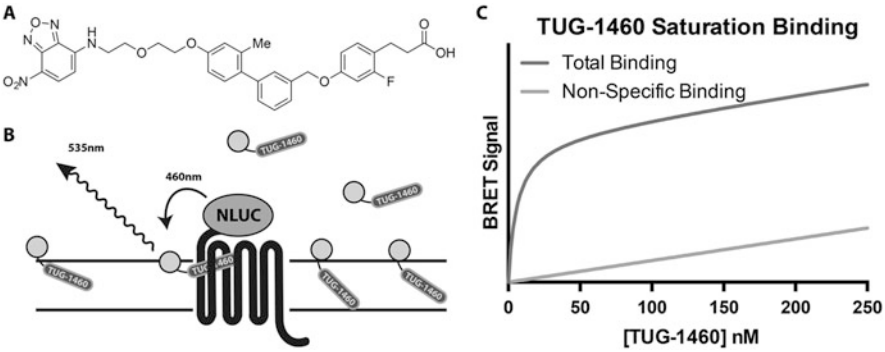

Fig. 3 A BRET-based ligand binding sensor for FFA1. The structure of TUG-1460, a fluorescent FFA1 agonist is shown in (**a**). Employing TUG-1460 in an intermolecular BRET biosensor utilizing NLUC-FFA1 allows for a BRET signal only from TUG-1460 bound to the receptor (**b**). This results in favourable specific to non-specific binding (simulated saturation binding data in **c**) despite the high lipophilicity of TUG-1460

overcome this, Christiansen et al. (2016) developed an intermolecular BRET biosensor approach to reduce the non-specific binding signal (Fig. 3).

By employing a system in which the FFA1 receptor was tagged at its N-terminus with the NLUC bioluminescent protein, BRET between the NLUC donor and the fluorescent tracer acceptor, TUG-1460, a high affinity FFA1 receptor agonist, should only be observed when the ligand is in close proximity to the receptor. This resulted in a very good specific to non-specific binding ratio for TUG-1460, despite its high lipophilicity. An additional benefit to this approach was that binding of the tracer to the receptor could be assessed in real time, which allowed Christenson et al. to measure the rapid binding kinetics of TUG-1460. In recent times there has been growing appreciation for the importance of binding kinetics in drug discovery (Swinney et al. 2015). Thus such BRET-sensor-based binding assays are likely to prove to be invaluable tools in the future. To date, FFA1 is the only FFA receptor for which such a BRET-binding assay has been reported; however, there is every likelihood that this approach can be extended to study the other members of the family if suitable fluorescent tracers can be developed.

4.1.2 G Protein Sensors

There are several RET-based biosensors designed to assess G protein interaction with GPCRs. These include: (1) intermolecular sensors where the receptor and G protein are tagged with either the RET donor or acceptor, and direct receptor/G protein interactions are assessed (Fig. 4a) (Ayoub et al. 2012); (2) intermolecular sensors where different subunits of the G protein αβγ heterotrimer are tagged with the RET donor and acceptor, allowing for dissociation/conformational rearrangement within the G protein to be measured (Fig. 4b) (Ayoub et al. 2012; Malik et al. 2013) or (3) intramolecular sensors where the receptor is tagged with both the RET donor and acceptor separated by a semi-flexible linker and by a peptide

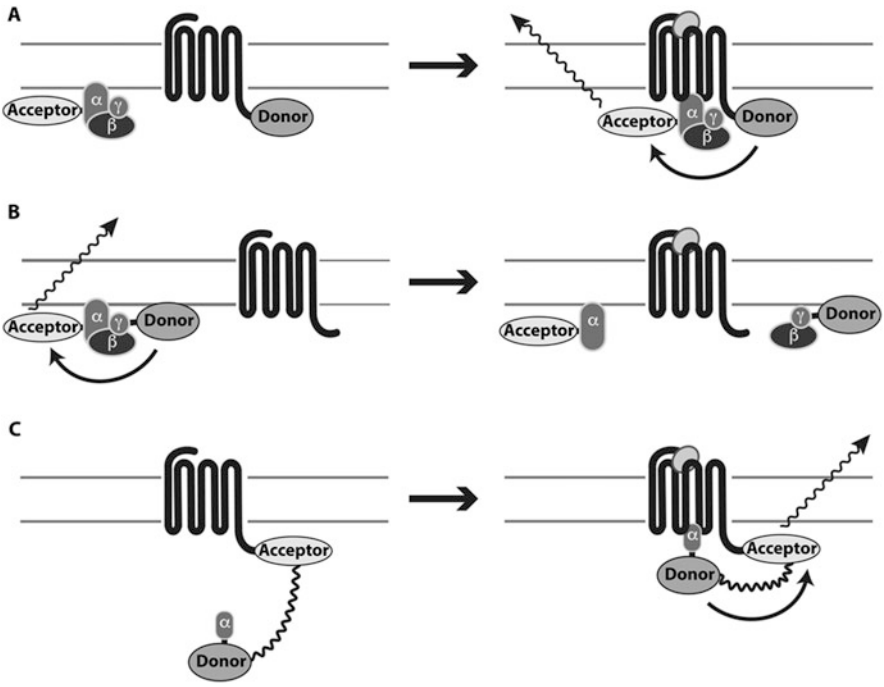

Fig. 4 Biosensor approaches to assess GPCR interactions with G proteins. (**a**) Intermolecular biosensors measure G protein recruitment through increased RET between tagged receptor and G protein. (**b**) Intermolecular biosensors where different components of the αβγ heterotrimer are labelled can measure dissociation of the G protein in response to activation as decreased RET. Although, the classic view of G protein signalling was that α subunit dissociates from the βγ upon activation, this may in reality only be a conformational rearrangement, which can equally be measured with this sensor as a change in RET. (**c**) An intramolecular RET sensor is shown where the receptor is tagged with the acceptor, which is then separated from the donor with a semi-flexible linker. The donor is then tagged with a peptide corresponding to the final 28 amino acids of a specific Gα subunit. Receptor activation leads to interaction between the Gα peptide and the receptor, resulting in increased RET. The specific locations of donor vs acceptor tags in this figure are used, for example, only, and in most cases these can be reversed

corresponding to the terminal residues of a specific Gα subunit; where if a ligand/receptor interaction leads to interaction between the G protein peptide and the receptor an increase in RET will be observed (Fig. 4c) (Malik et al. 2013).

To date, these approaches have not been widely employed in relation to the FFA receptors, with only one study using a BRET-based sensor to show Gq activation by the FFA1 receptor (Mancini et al. 2015). However, given that there is now good evidence that each of FFA1, FFA2 and FFA4 couple to multiple G proteins, and that this appears to vary depending on the specific cell type (Milligan et al. 2016), RET biosensors will be valuable tools to tease out factors that regulate G protein coupling preference (e.g., bias, cell type, oligomerization, etc.) of these receptors.

4.1.3 Arrestin Sensors

Arrestins were viewed historically as the 'off' switches of GPCR signalling, facilitating the removal of activated receptors from the cell surface. The fact that this process is conserved, and occurs with many different receptors has meant that arrestin interaction assays have become an important tool for GPCR drug discovery (Zhang and Xie 2012). In recent years, the importance of GPCR/arrestin interactions has only increased with the realization that arrestins not only turn off receptor signalling, but also generate their own G protein-independent signalling responses (Shukla et al. 2011). Although there are several assay formats able to assess GPCR/arrestin interactions, the most commonly employed biosensor capable of real time measurement of this interaction is an intermolecular BRET sensor, where the receptor and arrestin (either arrestin-2 or -3) are tagged with either of the BRET donor or acceptor (Fig. 5a). Over the years, this has become widely used as a key tool to characterize the pharmacology of several FFA family members, most notably for FFA4, which undergoes particularly robust interaction with arrestins-2 and -3.

One common application of the BRET arrestin-recruitment sensor has been to use this as a surrogate measure of agonist binding. As the sensor measures directly the interaction between receptor and arrestin, and this interaction should occur in a 1:1 stoichiometric ratio, the potency of an agonist to activate the BRET arrestin sensor is anticipated to be a good reflection of its affinity for the receptor (Hudson et al. 2011). This concept has been invoked as the basis for using a BRET arrestin-3-recruitment sensor to interrogate agonist binding at mutants intended to disrupt the orthosteric binding pockets of both FFA2 (Hudson et al. 2012, 2013a) and FFA4 (Hudson et al. 2013b, 2014). Likewise, this sensor has also been a useful tool in drug discovery efforts targeting FFA4, and indeed was the key assay used by Shimpukade et al. in identifying TUG-891 as the first potent and selective FFA4 agonist (Shimpukade et al. 2012).

The BRET arrestin-recruitment sensor has also proven useful in helping to define more complex aspects of FFA receptor pharmacology and function. This includes, for example, demonstrating arrestin bias agonism of certain synthetic agonists of FFA1 (Mancini et al. 2015) and FFA4 (Li et al. 2015), or confirming that arrestin recruitment to FFA4 occurs independently of $G_{q/11}$ activation (Schrage et al. 2015). The sensor has been particularly useful for defining the contribution of phosphorylated residues vs those possessing a fixed negative charge in the C-terminal tail of FFA4 for the recruitment of arrestin to the receptor (Butcher et al. 2014; Prihandoko et al. 2016).

Although one of the key advantages of using genetically encoded biosensors is the ability to assess receptor/arrestin interactions in real time, few studies have examined the kinetics of FFA receptor/arrestin interactions. While Watson et al. used a fluorescent protein fragment complementation sensor to study arrestin interaction with splice variants of the FFA4 receptor over time (Watson et al. 2012), it must be noted that the irreversible nature of this type of sensor makes interpretation of kinetic data challenging. More recently, Prihandoko et al. used a BRET arrestin-recruitment sensor to show the kinetics of arrestin interaction with mouse

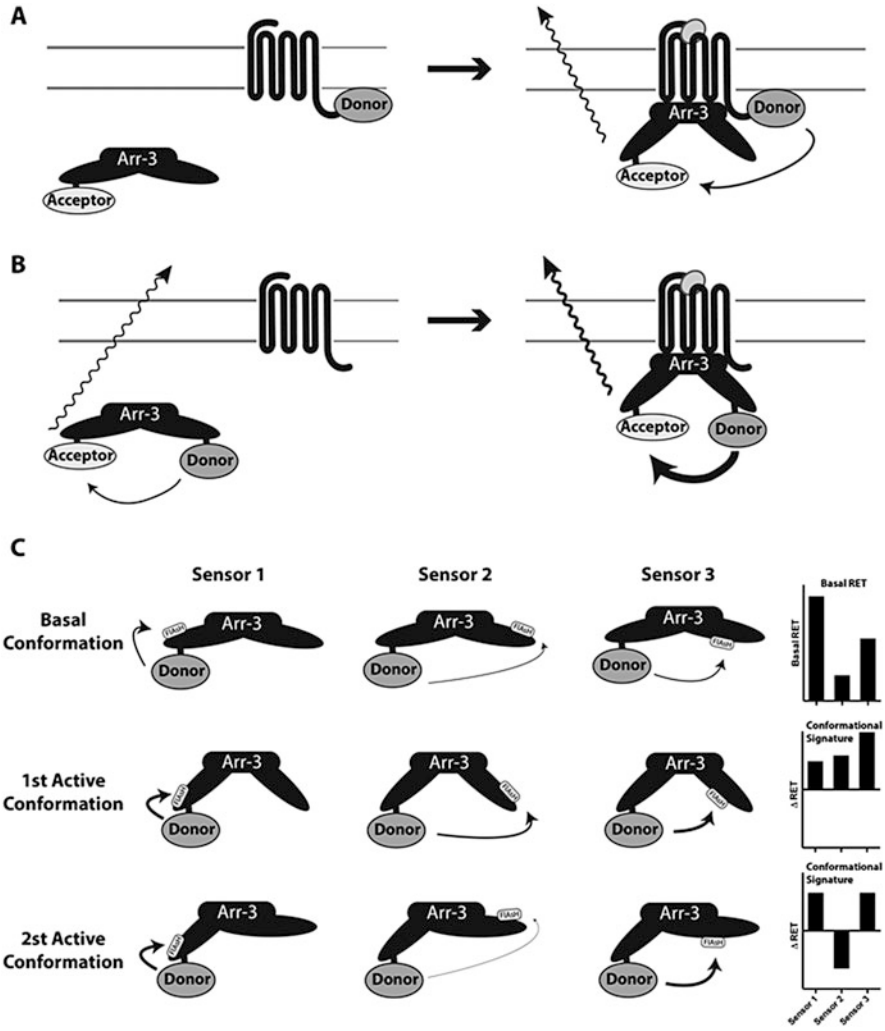

Fig. 5 Biosensors to assess GPCR interactions with an arrestin. (**a**) An intermolecular biosensor where the receptor and arrestin are tagged with RET donor and acceptor, respectively. Arrestin recruitment to the receptor increases RET. (**b**) An intramolecular arrestin sensor has the RET donor and acceptor at the N and C termini of arrestin. Interaction with an activated receptor results in a conformational change in arrestin, altering the RET. In (**c**), three examples of FlAsH-based arrestin conformational sensors are shown. In each case the six amino acid Cys-Cys-Pro-Gly-Cys-Cys sequence that binds the FlAsH fluorophore [4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein] is inserted into a different position of arrestin. Based on the location of FlAsH labelling, each sensor has a different basal RET between the donor and FlAsH acceptor. When arrestin adopts an active conformation (1st active conformation) the relative change in RET differs among the three sensors. The combined RET responses of all the sensors have been described as a conformational signature (Lee et al. 2016). If arrestin adopts a different active conformation (2nd active conformation), the conformational signature will be different, representative of differences in the relative distances between the RET donor and FlAsH acceptor between the two active conformations

FFA4 mutants lacking key phosphorylation sites (Prihandoko et al. 2016). Unravelling more details of the kinetics of FFA receptor/arrestin interactions is likely to be an important future application for these sensors.

In addition to sensors measuring receptor/arrestin interactions, there has also been some effort to develop intramolecular arrestin RET sensors able to assess the conformational changes that occur in arrestin itself in response to its interaction with an activated GPCR (Fig. 5b). This initially involved simple BRET-based sensors where arrestin-3 was tagged at its N-terminus with a luciferase and its C-terminus with a fluorescent protein (Charest et al. 2005). When the sensor interacted with an activated GPCR it resulted in a conformational change in arrestin, altering the relative distance between the two tags. Although simplistic, arrestin sensors based on this design were able to demonstrate that different ligands acting through the same receptor can generate distinct arrestin conformations (Shukla et al. 2008).

More recently, two groups independently developed more refined arrestin conformational sensors (one BRET- and one FRET-based) that employ the tetracysteine binding FlAsH fluorophore as the acceptor (Lee et al. 2016; Nuber et al. 2016). FlAsH binds to a six amino acid sequence, which provides more flexibility in terms of where within arrestin the acceptor can be inserted (Fig. 5c). This allowed both Nuber et al. and Lee at al. to generate multiple sensors reporting different aspects of the conformational changes that occurred after interaction with an activated GPCR (Lee et al. 2016; Nuber et al. 2016). Nuber et al. utilized their sensors to examine the conformational changes that occur in arrestin-3 after activation of the FFA4 receptor (Nuber et al. 2016). Of particular note, the conformational changes in arrestin-3 upon FFA4 activation were qualitatively different than those observed after β_2 adrenoceptor, or M_2 muscarinic receptor activation, providing evidence for receptor-specific arrestin active conformations. The full implications for this in terms of how it relates to arrestin-dependent signalling remain to be determined, but it is likely that these and similar arrestin conformational sensors will help define the finer details of FFA receptor/arrestin interactions and, potentially, signals generated.

4.2 Studying FFA Receptors with Non-genetically Encoded Biosensors

A key limitation to using genetically encoded biosensors is the need to heterologously express the sensor protein. Although not necessarily a requirement, in practice this means the majority of studies done using these sensors have been performed in common heterologous cell systems, such as HEK293 cells. Non-genetically encoded sensors have the potential to overcome this limitation, as at least conceptually, these should be suitable for use in any unmodified cell type. The most common of these approaches are the so-called label-free platforms, based on assessing either dynamic mass redistribution (DMR) or cellular impedance. These sensors have been important tools to study all four of the FFA family members. In addition to the label-free approaches, antibodies against residues

that alter phosphorylation status on addition of an agonist ligand may also be used as biosensors to assess GPCR activation and several studies have now developed these for the FFA4 receptor.

4.2.1 Label-Free Biosensors

Since label-free biosensors measure global cellular changes, they are a useful way to assess the effect of a drug-GPCR interaction without concern for the specific pathway that is activated. Schmidt et al. took advantage of this and used a DMR assay to assess a library of small carboxylic acids (SCAs) at the FFA2 and FFA3 receptors (Schmidt et al. 2011). This identified *trans*-2-methylcrotonic acid and cyclopent-1-enecarboxylic acid as orthosteric agonists selective for FFA2 over FFA3. It is interesting to note that although most of the SCAs tested produced responses, clear differences in the nature of the responses were observed for individual SCAs, even when acting through the same receptor (Schmidt et al. 2011). This highlights another key advantage of label-free sensors, in that they are very good at showing functional differences, or perhaps bias, between ligands. A particularly interesting example of this came from Grundmann et al. when they compared both impedance and DMR label-free responses obtained with the endogenous SCFA, C3, to those obtained with a reported allosteric agonist, 4-CMTB [4-chloro-α-(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide], at the FFA2 receptor (Grundmann et al. 2016). They noted that although C3 produced robust, rapid and sustained responses in both assays, 4-CMTB produced only a modest rapid response, followed by a robust sustained response. This demonstrated clear functional differences between the way these two ligands activate FFA2, which ultimately was attributed to an unusual ability of 4-CMTB to interact with the FFA2 orthosteric site to produce the rapid response, before producing its sustained response through interaction with a distinct allosteric site on the receptor (Grundmann et al. 2016).

In addition to demonstrating unique functional responses between two different ligands, label-free biosensors are also useful in demonstrating different responses to two different receptors, or receptor variants. Watson et al. used a DMR assay to show differences in the cellular response generated following activation of either short or long FFA4 splice variants (Watson et al. 2012). This difference was then attributed to an inability of the long variant to couple to $G_{q/11}$ pathways (Watson et al. 2012). The same DMR approach proved useful in demonstrating that a receptor activated solely by synthetic ligand (RASSL) variant of the FFA2 receptor displays signalling that is indistinguishable from the wild type receptor (Hudson et al. 2012), a critical property if this RASSL is to be used in future studies to help define FFA2 function (Bolognini et al. 2016).

Although the global nature of the label-free biosensor readout has advantages, it also can make it challenging to understand what exactly these sensors are measuring. To overcome this, a range of pharmacological inhibitors can be used to 'deconvolute' a label-free response into its individual components (Schroder et al. 2010). Such efforts, demonstrated that although FFA1 is traditionally viewed as a $G_{q/1}$-coupled receptor, treatment with a $G_{q/11}$ inhibitor, YM-254890, did not

fully eliminate the FFA1-DMR response (Schroder et al. 2010). Instead, it was only when cells were treated with both YM-254890 and the $G_{i/o}$ inhibitor, pertussis toxin, that the full FFA1-DMR response was eliminated (Schroder et al. 2010). This provided key support for the concept that FFA1 couples to multiple G protein families.

Despite a key advantages of the label-free biosensors being that they can be used in any cell type, to date the vast majority of work using these sensors on the FFA family has continued to utilize cells heterologously overexpressing the desired receptor. However, it has been shown that DMR can be used in MIN-6 and INS-1E insulinoma cell lines, as well as with primary rat islets, in order to assess endogenous FFA1 function (Grundmann and Kostenis 2015). Indeed, extending the use of these label-free biosensors to study endogenously expressed FFA receptors is likely to be a key use for these technologies.

4.2.2 Antibody Biosensors

There is an appreciation that antibodies able to recognize phosphorylated forms of a GPCR can, in effect, serve as biosensors for receptor activation (Butcher et al. 2016). Thus, it should be of note that antibodies specific to phosphorylated forms of both the mouse and human orthologues of the FFA4 receptor have now been described (Butcher et al. 2014; Prihandoko et al. 2016). To date, these antibodies have been employed only to show FFA4 activation in cells heterologously overexpressing the receptor. However, the antibody against mouse FFA4 was suitable to use in immunocytochemistry studies to visualize the location of activated receptor within cells (Prihandoko et al. 2016). Although still at an early stage, it will be exciting to see how these antibodies may be used in the future to assess FFA4 activation in cells and tissues endogenously expressing FFA4.

5 Conclusions and Future Perspectives

Biosensor technologies have proven to be important tools for uncovering the complex pharmacology of the FFA receptor family. This has included using sensors to identify new ligands, to define how these ligands interact with the receptors, to dissect signalling pathways and to understand the basis of receptor/arrestin interactions. There are, however, many questions that remain to be answered about the pharmacology and function of the FFA receptor family and biosensor technologies are likely to continue to be central to these efforts. Most notably, it remains unclear how the FFA receptors function under physiological conditions and how certain, rare fatty acids, for example, the omega-3 and fatty acid hydroxy fatty acids, that are believed to produce biological effects through the FFA receptors in vivo (Oh et al. 2010; Yore et al. 2014), are able to do so, even though these are at much lower levels in the body than the more common fatty acids that are also known to activate the receptors. Key to using biosensors to answer these questions will be to begin employing the sensors, both genetically encoded and otherwise, in more physiological situations. The recent revolution in CRISPR

genome editing technologies should facilitate the use of genetically encoded sensors in a wider range of cell types and at more physiologically relevant expression levels, allowing for studies on the FFA receptors in, for example, model cells relevant to the regulation of metabolism and inflammation. There has also been increased interest in using sensors to study GPCR function in synthetic organs and ultimately in intact animals (van Unen et al. 2015), which may provide key answers to where, when and how the FFA receptors are activated in vivo. Taken together, the use of biosensor technologies has, and will continue to, provide exciting opportunities to help unravel the complex pharmacology of the FFA family of receptors.

References

Ashby MC, Ibaraki K, Henley JM (2004) It's green outside: tracking cell surface proteins with pH-sensitive GFP. Trends Neurosci 27:257–261

Ayoub MA, Al-Senaidy A, Pin JP (2012) Receptor-G protein interaction studied by bioluminescence resonance energy transfer: lessons from protease-activated receptor 1. Front Endocrinol (Lausanne) 3:82

Azad T, Tashakor A, Hosseinkhani S (2014) Split-luciferase complementary assay: applications, recent developments, and future perspectives. Anal Bioanal Chem 406:5541–5560

Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD (2003) beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. Proc Natl Acad Sci U S A 100:940–945

Bertrand R, Wolf A, Ivashchenko Y, Lohn M, Schafer M, Bronstrup M, Gotthardt M, Derdau V, Plettenburg O (2016) Synthesis and characterization of a promising novel FFAR1/GPR40 targeting fluorescent probe for beta-cell imaging. ACS Chem Biol 11:1745–1754

Bolognini D, Tobin AB, Milligan G, Moss CE (2016) The pharmacology and function of short chain fatty acid receptors. Mol Pharmacol 89:388–398

Bouschet T, Martin S, Henley JM (2005) Receptor-activity-modifying proteins are required for forward trafficking of the calcium-sensing receptor to the plasma membrane. J Cell Sci 118:4709–4720

Boute N, Jockers R, Issad T (2002) The use of resonance energy transfer in high-throughput screening: BRET versus FRET. Trends Pharmacol Sci 23:351–354

Briddon SJ, Kellam B, Hill SJ (2011) Design and use of fluorescent ligands to study ligand-receptor interactions in single living cells. Methods Mol Biol 746:211–236

Butcher AJ, Hudson BD, Shimpukade B, Alvarez-Curto E, Prihandoko R, Ulven T, Milligan G, Tobin AB (2014) Concomitant action of structural elements and receptor phosphorylation determines arrestin-3 interaction with the free fatty acid receptor FFA4. J Biol Chem 289:18451–18465

Butcher AJ, Bradley SJ, Prihandoko R, Brooke SM, Mogg A, Bourgognon JM, Macedo-Hatch T, Edwards JM, Bottrill AR, Challiss RA, Broad LM, Felder CC, Tobin AB (2016) An antibody biosensor establishes the activation of the M1 muscarinic acetylcholine receptor during learning and memory. J Biol Chem 291:8862–8875

Charest PG, Terrillon S, Bouvier M (2005) Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. EMBO Rep 6:334–340

Christiansen E, Hudson BD, Hansen AH, Milligan G, Ulven T (2016) Development and characterization of a potent free fatty acid receptor 1 (FFA1) fluorescent tracer. J Med Chem 59 (10):4849–4858

Cooper MA (2006) Non-optical screening platforms: the next wave in label-free screening? Drug Discov Today 11:1068–1074

Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE, Monaghan AE, Liew WC, Mpamhanga CP, Bonner TI, Neubig RR, Pin JP, Spedding M, Harmar AJ (2013) International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. Pharmacol Rev 65:967–986

De A, Gambhir SS (2005) Noninvasive imaging of protein-protein interactions from live cells and living subjects using bioluminescence resonance energy transfer. FASEB J 19:2017–2019

Emami-Nemini A, Roux T, Leblay M, Bourrier E, Lamarque L, Trinquet E, Lohse MJ (2013) Time-resolved fluorescence ligand binding for G protein-coupled receptors. Nat Protoc 8:1307–1320

Fang Y (2006) Label-free cell-based assays with optical biosensors in drug discovery. Assay Drug Dev Technol 4:583–595

Fang Y (2013) Troubleshooting and deconvoluting label-free cell phenotypic assays in drug discovery. J Pharmacol Toxicol Methods 67:69–81

Fang Y, Ferrie AM, Fontaine NH, Mauro J, Balakrishnan J (2006) Resonant waveguide grating biosensor for living cell sensing. Biophys J 91:1925–1940

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63:1256–1272

Grundmann M, Kostenis E (2015) Label-free biosensor assays in GPCR screening. Methods Mol Biol 1272:199–213

Grundmann M, Tikhonova IG, Hudson BD, Smith NJ, Mohr K, Ulven T, Milligan G, Kenakin T, Kostenis E (2016) A molecular mechanism for sequential activation of a G protein-coupled receptor. Cell Chem Biol 23:392–403

Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, Wood KV (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chem Biol 7:1848–1857

Hara T, Hirasawa A, Sun Q, Koshimizu TA, Itsubo C, Sadakane K, Awaji T, Tsujimoto G (2009) Flow cytometry-based binding assay for GPR40 (FFAR1; free fatty acid receptor 1). Mol Pharmacol 75:85–91

Hattori M, Ozawa T (2014) Split luciferase complementation for analysis of intracellular signaling. Anal Sci 30:539–544

Hochreiter B, Garcia AP, Schmid JA (2015) Fluorescent proteins as genetically encoded FRET biosensors in life sciences. Sensors 15:26281–26314

Hudson BD, Smith NJ, Milligan G (2011) Experimental challenges to targeting poorly characterized GPCRs: uncovering the therapeutic potential for free fatty acid receptors. Adv Pharmacol 62:175–218

Hudson BD, Christiansen E, Tikhonova IG, Grundmann M, Kostenis E, Adams DR, Ulven T, Milligan G (2012) Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. FASEB J 26:4951–4965

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, Tikhonova IG, Ulven T, Milligan G (2013a) Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288:17296–17312

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, Heathcote H, Tobin AB, Ulven T, Milligan G (2013b) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 84:710–725

Hudson BD, Shimpukade B, Milligan G, Ulven T (2014) The molecular basis of ligand interaction at free fatty acid receptor 4 (FFA4/GPR120). J Biol Chem 289:20345–20358

Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, von Zastrow M (2013) Conformational biosensors reveal GPCR signalling from endosomes. Nature 495:534–538

Kitayama A, Yoshizaki H, Ohmiya Y, Ueda H, Nagamune T (2003) Creation of a thermostable firefly luciferase with pH-insensitive luminescent color. Photochem Photobiol 77:333–338

Kneen M, Farinas J, Li YX, Verkman AS (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys J 74:1591–1599

Lane JR, Henderson D, Powney B, Wise A, Rees S, Daniels D, Plumpton C, Kinghorn I, Milligan G (2008) Antibodies that identify only the active conformation of G(i) family G protein alpha subunits. FASEB J 22:1924–1932

Lee MH, Appleton KM, Strungs EG, Kwon JY, Morinelli TA, Peterson YK, Laporte SA, Luttrell LM (2016) The conformational signature of beta-arrestin2 predicts its trafficking and signalling functions. Nature 531:665–668

Li A, Yang D, Zhu M, Tsai KC, Xiao KH, Yu X, Sun J, Du L (2015) Discovery of novel FFA4 (GPR120) receptor agonists with beta-arrestin2-biased characteristics. Future Med Chem 7:2429–2437

Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J, Vimolratana M, Jiao XY, Wang Y, Birdsall NJ, Swaminath G (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859

Lohse MJ, Nuber S, Hoffmann C (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. Pharmacol Rev 64:299–336

Machleidt T, Woodroofe CC, Schwinn MK, Mendez J, Robers MB, Zirnmerman K, Otto P, Daniels DL, Kirkland TA, Wood KV (2015) NanoBRET – a novel BRET platform for the analysis of protein-protein interactions. ACS Chem Biol 10:1797–1804

Malik RU, Ritt M, DeVree BT, Neubig RR, Sunahara RK, Sivaramakrishnan S (2013) Detection of G protein-selective G protein-coupled receptor (GPCR) conformations in live cells. J Biol Chem 288:17167–17178

Mancini AD, Bertrand G, Vivot K, Carpentier E, Tremblay C, Ghislain J, Bouvier M, Poitout V (2015) beta-Arrestin recruitment and biased agonism at free fatty acid receptor 1. J Biol Chem 290:21131–21140

Marsango S, Varela MJ, Milligan G (2015) Approaches to characterize and quantify oligomerization of GPCRs. Methods Mol Biol 1335:95–105

Maurel D, Comps-Agrar L, Brock C, Rives ML, Bourrier E, Ayoub MA, Bazin H, Tinel N, Durroux T, Prezeau L, Trinquet E, Pin JP (2008) Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat Methods 5:561–567

May LT, Leach K, Sexton PM, Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. Annu Rev Pharmacol Toxicol 47:1–51

Milligan G (2013) The prevalence, maintenance, and relevance of G protein-coupled receptor oligomerization. Mol Pharmacol 84:158–169

Milligan G, Shimpukade B, Ulven T, Hudson B (2016) Complex pharmacology of free fatty acid receptors. Chem Rev. doi:10.1021/acs.chemrev.6b00056

Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. Nat Biotechnol 19:137–141

Nuber S, Zabel U, Lorenz K, Nuber A, Milligan G, Tobin AB, Lohse MJ, Hoffmann C (2016) beta-Arrestin biosensors reveal a rapid, receptor-dependent activation/deactivation cycle. Nature 531:661–664

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698

Pfleger KDG, Eidne KA (2006) Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). Nat Methods 3:165–174

Prihandoko R, Alvarez-Curto E, Hudson BD, Butcher AJ, Ulven T, Miller AM, Tobin AB, Milligan G (2016) Distinct phosphorylation clusters determine the signaling outcome of free fatty acid receptor 4/G protein-coupled receptor 120. Mol Pharmacol 89:505–520

Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF, George SR (2007) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. Proc Natl Acad Sci U S A 104:654–659

Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK (2011) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. Nature 469:175–180

Ren XM, Cao LY, Zhang J, Qin WP, Yang Y, Wan B, Guo LH (2016) Investigation of the binding interaction of fatty acids with human G protein-coupled receptor 40 using a site-specific fluorescence probe by flow cytometry. Biochemistry 55:1989–1996

Robers MB, Binkowski BF, Cong M, Zimprich C, Corona C, McDougall M, Otto G, Eggers CT, Hartnett J, Machleidt T, Fan F, Wood KV (2015) A luminescent assay for real-time measurements of receptor endocytosis in living cells. Anal Biochem 489:1–8

Rose RH, Briddon SJ, Holliday ND (2010) Bimolecular fluorescence complementation: lighting up seven transmembrane domain receptor signalling networks. Br J Pharmacol 159:738–750

Salahpour A, Espinoza S, Masri B, Lam V, Barak LS, Gainetdinov RR (2012) BRET biosensors to study GPCR biology, pharmacology, and signal transduction. Front Endocrinol (Lausanne) 3:105

Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, Ward RJ, Drewke C, Milligan G, Kostenis E, Ulven T (2011) Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. J Biol Chem 286:10628–10640

Schrage R, Schmitz AL, Gaffal E, Annala S, Kehraus S, Wenzel D, Bullesbach KM, Bald T, Inoue A, Shinjo Y, Galandrin S, Shridhar N, Hesse M, Grundmann M, Merten N, Charpentier TH, Martz M, Butcher AJ, Slodczyk T, Armando S, Effern M, Namkung Y, Jenkins L, Horn V, Stossel A, Dargatz H, Tietze D, Imhof D, Gales C, Drewke C, Muller CE, Holzel M, Milligan G, Tobin AB, Gomeza J, Dohlman HG, Sondek J, Harden TK, Bouvier M, Laporte SA, Aoki J, Fleischmann BK, Mohr K, Konig GM, Tuting T, Kostenis E (2015) The experimental power of FR900359 to study Gq-regulated biological processes. Nat Commun 6:10156

Schroder R, Janssen N, Schmidt J, Kebig A, Merten N, Hennen S, Muller A, Blattermann S, Mohr-Andra M, Zahn S, Wenzel J, Smith NJ, Gomeza J, Drewke C, Milligan G, Mohr K, Kostenis E (2010) Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. Nat Biotechnol 28:943–949

Scott CW, Peters MF (2010) Label-free whole-cell assays: expanding the scope of GPCR screening. Drug Discov Today 15:704–716

Sergeev E, Hansen AH, Pandey SK, MacKenzie AE, Hudson BD, Ulven T, Milligan G (2016) Non-equivalence of Key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. J Biol Chem 291:303–317

Shimpukade B, Hudson BD, Hovgaard CK, Milligan G, Ulven T (2012) Discovery of a potent and selective GPR120 agonist. J Med Chem 55:4511–4515

Shukla AK, Violin JD, Whalen EJ, Gesty-Palmer D, Shenoy SK, Lefkowitz RJ (2008) Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors. Proc Natl Acad Sci U S A 105:9988–9993

Shukla AK, Xiao K, Lefkowitz RJ (2011) Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. Trends Biochem Sci 36:457–469

Steen A, Larsen O, Thiele S, Rosenkilde MM (2014) Biased and g protein-independent signaling of chemokine receptors. Front Immunol 5:277

Stoddart LA, Smith NJ, Milligan G (2008) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev 60:405–417

Stoddart LA, Johnstone EKM, Wheal AJ, Goulding J, Robers MB, Machleidt T, Wood KV, Hill SJ, Pfleger KDG (2015) Application of BRET to monitor ligand binding to GPCRs. Nat Methods 12:661–663

Swinney DC, Haubrich BA, Van Liefde I, Vauquelin G (2015) The role of binding kinetics in GPCR drug discovery. Curr Top Med Chem 15:2504–2522

van Unen J, Woolard J, Rinken A, Hoffmann C, Hill SJ, Goedhart J, Bruchas MR, Bouvier M, Adjobo-Hermans MJ (2015) A perspective on studying G-protein-coupled receptor signaling with resonance energy transfer biosensors in living organisms. Mol Pharmacol 88:589–595

Watson SJ, Brown AJ, Holliday ND (2012) Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 81:631–642

Wehr MC, Rossner MJ (2016) Split protein biosensor assays in molecular pharmacological studies. Drug Discov Today 21:415–429

Xu XD, Soutto M, Xie Q, Servick S, Subramanian C, von Arnim AG, Johnson CH (2007) Imaging protein interactions with bioluminescence resonance energy transfer (BRET) in plant and mammalian cells and tissues. Proc Natl Acad Sci U S A 104:10264–10269

Yore MM, Syed I, Moraes-Vieira PM, Zhang T, Herman MA, Homan EA, Patel RT, Lee J, Chen S, Peroni OD, Dhaneshwar AS, Hammarstedt A, Smith U, McGraw TE, Saghatelian A, Kahn BB (2014) Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. Cell 159:318–332

Zhang R, Xie X (2012) Tools for GPCR drug discovery. Acta Pharmacol Sin 33:372–384

Key Questions for Translation of FFA Receptors: From Pharmacology to Medicines

Arthur T. Suckow and Celia P. Briscoe

Contents

Abstract

The identification of fatty acids as ligands for the G-protein coupled free fatty acid (FFA) receptor family over 10 years ago led to intensive chemistry efforts to find small-molecule ligands for this class of receptors. Identification of potent, selective modulators of the FFA receptors and their utility in medicine has proven challenging, in part due to their complex pharmacology. Nevertheless, ligands have been identified that are sufficient for exploring the therapeutic potential of this class of receptors in rodents and, in the case of FFA1, FFA2, FFA4, and GPR84, also in humans. Expression profiling, the phenotyping of

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FFA receptor knockout mice, and the results of studies exploring the effects of these ligands in rodents have uncovered a number of indications where engagement of one or a combination of FFA receptors might provide some clinical benefit in areas including diabetes, inflammatory bowel syndrome, Alzheimer's, pain, and cancer. In this chapter, we will review the clinical potential of modulating FFA receptors based on preclinical and in some cases clinical studies with synthetic ligands. In particular, key aspects and challenges associated with small-molecule ligand identification and FFA receptor pharmacology will be addressed with a view of the hurdles that need to be overcome to fully understand the potential of the receptors as therapeutic targets.

Keywords
Diabetes • FFA • GPR120 • GPR40 • GPR41 • GPR43 • IBD

1 Introduction

Free fatty acids (FFAs) of varying chain lengths are known to play key roles in disease, contextually playing either a protective or causative role. For example, FFAs play a role in modulating the function of the immune system (Kim et al. 2014), in regulating pancreatic insulin secretion (Greenough et al. 1967), and in mediating the positive benefits of commensal gut microbiota (Montalban-Arques et al. 2015). They also may prevent the progression of neurodegenerative diseases (Song et al. 2016), reduce pain associated with neuropathy and fibromyalgia (Khan and He 2015), and inhibit the proliferation of cancer cells (Hopkins et al. 2016). On the contrary, high levels of certain species of fatty acids are associated with a proinflammatory state (Baer et al. 2004; Lopez-Garcia et al. 2005), leading to an increased risk for several diseases including cancer, autoimmunity, heart disease, obesity, and diabetes (de Souza et al. 2015). These positive and negative effects of FFAs are likely mediated through a combination of cell surface receptors, nuclear receptors, and intracellular metabolism. This review will primarily focus on the therapeutic potential of targeting the family of cell surface receptors, known as free fatty acid receptors (FFA receptors), which are engaged endogenously by free fatty acids.

The discovery of FFAs as agonists for orphan G-protein coupled receptors during the period between 2003 and 2006 clearly established FFAs as cell surface signaling molecules. Aside from GPR84, the receptors activated by FFAs are now classified by the International Union of Pharmacology (IUPHAR) into the FFA receptor family (Stoddart et al. 2008) as described in Table 1. These receptors are differentially activated by both saturated and unsaturated FFAs of short, medium, and long carbon chain lengths (SCFA, MCFA, and LCFA, respectively) and signal via distinct G-proteins as well as through β-arrestin dependent mechanisms (Briscoe et al. 2003; Brown et al. 2003; Hirasawa et al. 2005; Itoh et al. 2003; Wang et al. 2006). Table 1 summarizes the fatty acid ligands, the G-protein

Table 1 Summary of the free fatty acid activated GPCRs, their coupling, and their expression

Receptor	FFA1 (GPR40)	FFA2 (GPR43)	FFA3 (GPR41)	FFA4 (GPR120)	GPR84
Activating fatty acid (carbon length)	C10–C22, saturated and unsaturated	C1–C6	C1–C6	C12–C22, saturated and unsaturated	C8–C14 saturated
Potency EC_{50} (recombinantly expressing cells)	1–30 μM[1–3]	0.1–1 mM[4–6]	0.1–1 mM[4,5,7]	1–20 μM[8–11]	1–70 μM[12]
Levels of endogenous ligand (total)[a]	Plasma; 200-500 μM[14]	Gut; 80–130 mM/kg Plasma; 80–375 μM[13–15]	Gut; 80–130 mM/kg Plasma; 80–375 μM[13–15]	Plasma; 200–500 μM[14]	Plasma; 200–500 μM[14]
G protein coupling	$G\alpha_{q/11}$	$G\alpha_{i/o}$ and $G\alpha_{q/11}$	$G\alpha_{i/o}$	β-arrestin, $G\alpha_{q/11}$	Gαi/o
% Identity/similarity human vs mouse	83/95	85/96	77/90	83/93	86/94
Expression	Intestine, islets, brain and monocytes	Immune, adipocytes, intestine, islets, bone marrow, spleen	Adipose, spleen, lymph node, bone marrow, PBMC	Intestine, adipose, islets, lung, macrophage, taste bud	Bone marrow, lung, BMC

Source: [1]Briscoe et al. (2003), [2]Itoh et al. (2003), [3]Kotarsky et al. (2003), [4]Brown et al. (2003), [5]Le Poul et al. (2003), [6]Nilsson et al. (2003), [7]Xiong et al. (2004), [8]Hirasawa et al. (2005), [9]Briscoe et al. (2006), [10]Shimpukade et al. (2012), [11]Watson et al. (2012), [12]Wang et al. (2006), [13]Cummings et al. (1987), [14]Jouven et al. (2001), [15]van Eijk et al. (2009)

% Identity based on pair-wise alignment of protein sequences using http://pir.georgetown.edu/

[a]Total amount of FFA reported including that bound to proteins such as albumin

Table 2 Summary of current clinical development for FFA-receptor based therapeutics

Target	NCE	Company	Stage/status	Indication	Results
FFA1 agonist	TAK-875 (Fasiglifam)	Takeda	Terminated 2013 in Ph3 (liver toxicity)	Type 2 diabetes	Ph2[1–4]: Ph3[5] ↑insulin ↓A1c ↓FPG
	AMG837	Amgen	Ph1/No further development	Type 2 diabetes	No change glucose or insulin[6,7]
	LY2881835	Lilly	Ph1/No further development[8]	Type 2 diabetes	
	JIT-851	Japan Tobacco	Ph2[9]	Type 2 diabetes	
	P11187	Piramal	Ph1[10]	Type 2 diabetes	
FFA2 antagonist	GLPG0974	Galapagos/ GSK	Ph1[11], Ph2[12]	IBD/ ulcerative colitis	Failed to meet endpoint
FFA4/ PPARγ agonist	KDT501[13]	Kindex	Ph2[14]	Type 2 diabetes	
GPR84 antagonist	GLPG1205[15]	Galapagos	Ph2/No further development	Ulcerative colitis	Failed to meet endpoint

Source: [1]Araki et al. (2012), [2]Burant et al. (2012), [3]Burant (2013), [4]Kaku et al. (2013), [5]Kaku et al. (2015), [6]Houze et al. (2012), [7]Luo et al. (2012), [8]Defossa and Wagner (2014), Hamdouchi et al. 2016, [9]www.jt.com/about/division/pharma/, [10]www.clinicaltrials.gov accessed November 11th 2016, [11]Namour et al. (2016), [12]www.clinicaltrials.gov accessed November 11th 2016, [13]Konda et al. (2014), [14]www.kindexpharmaceuticals.com/, [15]http://www.glpg.com/

coupling mechanism, and the tissue mRNA expression profile for each of the known FFA receptors. Notably, the FFA receptors are activated by free fatty acids that are not bound to albumin since the presence of albumin decreases their potency at the receptors (Itoh et al. 2003). The identification of ligands for FFA receptors opened up the possibility of developing synthetic molecules that could be used to elucidate the physiological role of each receptor. Based on this knowledge, it is anticipated that opportunities may be identified to develop therapeutics that selectively promote the beneficial effects of fatty acids.

To date, discovery and development of small-molecules for FFA have focused mainly on approaches aimed to treat indications that include type 2 diabetes and inflammatory disease. The strongest proof of concept of the utility of targeting FFA receptors comes from the FFA1 receptor where the selective agonist TAK-875 (fasiglifam) improved glucose control in patients with diabetes (Kaku et al. 2015) (Table 2). Synthetic ligands to other FFA receptors are either in early proof of concept studies in humans or are still being validated in preclinical studies. As will be highlighted throughout this discussion, finding potent and selective agonists or antagonists, either orthosteric or allosteric, to this family of receptors has proven quite challenging. In this chapter, we will discuss generally what the challenges

associated with developing synthetic ligands to FFA receptors are. We will also discuss the relevant genetic data supporting the development of synthetic ligands to each of the receptors for a given indication, the status of the development of synthetic ligands, preclinical data supporting further development of synthetic ligands and where available the clinical data evaluating the potential of synthetic FFA ligands. With the caveat of limited data and several outstanding questions, we aim to speculate what the future may look like for therapeutics based on the FFA receptors.

2 Challenges in Identification and Development of Pharmacological Molecules for FFA Receptors

There are a number of factors influencing both the identification of small-molecule modulators of FFA receptors and the determination of the target disease area where an FFA receptor-based therapeutic is most likely to be successful. A summary of these factors are highlighted in Table 3. The questions fall into four major groups

Table 3 Key questions relating to FFA receptors in identifying and validating molecules as potential therapeutics

Challenges	Key questions
Receptor tone	• What is the level of receptor tone due to endogenous ligands? • What is the degree of constitutive activity of the receptor? • Are there differences in receptor constitutive activity across species?
Identification of potent, selective agonists/antagonists	• Is the molecule sufficiently selective to allow identification of the receptor responsible for observed efficacy? • Is the molecule's potency and selectivity the same across species? • Are there differences in G-protein coupling between ligands and/or between species which could lead to differences in biological responses?
Agonist receptor pharmacology and tachyphylaxis	• Is efficacy durable? • Which signaling pathway is important for desired efficacy? • Does the molecule preferentially activate the β-arrestin pathway? – Would a biased ligand be desirable?
Disease association	• Are there orthologue differences in receptor expression at the mRNA and/or protein level? • Does the phenotyping of the KO mouse support a role for the receptor in the disease of interest? • Are there human polymorphisms associated with disease?

and will be discussed more specifically as they relate to development of synthetic ligands for FFA receptors and associated disease areas.

2.1 Endogenous Receptor Tone and Constitutive Receptor Activity

The potency of FFAs for their respective GPCRs is in the micromolar (μM) range (Table 1) and initially raised questions as to whether FFAs were bona fide endogenous ligands for the receptors. Levels of total bound (predominantly to albumin) and unbound FFAs are present in the circulation, but concentrations in healthy individuals remain unclearly defined and vary with methodology and population size examined. In general, however, total FFAs circulate at concentrations up to 500 μM (Jouven et al. 2001; van Eijk et al. 2009). The majority of FFAs are bound to albumin and only a small fraction of the total FFAs, especially the more insoluble LCFAs (carbon chain >14), circulate as unbound FFAs with dissociation constants dependent on the fatty acid (Demant et al. 2002). Estimates of unbound FFAs in the circulation of healthy individuals are at or below 10 nanomolar (nM) (Apple et al. 2005; Azzazy et al. 2006; Richieri and Kleinfeld 1995; Richieri et al. 1992), suggesting that under normal physiological conditions FFA receptors are likely not activated based on potencies determined in cells recombinantly expressing the receptors (Table 1). Local tissue levels of total FFAs may however be higher than those in the circulation. Notably in the gut, where SCFAs are produced from microbiota fermentation of dietary fiber, levels are in the high millimolar range (Cummings et al. 1987) suggesting that the concentration of unbound SCFAs in the lower GI tract may be at or close to levels that could activate FFA2 and FFA3 receptors. Changes in circulating levels, such as following a period of fasting may lead to increases in local tissue FFA levels that could be sufficient for receptor activation. For example, activation of FFA1 in the pancreatic islet is thought to play a role in potentiation of insulin secretion by FFAs following a period of fasting (Dobbins et al. 1998). However, there remains some debate as to what extent endogenous FFAs would activate FFA1, FFA4, and GPR84. For FFA receptors where therapeutic agonists are desirable, the theoretical possibility of endogenous tone at these receptors could limit the ability of synthetic ligands to meaningfully enhance signaling. Allosteric ligands that modulate or enhance signaling in the presence of an orthosteric ligand may be more desirable, especially if they can enhance signaling to a greater extent than the orthosteric ligand alone. In situations where increased receptor tone could be detrimental to an individual, an antagonist may be a more appropriate approach.

All GPCRs are in equilibrium between an inactive state and active state even in the absence of ligand, the balance of which determines their basal activity (Sato et al. 2016). In a situation where the level of basal or constitutive activity contributes to the pathogenesis of a disease, only an inverse agonist rather than a neutral antagonist would be effective in reducing the receptor activity. Moreover, variation of the receptor's constitutive activity across species would influence the potency of the inverse agonist in different species. Species differences in constitutive activity have been described for FFA2 and FFA3 receptors with highest constitutive activity for hFFA2 and mFFA3 relative to their respective orthologues (Hudson et al. 2012). However, since a selective inverse agonist that has activity across species has not been identified it has not been possible to examine the effect of the differences in constitutive activity on potency.

2.2 Identification of Potent, Selective Small-Molecule Agonists/Antagonists

The literature contains many examples where non-selective tool compounds or fatty acids themselves are used to try to link a member of the FFA receptor family to a disease area (Hopkins et al. 2016; Nakamoto et al. 2015; Tang et al. 2015). For example, GW9508 is often used as a tool compound at 10 μM to investigate the involvement of FFA1 or FFA4 in a process. GW9508 has an EC_{50} of 50 nM for GPR40 and 5 μM for GPR120 (Briscoe et al. 2006). GW9508 is frequently used at concentrations 100-fold higher than its EC_{50} for FFA1, at these concentrations it may signal through other receptors and intracellular pathways in addition to FFA4, hence the ability to conclude whether the FFA1 receptor is the major player in the biological end-point examined is compromised. In particular, where non-selective fatty acids are used in vitro, they not only need to be presented at a physiologically relevant concentration in the presence of albumin, but studies using genetic or pharmacological tools are important to ensure effects are not a result of intracellular metabolism, engagement of nuclear receptors, or cytotoxicity, and to elucidate which receptor is involved. In studies involving a tissue or cells where FFA receptors are co-expressed, the use of selective agonists or antagonists is particularly important in characterizing changes associated with a single receptor. This can be complicated further by species differences in ligand selectivity. For example acetate is non-selective for FFA2 and FFA3 in mouse although 20-fold more selective for human FFA2 over FFA3 (Hudson et al. 2012). Thus the interpretation of studies where multiple receptors are expressed such as in the intestine is challenging when non-selective ligands are used.

Generating small molecule agonists/antagonists that are truly selective for one FFA receptor subtype has been difficult in general, especially for subtypes that respond to the same classes of free fatty acids. Although the percent similarity between the mouse and human FFA receptors is higher than 90% at the protein level (Table 1), species differences in key residues involved in ligand interactions are common, leading to alterations in potency and selectivity of small-molecule

agonists and antagonists between species. Clearly where potency and/or selectivity is reduced in the mouse, interpretation of in vivo studies and clinical translation for lead compounds becomes an issue (Hudson et al. 2013b). By way of example, TUG-891 is a potent and selective FFA4 receptor agonist at the human receptor, but selectivity for the FFA4 receptor over the FFA1 receptor is reduced at the mouse receptor (Hudson et al. 2013b, c). Selectivity issues are also observed for antagonists, with Pfizer FFA1 receptor antagonists reported to be more selective at the human receptor (Hudson et al. 2013b; Humphries et al. 2009). In addition the majority of FFA1 agonists are more potent at the human receptor than at the mouse (Christiansen et al. 2012, 2013; Lin et al. 2011, 2012). Choosing the most selective agonist may also be dependent on the choice of in vitro signaling assay. TUG891 for example was more selective for the FFA4 receptor when tested in an assay looking at β-arrestin signaling compared to Ca^{2+} mobilization (Shimpukade et al. 2012).

Translation of pharmacology from species to species is further complicated by the observation that the human and mouse receptors for the FFA2 and FFA1 receptors, couple to different G-proteins, engagement of which leads to different biological responses in the case of insulin secretion (Priyadarshini et al. 2015). A final related issue is the finding that several FFA receptor family members, including FFA1 and FFA2, can couple to distinct G-proteins depending on the ligand that is used to engage the receptor. As an example, synthetic ligands to FFA1 have been generated that result in FFA1 coupling solely to Gαq or to both the Gαs and Gαq pathways. These differences in compound selectivity across species, species-dependent receptor signaling, and promiscuous G-protein coupling for some FFA receptors introduce factors that need to be carefully considered and underpins the basis of selecting candidates for preclinical to clinical progression.

2.3 Agonist Receptor Pharmacology and Tachyphylaxis

Receptor desensitization following activation with an agonist, commonly referred to as tachyphylaxis, is a concern for any GPCR since this could lead to loss of efficacy over time. For example, tachyphylaxis has been suggested as one possibility in the failure to observe sustained clinical efficacy in type 2 diabetes trials with GPR119 agonists (Kang 2013). Receptor-mediated activation of the β-arrestin pathway is not only a key component in termination of the signaling from agonist-occupied GPCRs, leading to receptor internalization, but is established as an important effector of G-protein independent signaling pathways (Rajagopal et al. 2010). In particular, the FFA4 receptor is known to couple strongly to the β-arrestin 2 pathway which is thought to be responsible for the anti-inflammatory effects of FFA4 agonists (Oh et al. 2010), whereas the $G\alpha_{q/11}$ pathway is likely associated with increases in glucagon-like peptide-1 (GLP-1) and glucose-uptake in adipocytes (Hudson et al. 2013c). As would be anticipated therefore, FFA4 receptor agonists including TUG891 result in rapid desensitization of signaling and internalization followed by resensitization of the G-protein mediated signaling after

removal of the agonist (Hudson et al. 2013c). Currently, it remains unclear for several of the FFA receptors whether biased agonists toward the G-protein signaling cascade are necessary for long-term benefits. Importantly it is thought that the receptor/β-arrestin complex can continue to signal despite internalization (Luttrell and Gesty-Palmer 2010). Indeed the finding that enhancement of insulin secretion following FFA1 receptor activation requires the β-arrestin pathway potentially supports the development of a biased FFA1 receptor agonist for type 2 diabetes (Mancini et al. 2015).

2.4 Establishing Precedence for Disease Association

With fatty acids linked to numerous diseases, the ability to form a hypothetical, testable link between a FFA receptor family member and therapeutic indication is paramount both in humans and in preclinical species. Unfortunately, learnings from knockout mouse phenotyping have been limited, with conflicting literature reports for several of the FFA receptors (Ang and Ding 2016; Bjursell et al. 2014; Brownlie et al. 2008; Lan et al. 2008). Differences in strain background and environmental conditions are likely a contributing factor to the variability (Tables 4 and 5). Additional support for disease association comes from expression of the receptor in relevant tissues and expression changes under diseased conditions. Researchers have relied heavily on mRNA expression, with a lack of available and reliable antibodies preventing investigators from confirming the expression of FFA receptor protein on the cell surface in relevant tissues. In the absence of mouse genetic data, and human and rodent protein expression information, drug development for the FFA receptors has focused predominantly on testing small-molecule ligands in a number of preclinical models, while using knockout models, or antagonists where available to confirm that effects are on target This approach clearly puts the emphasis on identifying potent and selective molecules in order to connect the FFA receptor target with the resultant efficacy. For example from studies using the FFA1 agonist tool compound GW9508, the receptor has been suggested to play a role in other diseases aside from diabetes including pain (Nakamoto et al. 2015) and Alzheimer's (Khan and He 2015). Developing confidence in clinical translatability for an FFA1 receptor agonist to treat these indications will require rigorous preclinical testing with more potent, selective agonists and preferably an understanding of the expression of the receptor in the respective human disease settings. Indeed since it has been a challenge to identify highly potent and selective synthetic ligands for several of the FFA receptor family members, the risk of not being able to translate findings with existing molecules from preclinical models into the clinic remains higher than desirable.

Table 4 Reported metabolic phenotypes of FFA family member knockout mice

Receptor knockout	Metabolic phenotyping supports *agonist*	Metabolic phenotyping supports *antagonist*
FFA1	Reduced potentiation of insulin secretion by fatty acids.[1,2] No protection from diet-induced obesity[1,3]	↓Diet-induced obesity and insulin resistance[2]
FFA2	↓SCFA-stimulated GLP-1 and worsened glucose tolerance[4] ↑Obesity[5] Worsened glucose tolerance due to defect in insulin secretion. Reduction in β-cell mass[6] No change in glucose tolerance, slight defect insulin secretion[7] ↑Lipolysis and FFA[8]	↓Diet-induced obesity and improved glucose tolerance due to increased energy expenditure[9]
FFA3	↑Obesity, decreased energy expenditure in males[10]	↑ GSIS from KO islets[11] ↓PYY, lean (phenotype disappears under germ-free conditions)[12]
FFA2/3		↑ GSIS from KO islets Improved glucose tolerance in diet-induced obese mice[13]
FFA4	Worsened glucose tolerance ↑obesity ↑insulin, glucose[14–16] ↑glucagon secretion following arginine challenge[16]	

Source: [1]Kebede et al. (2008), [2]Steneberg et al. (2005), [3]Lan et al. (2008), [4]Tolhurst et al. (2012), [5]Kimura et al. (2013), [6]McNelis et al. (2015), [7]Priyadarshini et al. (2015), [8]Ge et al. (2008), [9]Bjursell et al. (2011), [10]Bellahcene et al. (2013), [11]Priyadarshini and Layden (2015), [12]Samuel et al. (2008), [13]Tang et al. (2015), [14]Ichimura et al. (2012), [15]Oh et al. (2010), [16]Suckow et al. (2014)

Table 5 Reported phenotypes in inflammatory disease models of knockout mice

Receptor	Inflammatory model phenotyping supports *agonist*	Inflammatory model phenotyping supports *antagonist*
FFA2	↑Colitis, arthritis, asthma. ↑Immune cell recruitment and inflammatory mediators[1] ↑DSS-colitis[2,3] ↑Colitis (T-cell transfer model)[4]	↓DSS-colitis mediated tissue destruction (↑*neutrophil recruitment*)[5] ↓Intestinal inflammation following TNBS or ethanol administration[6] ↓MSU-stimulated gout[7]
FFA3	↑Asthma[8]	↓Intestinal inflammation following TNBS or ethanol administration[6]
GPR84		Enhanced IL-4 production in activated T-cells[9]

Source; [1]Maslowski et al. (2009), [2]Macia et al. (2015), [3]Masui et al. (2013), [4]Smith et al. (2013), [5]Sina et al. (2009), [6]Kim et al. (2013), [7]Vieira et al. (2015), [8]Trompette et al. (2014), [9]Venkataraman and Kuo (2005)

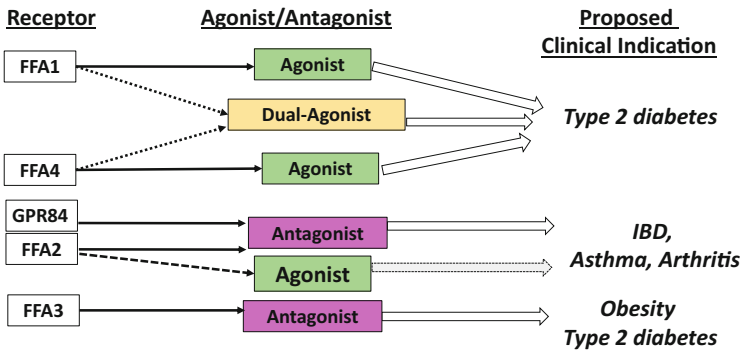

Fig. 1 Currently targeted indications for agonists or antagonist for FFA receptors and GPR84. It remains unclear whether an agonist or antagonist of FFA2 receptor would be effective in IBD

3 FFA Receptors and Therapeutic Indications

To date small-molecule clinical development for FFA receptors has focused on type 2 diabetes and inflammatory disease. Progress in these areas and in other more recently suggested indications are described in more detail below and summarized in Fig. 1.

3.1 Type 2 Diabetes

Type 2 diabetes is characterized by loss of pancreatic β cell function and development of peripheral tissue insulin resistance. These pathophysiologies lead to hyperglycemia and ultimately to complications such as cardiovascular disease, neuropathy, retinopathy, and kidney damage that significantly reduce life expectancy. Outcome studies in patients having undergone bariatric surgery, the only known treatment that results in diabetes remission, demonstrate impressive improvements in mortality and cardiovascular risk (Cummings et al. 2016; Eliasson et al. 2015). Non-surgical treatment paradigms typically include a combination of metformin, a drug that acts on the liver to lower glucose production, with drugs that enhance β cell function (e.g., GLP-1 receptor agonists or dipeptidyl peptidase-IV (DPP-IV) inhibitors that reduce the degradation of GLP-1), those that improve insulin sensitivity in adipose tissue (e.g., peroxisome proliferator-activated receptor gamma (PPARγ) agonists), or those that enhance the ability of the kidneys to excrete glucose (sodium-glucose co-transporter inhibitors (SGLT2 inhibitors)). Emerging evidence suggests that the inclusion of the more recently approved SGLT2 inhibitors and injectable GLP-1 analogs does provide some cardiovascular benefit (Hiatt et al. 2013; Pfeffer et al. 2015); however, the currently available data does not suggest they result in the same degree in improvement in glucose control or associated comorbidities as bariatric surgery. A drug or combination of drugs

that more closely mimics the benefits of bariatric surgery is highly desirable since surgery is invasive and can be associated with significant complications. Preclinical and early clinical studies suggest that FFA1 receptor and/or FFA4 receptor agonists may have potential to further improve glucose homeostasis and reduce complications/comorbidities in patients with type 2 diabetes.

3.1.1 FFA1 for Diabetes Treatment

The first studies characterizing the FFA1 receptor, in which long-chain fatty acids (LCFAs) were identified as FFA1 receptor agonists, suggested a role for the receptor in the potentiation of glucose-stimulated insulin secretion (GSIS) from pancreatic β cells (Briscoe et al. 2003; Itoh et al. 2003). These data are supported by the identification of several polymorphisms in the FFA1 gene that are associated with reduced insulin secretion, although these findings have not been reproducible (Hamid et al. 2005; Kalis et al. 2007; Smith et al. 2009; Vettor et al. 2008). The confirmation that LCFA-potentiated GSIS is significantly attenuated in islets from FFA1 receptor knockout mice (Latour et al. 2007; Steneberg et al. 2005), together with the finding that the synthetic FFA1 receptor agonist GW9508 potentiates insulin secretion, led to intensive efforts across the pharmaceutical industry to develop potent, selective, and orally bioavailable FFA1 receptor agonists for the treatment of diabetes. The available data suggests that the effective mechanism of action of an FFA1 receptor agonist would be very similar to that of DPP-IV inhibitors and GLP-1R agonists, in that they lead to glucose-dependent insulin release from pancreatic β cells. The potential differentiation from DPPIV inhibitors based on currently available data is somewhat limited, although FFA1 receptor agonists, like GLP-1R agonists, act directly on β cells rather than by indirect means (Omar and Ahrén 2014). An advantage of FFA1 receptor agonists over GLP-1R agonists is that they have the potential to be delivered orally rather than via injection. A significant disadvantage versus the GLP-1R class is that an FFA1 receptor agonist is not expected to result in weight loss, a desirable benefit in most patients with type 2 diabetes. Takeda, Amgen, Japan Tobacco, GSK, Janssen, Eli Lilly, and several other small biotechnology companies all have or have had active programs aimed at developing FFA1 receptor agonists.

The FFA1 receptor agonist that has progressed furthest in the clinic is an ago-allosteric modulator referred to as TAK-875 (Fasiglifam), which was identified and characterized by Takeda Pharmaceuticals (Negoro et al. 2010). The development program for TAK-875 reached phase 3 clinical trials before being terminated in late 2013 due to liver toxicity concerns (Kaku et al. 2015). Since the FFA1 receptor does not appear to be expressed in the liver (Briscoe et al. 2003), the toxicity concerns are likely specific to the TAK-875 scaffold rather than a receptor-mediated phenomenon (Mancini and Poitout 2015). The efficacy with respect to lowering of HbA1c, a measure of glucose control over longer time periods, in two 12-week phase 2 dose-range finding studies and a 24-week phase 3 study suggests that TAK-875 markedly improves glucose control. The degree of improvement for a 50 mg dose varied from approximately 0.8% to 1.3% across studies and was highly dependent on the starting HbA1c. The improvement in HbA1c is comparable

to that observed in similar studies with other recently approved therapies (Inagaki et al. 2013; Terauchi et al. 2014). However, whether TAK-875 could have been competitive as a monotherapy against existing therapies cannot be judged in the absence of head-to-head studies. Aside from the liver toxicity, TAK-875 was generally well tolerated with very low rates of hypoglycemia. Other companies maintain an interest in the development of FFA1 receptor agonists for type 2 diabetes and are currently testing small-molecules in the clinic (Table 2).

Despite positive efficacy in clinical trials, there are several outstanding questions with respect to the development of FFA1 receptor agonists for the treatment of diabetes. The most significant question relates to whether or not drug hunters should focus on developing full agonists, partial agonists, or allosteric modulators. Recent data suggest that the FFA1 receptor has at least three distinct binding sites which when occupied appear to result in different degrees of insulin secretion from pancreatic β-cells (Lin et al. 2012; Yabuki et al. 2013). Amgen has recently identified both full and partial agonists, termed AMG1638 and AMG837 respectively, which bind to sites that are distinct from the site at which LCFAs engage the FFA1 receptor. AMG1638 appears to have significantly better efficacy with respect to the potentiation of insulin secretion from islets and in lowering glucose excursion during a glucose tolerance test in mice, compared to that observed with AMG837. Further studies with AMG837 revealed that the combination of AMG837 with the LCFA docosahexaenoic acid (DHA) enhanced insulin secretion to levels that exceeded those following treatment with either AMG837 or DHA alone, suggesting that the molecule is an allosteric modulator (Lin et al. 2012). Although not compared in a head-to-head study, the potentiation of insulin secretion in isolated islets observed with the AMG837/LCFA combination appears similar to that observed with the full agonist AMG1638 (Luo et al. 2012).

Studies with TAK-875 revealed that, like AMG837, it was also an ago-allosteric modulator that resulted in an impressive increase in insulin secretion from islets when combined with a LCFA (Yabuki et al. 2013). The fact that an ago-allosteric modulator was efficacious in the clinic supports the hypothesis that local tissue levels of LCFAs are indeed sufficient to engage the receptor to some extent. It is not yet clear, however, whether there might be additional benefit to the patient from a full agonist such as AMG1638, since preclinical repeat dosing studies comparing a full agonist to an ago-allosteric modulator in diabetic rodents are yet to be disclosed. There are some hints from functional in vitro assays that full agonists differentially stimulate signaling from the FFA1 receptor (Hauge et al. 2015). Single dose studies in rodents have shown that full agonists result in the additional benefit of releasing enteroendocrine hormones, such as GLP-1 and gastric-inhibitory polypeptide (GIP) (Luo et al. 2012; Xiong et al. 2013). However, the therapeutic potential of promoting insulin secretion through both direct stimulation of the β-cells and via GLP-1 comes with the caveat that a full agonist might stimulate differential signaling that may affect the internalization of the receptor and result in tachyphylaxis. Notably, the FFA1 receptor has recently been demonstrated to stimulate insulin secretion through both a $G\alpha_{q/11}$ and β-arrestin

2 pathways suggesting the additional possibility of developing biased agonists (Mancini et al. 2015).

Although there are several outstanding questions regarding FFA1 receptor agonists for diabetes therapy, and several companies are currently testing molecules in the clinic (Table 2), the potential of FFA1 receptor agonists as a standalone treatment is fairly limited given that receptor activation does not result in weight loss and only addresses one component, β-cell function, in a multi-factorial disease Hamdouchi et al. 2016. It also appears unlikely based on the TAK875 data that the efficacy of an FFA1 receptor agonist, at least an ago-allosteric modulator, will be superior with respect to effects on glucose control to currently approved monotherapies. The potential for FFA1 receptor agonists is likely to be in identifying other drugs that complement its mechanism of action, with combinations possibly resulting in additional improvements in glucose control and associated comorbidities. For example combinations of FFA1 receptor agonists with SGLT2 inhibitors or orally delivered GLP-1R agonists might result in further improvements in glucose control as well as reductions in body weight. One unique way of achieving a differentiated FFA1 receptor-based therapy is by developing single molecules with multiple activities. Notably, a recent publication has disclosed dual FFA1/PPARγ agonists as offering the potential of an insulin sensitizer and insulin secretagogue (Darwish et al. 2016). Dual single molecule FFA1/FFA4 agonists may also be feasible given the endogenous ligands are identical, although the amino acids involved in recognition of fatty acids in the FFA1 receptor are closer to those in the FFA2 and FFA3 receptors than in the FFA4 receptor (Milligan et al. 2015). Molecules that have dual activity at the FFA1 and FFA4 receptors such as GW9508 (Briscoe et al. 2006) and TUG891 (dual activity at the mouse receptors) (Hudson et al. 2013c) demonstrate that the concept of a dual agonist is certainly possible, although whether a dual agonist suitable for the clinic can be identified awaits further research.

3.1.2 FFAR4 Agonists for Treatment of Metabolic Disease

Preclinical studies using LCFAs as FFA4 receptor agonists suggest that synthetic and selective FFA4 receptor agonists might improve diabetes via mechanisms that result in enhanced β-cell function, increased insulin sensitivity, and reduced body weight. LCFAs, through engagement of the FFA4 receptor, were originally reported to act directly on enteroendocrine L-cells to induce GLP-1 secretion, and thus have the potential to promote insulin secretion indirectly from pancreatic β cells in a glucose-dependent manner (Hirasawa et al. 2005). Furthermore, a role for the FFA4 receptor in inflammation associated with obesity has been proposed on the basis that DHA represses macrophage-induced tissue inflammation in a diet-induced obese (DIO) mouse model in a FFA4 receptor-dependent manner, resulting in enhancement of insulin sensitivity and improved glucose disposal (Oh et al. 2010). Support for a role of the FFA4 receptor in obesity comes from the identification of a mutation in the FFA4 receptor gene that results in attenuated signaling and an increased risk of obesity in European populations (Ichimura et al. 2012). Although there are no reports of LCFAs inducing weight loss in an FFA4 receptor-

dependent manner, mice lacking the FFA4 gene are obese and glucose intolerant (Ichimura et al. 2012; Suckow et al. 2014). Taken in combination, our existing knowledge of FFA4 mRNA expression (Table 1), the preclinical data with LCFAs and genetic data provide for a very compelling rationale to develop and test potent and selective FFA4 receptor agonists in the clinic. FFA4 receptor agonists have the potential to be quite differentiated from anything else currently on the market or in development in that they would address, via a single target, the two major pathophysiologies driving diabetes (insulin secretion and insulin resistance) with the additional potential for weight loss.

Based on the scarcity of publications disclosing the development and characterization of FFA4 receptor agonists and the time that has lapsed since the deorphanization of the receptor, the reality of identifying and developing agonists suitable even for proof of concept studies in vivo appears to be quite challenging. Kindex reportedly has a dual GPR120/PPARγ agonist in phase 2 clinical trials. Although the trial was completed in November 2015 (www.clinicaltrial.gov, accessed 4/23/2016), no data are currently available from this study. The only in vivo study characterizing the long-term effects of a selective GPR120 agonist, termed CpdA, demonstrated an improvement in glucose tolerance and insulin sensitivity in a DIO mouse model (Oh et al. 2014). The effects on glycemic control appear quite modest, although a positive comparator was not included, and CpdA did not result in increased GLP-1 secretion or body weight loss as might have been predicted. Moreover, only a single dose level was used in the study, leaving open the possibility that a higher dose may have produced a different or more pronounced response. Other FFA4 receptor agonists such as TUG-891 have been developed that appear selective at the human FFA4 receptor, but lack orthologue selectivity. Since TUG891 lacks selectivity for FFA4 over FFA1 at the mouse receptor (Hudson et al. 2013c) mechanistic interpretation of any in vivo studies is challenging. Whether selectivity over the FFA1 receptor is in fact desirable remains a point for consideration. A potent, orally bioavailable, dual FFA1/FFA4 agonist has the potential to deliver efficacy that exceeds that which can be achieved via either mechanism alone as previously discussed (Sect. 3.1.1). TUG-891 has not been evaluated in vivo and it is not clear from the available data whether TUG-891 would have suitable properties for a proof of concept study. In cell lines endogenously expressing the FFA4 receptor, however, TUG-891 increases GLP-1 secretion (enteroendocrine cells), enhances glucose uptake (3 T3-L1 adipocytes), and inhibits TNFα secretion (RAW264.7 macrophages). These in vitro observations support the potential of an FFA4 receptor agonist to intervene in multiple pathways that together might result in a meaningful clinical benefit. Whether these effects will translate to efficacy in preclinical diabetes models or in the clinic awaits identification of suitable molecules.

The paucity of potent and selective agonists, or even antagonists, for the FFA4 receptor leaves open a number of questions with respect to the pharmacology of FFA4 receptor agonists. A key potential issue is whether or not the tight coupling of FFA4 to β-arrestin (Hudson et al. 2014b) will result in tachyphylaxis and failure to sustain efficacy. A separate, but related set of questions involves elucidating which

of the effects of the receptor are mediated through G protein-coupled signaling versus β-arrestin signaling and whether or not biased agonists that favor one signaling cascade over the other are desirable if they can be developed. With the assumption that FFA4, like FFA1 may have some endogenous tone under certain conditions it is possible that multiple binding sites on the FFA4 receptor exist, as for the FFA1 receptor, that might provide an avenue to the development of allosteric modulators to enhance the efficacy of the endogenous ligands. The answers to these questions will require a breakthrough in chemistry that results in the development of additional FFA4 receptor agonist tools, which hopefully are not only highly potent and selective, but which have sufficient pharmacokinetic properties suitable for dosing studies in rodent models.

3.1.3 FFA2 and FFA3 for Metabolic Disease

Although a body of research suggests a potential role for both FFA2 and FFA3 receptors as targets for the treatment of metabolic disease (Kimura 2014), the main focus in development of small-molecules acting at the FFA2 receptor has been in identification of antagonists for the treatment of chronic inflammatory disease as discussed in Sect. 3.2. The connection between the FFA2 and FFA3 receptors with obesity and type 2 diabetes stems from the association of microbiota in the GI tract with metabolic disease (Palau-Rodriguez et al. 2015) and their ability to produce SCFAs. The SCFAs may act via the FFA2 receptor and possibly FFA3 receptors to modulate inflammatory processes as well as to affect hormone secretion from enteroendocrine cells (Kuwahara 2014). Additional interest in their role in metabolism comes from the finding that these receptors are expressed by pancreatic β cells and appear to be critical for modulating the inhibitory effects of acetate on insulin secretion (Tang et al. 2015). The expression and role of the FFA2 and FFA3 receptors in adipose have been controversial, further complicated by the finding that FFA3 knockdown results in concomitant reduction (but not ablation) of the FFA2 receptor in mouse adipose. The more recent studies have demonstrated that only the FFA2 receptor is in adipose and is responsible for SCFA-stimulated leptin production from adipocytes (Zaibi et al. 2010).

Whilst the phenotype of mice lacking either the FFA2 or FFA3 receptors is largely supportive of a role for these receptors in metabolism (Table 4), it does not provide much clarity around the question of whether an agonist or antagonist is desirable. FFA3 receptor knockout mice are glucose intolerant and obese, but as described above the concomitant reduction of the FFA2 receptor in adipose complicates interpretation. Reports on FFA2 receptor knockout mice are variable with respect to the effects of deletion on glucose tolerance, gut hormone secretion, β-cell function, and adiposity. The majority of reports suggest that FFA2 receptor deletion leads to metabolic dysfunction including increased lipolysis, glucose intolerance, and obesity (Ge et al. 2008; Kimura et al. 2013; McNelis et al. 2015; Tolhurst et al. 2012). However, at least one report suggests FFA2 receptor deletion results in improved glucose homeostasis and reduced adiposity (Bjursell et al. 2011). Islets from FFA2 receptor KO mice have impaired glucose-stimulated insulin secretion (Priyadarshini et al. 2015), whereas islets from FFA3 receptor

KO mice have improved glucose-stimulated insulin secretion (Priyadarshini and Layden 2015). A double knockout of both the FFA2 and FFA3 receptors revealed an increase in insulin secretion and improvement in glucose tolerance in mice fed a high-fat diet (Tang et al. 2015). Although the KO characterization data, as well as the data with SCFAs, supports the development of an antagonist or inverse agonist for the FFA3 receptor, the fact that the FFA3 receptor is promiscuous and thus can interact with Gαq and Gαi depending on the ligand or cell type needs to be considered as drug-hunters identify and characterize ligands to these targets.

For both FFA2 and FFA3 receptors, potent and selective agonists and antagonists at human and rodent receptors, whether allosteric or orthosteric, have been difficult to identify. The few reports available suggest that the relative benefit of an agonist or antagonist/inverse agonist is yet to be decided with studies frequently limited to those in vitro due to poor selectivity or pharmacokinetic properties. The complexity of the responses resulting from a FFA2 receptor agonist is indeed borne out in the literature. Selective allosteric and orthosteric agonists have been reported to suppress lipolysis in vitro (Hudson et al. 2013a) and in vivo in mice (Wang et al. 2010). Effects on GLP-1 have been confusing with the selective orthosteric ligand stimulating GLP-1 secretion in vitro (Hudson et al. 2013a), while a human selective FFA2 inverse agonist potentiated GLP-1 secretion in vitro (Park et al. 2016) through elevation of cAMP pathways, despite reductions in SCFA-stimulated Ca^{2+} flux. The selective FFA2 agonist "cmpd 1" (Forbes et al. 2015) decreased food intake and body weight in a FFA2 receptor-dependent manner and slowed gastrointestinal (GI) transit. However, data from this study suggested that "cmpd 1" would not be suitable for treating diabetics since a worsening in glucose tolerance and suppression of insulin secretion was observed. The phenylacetamide FFA2 receptor agonist compound (*S*)-2-(4-chlorophenyl)-3,3-dimethyl-*N*-(5-phenylthiazol-2-yl) butanamide on the other hand was able to stimulate insulin secretion in mouse and human islets in a Gαq-dependent manner (McNelis et al. 2015). Not only is the insulin response to an FFA2 receptor agonist complicated by bias between agonists, but also by orthologue differences in G-protein coupling (Priyadarshini et al. 2015). In summary, current information suggests that translation of an FFA2 receptor agonist with potential as an insulin secretagogue with anti-lipolytic potential will require rigorous pharmacological examination and in the balance the risk may be higher than the potential benefit given the efficacy of the drugs currently approved for type 2 diabetes. Whether an antagonist against the FFA2 receptor, such as those tested for IBD, has the potential to exacerbate metabolic disease clinically is unclear and likely awaits longer term clinical studies.

Agonists for the FFA3 receptor are limited to few reports that include allosteric modulators (Hudson et al. 2014a). The FFA3 agonist AR4206526 has been shown to stimulate GLP-1 from mouse crypt cells and decrease ghrelin from gastric cells (Engelstoft et al. 2013; Nøhr et al. 2013), but there have been no in vivo studies with FFA3 agonists reported. Clearly challenges in SAR and pharmacology have hindered our understanding of whether FFA2 or FFA3 receptors are plausible targets for type 2 diabetes. With the increasing discovery of more tractable agonists it is hoped that this question may be resolved in the not too distant future.

3.2 FFAs, Inflammatory Disease, and the GI Tract

Inflammatory bowel disease (IBD) covers a number of inflammatory diseases in the gastrointestinal tract and is characterized by periods of remission and outbreaks. The major forms of IBD are Crohn's disease (CD), which can encompass the entire intestine, and ulcerative colitis (UC), which is limited to the colon and rectum. Current therapy includes immunosuppressants, steroids, and 5-aminosalicylic acid. Biologics such as anti-tumor necrosis factor α (anti-TNFα) antibodies and anti-α 4β7 antibodies are also approved and used in therapy of CD and UC, but 20–40% of patients do not respond to these therapies (Park and Jeen 2015). Moreover, a significant proportion of patients with IBD will require surgery to remove parts of their intestine. An active area of clinical development is focused on developing novel biologics including those antagonizing integrins and inhibiting IL-12p40 or IL-23 or small molecules inhibiting Janus kinases (JNK). The short-chain and medium-chain fatty acid ligands for FFA2, FFA3, and GPR84 receptors are present in high levels (millimolar) in the gut due to colonic fermentation of dietary fiber (Bergman 1990; Bloemen et al. 2009; Cummings and Macfarlane 1997). Given the presence of FFA2, FFA3 receptors and GPR84 in cells of the immune system and intestine (Le Poul et al. 2003, Brown et al. 2003, Nilsson et al. 2003, Stoddart et al. 2008), this subset of the free fatty acid activated receptor family offers the potential of a novel method of treating the disease using an orally available small molecule. However, conflicting reports on the response of the FFA2 and FFA3 receptor knockout mice to immune challenges (Table 5) have created questions as to whether an agonist or antagonist approach should be taken (Ang and Ding 2016).

Although a number of companies, notably Euroscreen and Galapagos, have identified FFA2 receptor antagonists and agonists, to date only Galapagos have taken both GPR84 and FFA2 receptor antagonists into the clinic. No clinical development for FFA3 receptor antagonists has been reported. GLPG0974 (Pizzonero et al. 2014) is the first FFA2 receptor antagonist to have been tested in humans. To date in two Phase 1 clinical trials conducted in 2012 and 2013 the molecule has been safe and well tolerated in once and twice daily dosing regimens. Target engagement up to a 24 h period was demonstrated using the neutrophil marker CD11b as a biomarker and neutrophil activation was dose-dependently inhibited (www.FlandersBio.be), supporting preclinical observations (Pizzonero et al. 2014). However, in a Phase 2a study completed in 2014 in individuals with mild-to-moderate ulcerative colitis GLPG0974 did not meet clinical endpoints. Although biomarkers indicated a reduction in neutrophil activation and infiltration in GLPG0974-treated individuals compared to those that received placebo, there were no differences in clinical responses, Mayo score, or histopathology scoring of colon biopsies (Vermeire et al. 2015). GLPG1205, the GPR84 antagonist, was reported to be safe and well tolerated up to a 100 mg dose in Phase 1 trials in healthy volunteers with once-daily dosing resulting in a 24 h inhibition of GPR84 ligand binding in whole blood (www.glpg.com). Galapagos continued into a 12-week phase II trial with GLPG1205 in individuals with moderate-to-severe UC (ORIGIN study). However results announced early in 2016 revealed that

patients receiving GLPG1205 did not alter their Mayo score compared to those receiving placebo and thus the trial failed to meet its primary endpoints (www.glpg.com). Currently development of GLPG1205 for other indications is reported to being under evaluation.

It remains unclear why promising preclinical anti-inflammatory effects of candidate molecules targeting GPR84 and FFA2 receptors have not translated into a more robust clinical signal. The lack of success in the clinic for UC, albeit based only on the two Galapagos molecules, raises questions as to whether it was due to the molecule and differences in potency at the human receptor in the diseased state, whether the receptors in humans are not the predominant regulators of inflammation in IBD, or if inhibiting neutrophil infiltration and activation is not an effective approach for the disease. Studies have demonstrated that there appears to be a difference in constitutive activity between the human and mouse FFA2 receptor due to a residue in extracellular-loop 2 that is missing in the human receptor, which results in an elevation in agonist-independent activity (Hudson et al. 2012). Certainly conflicting results surrounding the KO models and their phenotypes (Ang and Ding 2016) casts uncertainty as to whether an agonist or antagonist is needed for benefits in UC. The FFA2 receptor was shown to be essential for polymorphonuclear leukocytes (PMN) recruitment based on the phenotype of the KO mouse in acute and chronic colitis with reduced inflammation observed in the chronic model (Sina et al. 2009). However, although an FFA2 receptor antagonist may inhibit neutrophil migration, it is not entirely clear whether neutrophils play a beneficial or detrimental role in IBD and an FFA2 receptor antagonist may therefore be inhibiting a beneficial pathway (Williams and Parkos 2007). Moreover, Maslowski et al. (Maslowski et al. 2009) reported that FFA2 receptor knockout mice had exacerbated inflammation and increased production of inflammatory mediators in models of DSS-induced or TNBS-induced colitis, and worsening of inflammation in the K/BxN serum-induced model of arthritis and ovalbumin (OVA)-induced model of allergic airway inflammation. Notably, since several series of FFA2 receptor antagonists are inactive against the mouse FFA2 receptor, such as GLPG0974 (Sergeev et al., JBC 2016) and CATPB (Hudson et al. 2012) this has limited the breadth of preclinical studies that can be undertaken. Whether FFA2 receptor agonists may, as a significant portion of researchers have proposed, be a better approach for IBD awaits identification of a clinical candidate and human trials.

3.3 Other Indications

Although it is too early to know whether therapeutics directed against FFA receptors may have a place in other indications, there is some justifiable rationale, at least preclinically, for an FFA receptor-based treatment on the horizon in diseases such as neurodegeneration, pain, and cancer.

3.3.1 Neuronal

Recent research has suggested that modulation of GPR84 may be a novel therapeutic approach for neuronal degenerative diseases such as Alzheimer's. GPR84 is expressed in microglia and expression is increased in a mouse model of Alzheimer's. Moreover, mice lacking GPR84 exhibited accelerated dendrite degeneration and reduced microgliosis, consistent with the hypothesis that β-amyloid induced microgliosis is required for dendritic homeostasis (Audoy-Rémus et al. 2015). Amongst the known ligands for GPR84, 2-Hydroxy-FAs are present in several tissues, especially in the brain, and are formed by fatty acid 2-hydroxylase (FA2H) and degraded by α-oxidation (Guo et al. 2012). FA2H itself has been found to be important in a number of neurodegenerative diseases in mice and humans (Potter et al. 2011; Zöller et al., J. Neurosci. 2008). In addition it is possible that fatty acid metabolites generated as a result of β-amyloid production could result in GPR84 activation, thus mounting a protective response. These data would suggest that an agonist would be desirable for this indication; however at present no GPR84 small-molecule agonists have been revealed in the public domain. Moreover, it raises speculation as to whether long-term administration of GPR84 antagonists being study for UC, should they be brain penetrant, may accelerate the progression of Alzheimer's. Very recently studies with the FFA1/FFA4 receptor agonist GW9508 in a model of Alzheimer's improved cognitive performance (Khan et al. 2016). While the FFA1 receptor is expressed in areas of the brain involved in learning and memory (Briscoe et al. 2003), further association of the receptor with Alzheimer's requires testing of more selective agonists.

3.3.2 Cancer

Although controversial, some reports have suggested that ω-3 fatty acids may play a protective role in a number of cancers (Gerber 2012), others have concluded that there is either no effect (MacLean et al. 2006) or that they may promote cancer risk (Brasky et al. 2013). Recent preclinical reports using the selective small-molecule FFA4 receptor agonist TUG-891 suggests that FFA4 activation may inhibit proliferation of human prostate and breast cancer cells in response to mitogens (Hopkins et al. 2016; Liu et al. 2015). While there have been some reports of association between the microbiota, SCFAs, and colorectal cancer (Hester et al. 2015), there are no reports of selective small molecules for FFA2 or FFA3 receptors having been tested preclinically in a relevant model.

3.3.3 Pain

There is speculation as to whether the FFA1 receptor may play a role in pain based on its expression in brain in rodents and humans (Nakamoto et al. 2012), as well as the antinociceptive properties of ω-3 PUFAs (Tokuyama and Nakamoto 2011). Preclinical evidence to support the rationale for an FFA1 receptor agonist in the treatment of pain comes from studies using the tool agonist GW9508 and the FFA1 receptor antagonist GW1100 in various pain models, including formalin-induced pain behavior, Freund's adjuvant-induced mechanical allodynia, thermal hyperalgesia (Nakamoto et al. 2013), and in descending pain control (Nakamoto

et al. 2015). Further progress towards understanding how the treatment of pain could translate will benefit from testing of more selective and potent agonists.

4 Recommendations and the Therapeutic Future for FFAs

The theme underlying this chapter's discussion has focused on the drive to unlock the therapeutic potential of the FFA family of receptors and GPR84. Key issues in moving from basic pharmacology to medicine are summarized by the following points:

- Identification of potent and selective compounds has been challenging
- Species variation in receptor pharmacology has made comparative or translational studies difficult
- Development of potent/selective compounds is essential to characterize the receptor pharmacology and physiological role of the receptors
- It is unclear based on existing clinical data whether any of the FFA receptors can be the target of a translational therapy in any disease area

In particular, the regulation of the immune system by FFAs requires a greater understanding in order to target the relevant chronic inflammatory diseases appropriately (Ang and Ding 2016). This point is reinforced by clinical trials with FFA2 receptor antagonists where robust engagement of the target biomarker was reported, but this did not translate to efficacy. Utilization of better tools may contribute to progress in this field, such as the computer-based evidence-based translation to help translate gene expression changes observed in murine inflammation models to human (Seok 2015). Taking advantage of more recent homology models of FFA receptors (Tikhonova and Poerio 2015) and recently developed technologies such as the FFA1 receptor tracer to characterize ligands in competition-binding studies (Christiansen et al. 2016) may also accelerate the pace of drug development. With respect to regulation of metabolism, the FFA1 receptor stands out as an example of the FFA family where translation of preclinical efficacy has shown promise. Based on the clinical efficacy of TAK-875 in type 2 diabetics, a FFA1 receptor agonist holds potential for a new diabetes therapy, should a suitably safe molecule be identified. Moreover, the idea of a dual agonist of FFA1 and FFA4 receptors that would target pancreatic islets, adipose, and inflammation as a multi-pronged approach to type 2 diabetes could provide a novel medicine, should such a molecule be identified. More recently, suggestions have emerged that FFA receptors could play a role in indications such as Alzheimer's disease, cancer, and pain. Given that many of the links to these diseases are based on KO mouse models, and/or less than optimal tool compounds, it will require further detailed analysis of efficacy in relevant preclinical models, and ideally human ex vivo samples, to provide a solid foundation for translation.

To conclude, there is a large body of evidence to suggest association between free fatty acids and disease modulation. The implication that some of the effects of

fatty acids could be through a GPCR, the most successful drug target class, has paved the way for large discovery efforts in this area. Given that the initial deorphanization of the FFA1, 2, and 3 receptors was in 2003, with discovery of fatty acids as ligands for the FFA4 receptor and GPR84 in 2005 and 2006 respectively, the few small-molecules tested clinically reflects the difficulty in identifying suitable potent and selective agonists and antagonists for the receptors. We have highlighted a number of questions above that may underlie the future ability to translate receptor pharmacology into the clinic for the selected indications. Many of the current approaches used in the FFA receptor field to date to validate a proposed indication for the receptors can be improved, given awareness of the complexity of the receptor physiology and pharmacology. In particular as more selective small-molecules are identified reliance on phenotypes of knockout mouse models may decrease with more emphasis on testing in human ex vivo samples or primary cells to help position future drugs in the clinic. Promising clinical data with the FFA1 receptor in type 2 diabetes serves as a precedent that FFA receptors have the potential to be bona fide drug targets and the basis for novel therapeutics for several areas of unmet need.

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References

Ang Z, Ding JL (2016) GPR41 and GPR43 in obesity and inflammation – protective or causative? Front Immunol 7:28. doi:10.3389/fimmu.2016.00028

Apple FS, Wu AH, Mair J, Ravkilde J, Panteghini M, Tate J, Pagani F, Christenson RH, Mockel M, Danne O, Jaffe AS, Committee on Standardization of Markers of Cardiac Damage of the IFCC (2005) Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. Clin Chem 51:810–824. doi:10.1373/clinchem.2004.046292

Audoy-Rémus J, Bozoyan L, Dumas A, Filali M, Lecours C, Lacroix S, Rivest S, Tremblay ME, Vallières L (2015) GPR84 deficiency reduces microgliosis, but accelerates dendritic degeneration and cognitive decline in a mouse model of Alzheimer's disease. Brain Behav Immun 46:112–120. doi:10.1016/j.bbi.2015.01.010

Araki T, Hirayama M, Hiroi S, Kaku K (2012) GPR40-induced insulin secretion by the novel agonist TAK-875: first clinical findings in patients with type 2 diabetes. Diabetes Obes Metab 14(3):271–278. doi:10.1111/j.1463-1326.2011.01525.x

Azzazy HM, Pelsers MM, Christenson RH (2006) Unbound free fatty acids and heart-type fatty acid-binding protein: diagnostic assays and clinical applications. Clin Chem 52:19–29. doi:10.1373/clinchem.2005.056143

Baer DJ, Judd JT, Clevidence BA, Tracy RP (2004) Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized crossover study. Am J Clin Nutr 79:969–973

Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, Smith DM, Cawthorne MA, Stocker CJ, Arch JR (2013) Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. Br J Nutr 109:1755–1764. doi:10.1017/S0007114512003923

Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70:567–590

Bjursell M, Admyre T, Göransson M, Marley AE, Smith DM, Oscarsson J, Bohlooly-Y M (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. Am J Physiol Endocrinol Metab 300:E211–E220. doi:10.1152/ajpendo.00229.2010

Bjursell M, Xu X, Admyre T, Böttcher G, Lundin S, Nilsson R, Stone VM, Morgan NG, Lam YY, Storlien LH, Lindén D, Smith DM, Bohlooly-Y M, Oscarsson J (2014) The beneficial effects of n-3 polyunsaturated fatty acids on diet induced obesity and impaired glucose control do not require Gpr120. PLoS One 9, e114942. doi:10.1371/journal.pone.0114942

Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, Dejong CH (2009) Short chain fatty acids exchange across the gut and liver in humans measured at surgery. Clin Nutr 28:657–661. doi:10.1016/j.clnu.2009.05.011

Burant CF, Viswanathan P, Marcinak J, Cao C, Vakilynejad M, Xie B, Leifke E (2012) TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial. Lancet 379(9824):1403–1411. doi:10.1016/S0140-6736(11)61879-5

Burant CF (2013) Activation of GPR40 as a therapeutic target for the treatment of type 2 diabetes. Diabetes Care 36(Suppl 2):S175–S179. doi:10.2337/dcS13-2037

Brasky TM, Darke AK, Song X, Tangen CM, Goodman PJ, Thompson IM, Meyskens FL, Goodman GE, Minasian LM, Parnes HL, Klein EA, Kristal AR (2013) Plasma phospholipid fatty acids and prostate cancer risk in the SELECT trial. J Natl Cancer Inst 105:1132–1141. doi:10.1093/jnci/djt174

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311. doi:10.1074/jbc.M211495200

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 148:619–628. doi:10.1038/sj.bjp.0706770

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319. doi:10.1074/jbc.M211609200

Brownlie R, Mayers RM, Pierce JA, Marley AE, Smith DM (2008) The long-chain fatty acid receptor, GPR40, and glucolipotoxicity: investigations using GPR40-knockout mice. Biochem Soc Trans 36:950–954. doi:10.1042/BST0360950

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schröder R, Hudson BD, Milligan G, Cawthorne MA, Kostenis E, Kassack MU, Ulven T (2012) Free fatty acid receptor 1 (FFA1/GPR40) agonists: mesylpropoxy appendage lowers lipophilicity and improves ADME properties. J Med Chem 55:6624–6628. doi:10.1021/jm3002026

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schmidt J, Hansen SV, Hudson BD, Zaibi M, Markussen SB, Hagesaether E, Milligan G, Cawthorne MA, Kostenis E, Kassack MU, Ulven T (2013) Discovery of a potent and selective free fatty acid receptor 1 agonist with low lipophilicity and high oral bioavailability. J Med Chem 56:982–992. doi:10.1021/jm301470a

Christiansen E, Hudson BD, Hansen AH, Milligan G, Ulven T (2016) Development and characterization of a potent free fatty acid receptor 1 (FFA1) fluorescent tracer. J Med Chem. doi:10.1021/acs.jmedchem.6b00202

Cummings JH, Macfarlane GT (1997) Role of intestinal bacteria in nutrient metabolism. JPEN J Parenter Enteral Nutr 21:357–365

Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 28:1221–1227

Cummings DE, Arterburn DE, Westbrook EO, Kuzma JN, Stewart SD, Chan CP, Bock SN, Landers JT, Kratz M, Foster-Schubert KE, Flum DR (2016) Gastric bypass surgery vs intensive lifestyle and medical intervention for type 2 diabetes: the CROSSROADS randomised controlled trial. Diabetologia. doi:10.1007/s00125-016-3903-x

Darwish KM, Salama I, Mostafa S, Gomaa MS, Helal MA (2016) Design, synthesis, and biological evaluation of novel thiazolidinediones as PPARg/FFAR1 dual agonists. Eur J Med Chem 109:157–172. doi:10.1016/j.ejmech.2015.12.049

Defossa E, Wagner M (2014) Recent developments in the discovery of FFA1 receptor agonists as novel oral treatment for type 2 diabetes mellitus. Bioorg Med Chem Lett 24(14):2991–3000. doi:10.1016/j.bmcl.2014.05.019

de Souza RJ, Mente A, Maroleanu A, Cozma AI, Ha V, Kishibe T, Uleryk E, Budylowski P, Schünemann H, Beyene J, Anand SS (2015) Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies. BMJ 351:h3978

Demant EJ, Richieri GV, Kleinfeld AM (2002) Stopped-flow kinetic analysis of long-chain fatty acid dissociation from bovine serum albumin. Biochem J 363:809–815

Dobbins RL, Chester MW, Daniels MB, McGarry JD, Stein DT (1998) Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. Diabetes 47:1613–1618

Eliasson B, Liakopoulos V, Franzén S, Näslund I, Svensson AM, Ottosson J, Gudbjörnsdottir S (2015) Cardiovascular disease and mortality in patients with type 2 diabetes after bariatric surgery in Sweden: a nationwide, matched, observational cohort study. Lancet Diabetes Endocrinol 3:847–854. doi:10.1016/S2213-8587(15)00334-4

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nøhr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2:376–392. doi:10.1016/j.molmet.2013.08.006

Forbes S, Stafford S, Coope G, Heffron H, Real K, Newman R, Davenport R, Barnes M, Grosse J, Cox H (2015) Selective FFA2 agonism appears to act via Intestinal PYY to reduce transit and food intake but does not improve glucose tolerance in mouse models. Diabetes 64:3763–3771. doi:10.2337/db15-0481

Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, Tian H, Li Y (2008) Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 149:4519–4526. doi:10.1210/en.2008-0059

Gerber M (2012) Omega-3 fatty acids and cancers: a systematic update review of epidemiological studies. Br J Nutr 107(Suppl 2):S228–S239. doi:10.1017/S0007114512001614

Greenough WB, Crespin SR, Steinberg D (1967) Hypoglycaemia and hyperinsulinaemia in response to raised free-fatty-acid levels. Lancet 2:1334–1336

Guo L, Zhang X, Zhou D, Okunade AL, Su X (2012) Stereospecificity of fatty acid 2-hydroxylase and differential functions of 2-hydroxy fatty acid enantiomers. J Lipid Res 53:1327–1335. doi:10.1194/jlr.M025742

Hamdouchi C, Kahl SD, Patel Lewis A, Cardona GR, Zink RW, Chen K, Eessalu TE, Ficorilli JV, Marcelo MC, Otto KA, Wilbur KL, Lineswala JP, Piper JL, Coffey DS, Sweetana SA, Haas JV, Brooks DA, Pratt EJ, Belin RM, Deeg MA, Ma X, Cannady EA, Johnson JT, Yumibe NP, Chen Q, Maiti P, Montrose-Rafizadeh C, Chen Y, and Reifel Miller A (2016) The discovery, preclinical, and early clinical development of potent and selective GPR40 agonists for the treatment of type 2 diabetes mellitus (LY2881835, LY2922083, and LY2922470). J Med Chem doi: 10.1021/acs.jmedchem.6b00892

Hamid YH, Vissing H, Holst B, Urhammer SA, Pyke C, Hansen SK, Glümer C, Borch-Johnsen K, Jørgensen T, Schwartz TW, Pedersen O, Hansen T (2005) Studies of relationships between

variation of the human G protein-coupled receptor 40 gene and type 2 diabetes and insulin release. Diabet Med 22:74–80. doi:10.1111/j.1464-5491.2005.01505.x

Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di Salvo J, Weinglass AB, Engelstoft MS, Madsen AN, Lückmann M, Miller MW, Trujillo ME, Frimurer TM, Holst B, Howard AD, Schwartz TW (2015) GPR40 (FFAR1) – combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo. Mol Metab 4:3–14. doi:10.1016/j.molmet.2014.10.002

Hester CM, Jala VR, Langille MG, Umar S, Greiner KA, Haribabu B (2015) Fecal microbes, short chain fatty acids, and colorectal cancer across racial/ethnic groups. World J Gastroenterol 21:2759–2769. doi:10.3748/wjg.v21.i9.2759

Hiatt WR, Kaul S, Smith RJ (2013) The cardiovascular safety of diabetes drugs--insights from the rosiglitazone experience. N Engl J Med 369:1285–1287. doi:10.1056/NEJMp1309610

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94. doi:10.1038/nm1168

Hopkins MM, Zhang Z, Liu Z, Meier KE (2016) Eicosopentaneoic acid and other free fatty acid receptor agonists inhibit lysophosphatidic acid- and epidermal growth factor-induced proliferation of human breast cancer cells. J Clin Med 5. doi:10.3390/jcm5020016

Houze JB, Zhu L, Sun Y, Akerman M, Qiu W, Zhang AJ, Sharma R, Schmitt M, Wang Y, Liu J, Liu J, Medina JC, Reagan JD, Luo J, Tonn G, Zhang J, Lu JY, Chen M, Lopez E, Nguyen K, Yang L, Tang L, Tian H, Shuttleworth SJ, Lin DC (2012) AMG 837: a potent, orally bioavailable GPR40 agonist. Bioorg Med Chem Lett 22(2):1267–1270. doi:10.1016/j.bmcl.2011.10.118

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G (2012) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. J Biol Chem 287:41195–41209. doi:10.1074/jbc.M112.396259

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, Tikhonova IG, Ulven T, Milligan G (2013a) Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288:17296–17312. doi:10.1074/jbc.M113.455337

Hudson BD, Murdoch H, Milligan G (2013b) Minireview: The effects of species ortholog and SNP variation on receptors for free fatty acids. Mol Endocrinol 27:1177–1187. doi:10.1210/me.2013-1085

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, Heathcote H, Tobin AB, Ulven T, Milligan G (2013c) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 84:710–725. doi:10.1124/mol.113.087783

Hudson BD, Christiansen E, Murdoch H, Jenkins L, Hansen AH, Madsen O, Ulven T, Milligan G (2014a) Complex pharmacology of novel allosteric free fatty acid 3 receptor ligands. Mol Pharmacol 86:200–210. doi:10.1124/mol.114.093294

Hudson BD, Shimpukade B, Milligan G, Ulven T (2014b) The molecular basis of ligand interaction at free fatty acid receptor 4 (FFA4/GPR120). J Biol Chem 289:20345–20358. doi:10.1074/jbc.M114.561449

Humphries PS, Benbow JW, Bonin PD, Boyer D, Doran SD, Frisbie RK, Piotrowski DW, Balan G, Bechle BM, Conn EL, Dirico KJ, Oliver RM, Soeller WC, Southers JA, Yang X (2009) Synthesis and SAR of 1,2,3,4-tetrahydroisoquinolin-1-ones as novel G-protein-coupled receptor 40 (GPR40) antagonists. Bioorg Med Chem Lett 19:2400–2403. doi:10.1016/j.bmcl.2009.03.082

Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, Choquet H, Besnard P, Lecoeur C, Vivequin S, Ayukawa K, Takeuchi M, Ozawa K, Tauber M, Maffeis C, Morandi A, Buzzetti R, Elliott P, Pouta A,

Jarvelin MR, Körner A, Kiess W, Pigeyre M, Caiazzo R, Van Hul W, Van Gaal L, Horber F, Balkau B, Lévy-Marchal C, Rouskas K, Kouvatsi A, Hebebrand J, Hinney A, Scherag A, Pattou F, Meyre D, Koshimizu TA, Wolowczuk I, Tsujimoto G, Froguel P (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 483:350–354. doi:10.1038/nature10798

Inagaki N, Kondo K, Yoshinari T, Maruyama N, Susuta Y, Kuki H (2013) Efficacy and safety of canagliflozin in Japanese patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, 12-week study. Diabetes Obes Metab 15:1136–1145. doi:10.1111/dom.12149

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422:173–176. doi:10.1038/nature01478

Jouven X, Charles MA, Desnos M, Ducimetière P (2001) Circulating nonesterified fatty acid level as a predictive risk factor for sudden death in the population. Circulation 104:756–761

Kaku K, Araki T, Yoshinaka R (2013) Randomized, double-blind, dose-ranging study of TAK-875, a novel GPR40 agonist, in Japanese patients with inadequately controlled type 2 diabetes. Diabetes Care 36(2):245–250. doi:10.2337/dc12-0872

Kaku K, Enya K, Nakaya R, Ohira T, Matsuno R (2015) Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial. Diabetes Obes Metab 17(7):675–681. doi:10.1111/dom.12467

Kalis M, Levéen P, Lyssenko V, Almgren P, Groop L, Cilio CM (2007) Variants in the FFAR1 gene are associated with beta cell function. PLoS One 2, e1090. doi:10.1371/journal.pone.0001090

Kang SU (2013) GPR119 agonists: a promising approach for T2DM treatment? A SWOT analysis of GPR119. Drug Discov Today 18:1309–1315. doi:10.1016/j.drudis.2013.09.011

Kebede M, Alquier T, Latour MG, Semache M, Tremblay C, Poitout V (2008) The fatty acid receptor GPR40 plays a role in insulin secretion in vivo after high-fat feeding. Diabetes 57:2432–2437. doi:10.2337/db08-0553

Khan MZ, He L (2015) The role of polyunsaturated fatty acids and GPR40 receptor in brain. Neuropharmacology. doi:10.1016/j.neuropharm.2015.05.013

Khan MZ, Zhuang X, He L (2016) GPR40 receptor activation leads to CREB phosphorylation and improves cognitive performance in an Alzheimer's disease mouse model. Neurobiol Learn Mem 131:46–55. doi:10.1016/j.nlm.2016.03.006

Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH (2013) Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 145:396–406.e1-10. doi:10.1053/j.gastro.2013.04.056

Kim CH, Park J, Kim M (2014) Gut microbiota-derived short-chain Fatty acids, T cells, and inflammation. Immune Netw 14:277–288. doi:10.4110/in.2014.14.6.277

Kimura I (2014) Host energy regulation via SCFAs receptors, as dietary nutrition sensors, by gut microbiota. Yakugaku Zasshi 134:1037–1042

Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, Tani T, Takahashi T, Miyauchi S, Shioi G, Inoue H, Tsujimoto G (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829. doi:10.1038/ncomms2852

Konda VR, Desai A, Darland G, Grayson N, Bland JS (2014) KDT501, a derivative from hops, normalizes glucose metabolism and body weight in rodent models of diabetes. PLoS One 9, e87848. doi:10.1371/journal.pone.0087848

Kotarsky K, Nilsson NE, Olde B, Owman C (2003) Progress in methodology. Improved reporter gene assays used to identify ligands acting on orphan seven-transmembrane receptors. Pharmacol Toxicol 93:249–258

Kuwahara A (2014) Contributions of colonic short-chain fatty acid receptors in energy homeostasis. Front Endocrinol 5:144. doi:10.3389/fendo.2014.00144

Lan H, Hoos LM, Liu L, Tetzloff G, Hu W, Abbondanzo SJ, Vassileva G, Gustafson EL, Hedrick JA, Davis HR (2008) Lack of FFAR1/GPR40 does not protect mice from high-fat diet-induced metabolic disease. Diabetes 57:2999–3006. doi:10.2337/db08-0596

Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V (2007) GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. Diabetes 56:1087–1094. doi:10.2337/db06-1532

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278:25481–25489. doi:10.1074/jbc.M301403200

Lin DC, Zhang J, Zhuang R, Li F, Nguyen K, Chen M, Tran T, Lopez E, Lu JY, Li XN, Tang L, Tonn GR, Swaminath G, Reagan JD, Chen JL, Tian H, Lin YJ, Houze JB, Luo J (2011) AMG 837: a novel GPR40/FFA1 agonist that enhances insulin secretion and lowers glucose levels in rodents. PLoS One 6, e27270. doi:10.1371/journal.pone.0027270

Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J, Vimolratana M, Jiao XY, Wang Y, Birdsall NJ, Swaminath G (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859. doi:10.1124/mol.112.079640

Liu Z, Hopkins MM, Zhang Z, Quisenberry CB, Fix LC, Galvan BM, Meier KE (2015) Omega-3 fatty acids and other FFA4 agonists inhibit growth factor signaling in human prostate cancer cells. J Pharmacol Exp Ther 352:380–394. doi:10.1124/jpet.114.218974

Lopez-Garcia E, Schulze MB, Meigs JB, Manson JE, Rifai N, Stampfer MJ, Willett WC, Hu FB (2005) Consumption of trans fatty acids is related to plasma biomarkers of inflammation and endothelial dysfunction. J Nutr 135:562–566

Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, Vimolratana M, Houze JB, Wong S, Toteva M, Shan B, Li F, Zhuang R, Lin DC (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7, e46300. doi:10.1371/journal.pone.0046300

Luttrell LM, Gesty-Palmer D (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. Pharmacol Rev 62:305–330. doi:10.1124/pr.109.002436

Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR (2015) Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun 6:6734. doi:10.1038/ncomms7734

MacLean CH, Newberry SJ, Mojica WA, Khanna P, Issa AM, Suttorp MJ, Lim YW, Traina SB, Hilton L, Garland R, Morton SC (2006) Effects of omega-3 fatty acids on cancer risk: a systematic review. JAMA 295:403–415. doi:10.1001/jama.295.4.403

Mancini AD, Poitout V (2015) GPR40 agonists for the treatment of type 2 diabetes: life after 'TAKing' a hit. Diabetes Obes Metab 17:622–629. doi:10.1111/dom.12442

Mancini AD, Bertrand G, Vivot K, Carpentier É, Tremblay C, Ghislain J, Bouvier M, Poitout V (2015) β-Arrestin recruitment and biased agonism at free fatty acid receptor 1. J Biol Chem 290:21131–21140. doi:10.1074/jbc.M115.644450

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461:1282–1286. doi:10.1038/nature08530

Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, Izawa S, Kondo Y, Ito Y, Tamura Y, Yanamoto K, Noda H, Tanabe A, Okaniwa N, Yamaguchi Y, Iwamoto T, Kasugai K (2013) G protein-coupled receptor 43 moderates gut inflammation through cytokine

regulation from mononuclear cells. Inflamm Bowel Dis 19:2848–2856. doi:10.1097/01.MIB.0000435444.14860.ea

McNelis JC, Lee YS, Mayoral R, van der Kant R, Johnson AM, Wollam J, Olefsky JM (2015) GPR43 potentiates β-cell function in obesity. Diabetes 64:3203–3217. doi:10.2337/db14-1938

Milligan G, Alvarez-Curto E, Watterson KR, Ulven T, Hudson BD (2015) Characterizing pharmacological ligands to study the long-chain fatty acid receptors GPR40/FFA1 and GPR120/FFA4. Br J Pharmacol 172:3254–3265. doi:10.1111/bph.12879

Montalban-Arques A, De Schryver P, Bossier P, Gorkiewicz G, Mulero V, Gatlin DM, Galindo-Villegas J (2015) Selective manipulation of the gut microbiota improves immune status in vertebrates. Front Immunol 6:512. doi:10.3389/fimmu.2015.00512

Nakamoto K, Nishinaka T, Matsumoto K, Kasuya F, Mankura M, Koyama Y, Tokuyama S (2012) Involvement of the long-chain fatty acid receptor GPR40 as a novel pain regulatory system. Brain Res 1432:74–83. doi:10.1016/j.brainres.2011.11.012

Nakamoto K, Nishinaka T, Sato N, Mankura M, Koyama Y, Kasuya F, Tokuyama S (2013) Hypothalamic GPR40 signaling activated by free long chain fatty acids suppresses CFA-induced inflammatory chronic pain. PLoS One 8, e81563. doi:10.1371/journal.pone.0081563

Nakamoto K, Nishinaka T, Sato N, Aizawa F, Yamashita T, Mankura M, Koyama Y, Kasuya F, Tokuyama S (2015) The activation of supraspinal GPR40/FFA1 receptor signalling regulates the descending pain control system. Br J Pharmacol 172:1250–1262. doi:10.1111/bph.13003

Namour F, Galien R, Van Kaem T, Van der Aa A, Vanhoutte F, Beetens J, Van't Klooster G (2016) Safety, pharmacokinetics and pharmacodynamics of GLPG0974, a potent and selective FFA2 antagonist, in healthy male subjects. Br J Clin Pharmacol. doi:10.1111/bcp.12900

Negoro N, Sasaki S, Mikami S, Ito M, Suzuki M, Tsujihata Y, Ito R, Harada A, Takeuchi K, Suzuki N, Miyazaki J, Santou T, Odani T, Kanzaki N, Funami M, Tanaka T, Kogame A, Matsunaga S, Yasuma T, Momose Y (2010) Discovery of TAK-875: a potent, selective, and orally bioavailable GPR40 agonist. ACS Med Chem Lett 1:290–294. doi:10.1021/ml1000855

Nilsson NE, Kotarsky K, Owman C, Olde B (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 303:1047–1052

Nøhr MK, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, Han S, Jones RM, Offermanns S, Schwartz TW (2013) GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinology 154:3552–3564. doi:10.1210/en.2013-1142

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698. doi:10.1016/j.cell.2010.07.041

Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR, Sasik R, Hah N, Chi TJ, Cox JM, Powels MA, Di Salvo J, Sinz C, Watkins SM, Armando AM, Chung H, Evans RM, Quehenberger O, McNelis J, Bogner-Strauss JG, Olefsky JM (2014) A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med 20:942–947. doi:10.1038/nm.3614

Omar B, Ahrén B (2014) Pleiotropic mechanisms for the glucose-lowering action of DPP-4 inhibitors. Diabetes 63:2196–2202. doi:10.2337/db14-0052

Palau-Rodriguez M, Tulipani S, Isabel Queipo-Ortuño M, Urpi-Sarda M, Tinahones FJ, Andres-Lacueva C (2015) Metabolomic insights into the intricate gut microbial-host interaction in the development of obesity and type 2 diabetes. Front Microbiol 6:1151. doi:10.3389/fmicb.2015.01151

Park SC, Jeen YT (2015) Current and emerging biologics for ulcerative colitis. Gut Liver 9:18–27. doi:10.5009/gnl14226

Park BO, Kim SH, Kong GY, Kim da H, Kwon MS, Lee SU, Kim MO, Cho S, Lee S, Lee HJ, Han SB, Kwak YS, Lee SB, Kim S (2016) Selective novel inverse agonists for human GPR43 augment GLP-1 secretion. Eur J Pharmacol 771:1–9. doi:10.1016/j.ejphar.2015.12.010

Pfeffer MA, Claggett B, Diaz R, Dickstein K, Gerstein HC, Køber LV, Lawson FC, Ping L, Wei X, Lewis EF, Maggioni AP, McMurray JJ, Probstfield JL, Riddle MC, Solomon SD, Tardif JC, Investigators E (2015) Lixisenatide in patients with type 2 diabetes and acute coronary syndrome. N Engl J Med 373:2247–2257. doi:10.1056/NEJMoa1509225

Pizzonero M, Dupont S, Babel M, Beaumont S, Bienvenu N, Blanqué R, Cherel L, Christophe T, Crescenzi B, De Lemos E, Delerive P, Deprez P, De Vos S, Djata F, Fletcher S, Kopiejewski S, L'Ebraly C, Lefrançois JM, Lavazais S, Manioc M, Nelles L, Oste L, Polancec D, Quénéhen V, Soulas F, Triballeau N, van der Aar EM, Vandeghinste N, Wakselman E, Brys R, Saniere L (2014) Discovery and optimization of an azetidine chemical series as a free fatty acid receptor 2 (FFA2) antagonist: from hit to clinic. J Med Chem 57:10044–10057. doi:10.1021/jm5012885

Potter KA, Kern MJ, Fullbright G, Bielawski J, Scherer SS, Yum SW, Li JJ, Cheng H, Han X, Venkata JK, Khan PA, Rohrer B, Hama H (2011) Central nervous system dysfunction in a mouse model of FA2H deficiency. Glia 59:1009–1021. doi:10.1002/glia.21172

Priyadarshini M, Layden BT (2015) FFAR3 modulates insulin secretion and global gene expression in mouse islets. Islets 7, e1045182. doi:10.1080/19382014.2015.1045182

Priyadarshini M, Villa SR, Fuller M, Wicksteed B, Mackay CR, Alquier T, Poitout V, Mancebo H, Mirmira RG, Gilchrist A, Layden BT (2015) An acetate-specific GPCR, FFAR2, regulates insulin secretion. Mol Endocrinol 29:1055–1066. doi:10.1210/me.2015-1007

Rajagopal S, Rajagopal K, Lefkowitz RJ (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov 9:373–386. doi:10.1038/nrd3024

Richieri GV, Kleinfeld AM (1995) Unbound free fatty acid levels in human serum. J Lipid Res 36:229–240

Richieri GV, Ogata RT, Kleinfeld AM (1992) A fluorescently labeled intestinal fatty acid binding protein. Interactions with fatty acids and its use in monitoring free fatty acids. J Biol Chem 267:23495–23501

Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 105:16767–16772. doi:10.1073/pnas.0808567105

Sato J, Makita N, Iiri T (2016) Inverse agonism: the classic concept of GPCRs revisited. Endocr J. doi:10.1507/endocrj.EJ16-0084

Seok J (2015) Evidence-based translation for the genomic responses of murine models for the study of human immunity. PLoS One 10, e0118017. doi:10.1371/journal.pone.0118017

Sergeev E, Hansen AH, Pandey SK, Mackenzie AE, Hudson BD, Ulven T, Milligan G (2016) Non-equivalence of key positively charged residues of the free fatty acid 2 receptor in the recognition and function of agonist versus antagonist ligands. J Biol Chem 291(1):303–317. doi:10.1074/jbc.M115.687939

Shimpukade B, Hudson BD, Hovgaard CK, Milligan G, Ulven T (2012) Discovery of a potent and selective GPR120 agonist. J Med Chem 55:4511–4515. doi:10.1021/jm300215x

Sina C, Gavrilova O, Förster M, Till A, Derer S, Hildebrand F, Raabe B, Chalaris A, Scheller J, Rehmann A, Franke A, Ott S, Häsler R, Nikolaus S, Fölsch UR, Rose-John S, Jiang HP, Li J, Schreiber S, Rosenstiel P (2009) G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 183:7514–7522. doi:10.4049/jimmunol.0900063

Smith NJ, Stoddart LA, Devine NM, Jenkins L, Milligan G (2009) The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1. J Biol Chem 284:17527–17539. doi:10.1074/jbc.M109.012849

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341:569–573. doi:10.1126/science.1241165

Song C, Shieh CH, Wu YS, Kalueff A, Gaikwad S, Su KP (2016) The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: acting separately or synergistically? Prog Lipid Res 62:41–54. doi:10.1016/j.plipres.2015.12.003

Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H (2005) The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab 1:245–258. doi:10.1016/j.cmet.2005.03.007

Stoddart LA, Smith NJ, Milligan G (2008) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev 60:405–417. doi:10.1124/pr.108.00802

Suckow AT, Polidori D, Yan W, Chon S, Ma JY, Leonard J, Briscoe CP (2014) Alteration of the glucagon axis in GPR120 (FFAR4) knockout mice: a role for GPR120 in glucagon secretion. J Biol Chem 289:15751–15763. doi:10.1074/jbc.M114.568683

Tang C, Ahmed K, Gille A, Lu S, Gröne HJ, Tunaru S, Offermanns S (2015) Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nat Med 21:173–177. doi:10.1038/nm.3779

Terauchi Y, Satoi Y, Takeuchi M, Imaoka T (2014) Monotherapy with the once weekly GLP-1 receptor agonist dulaglutide for 12 weeks in Japanese patients with type 2 diabetes: dose-dependent effects on glycaemic control in a randomised, double-blind, placebo-controlled study. Endocr J 61:949–959

Tikhonova IG, Poerio E (2015) Free fatty acid receptors: structural models and elucidation of ligand binding interactions. BMC Struct Biol 15:16. doi:10.1186/s12900-015-0044-2

Tokuyama S, Nakamoto K (2011) Unsaturated fatty acids and pain. Biol Pharm Bull 34:1174–1178

Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM (2012) Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 61:364–371. doi:10.2337/db11-1019

Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, Marsland BJ (2014) Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20:159–166. doi:10.1038/nm.3444

van Eijk HM, Bloemen JG, Dejong CH (2009) Application of liquid chromatography-mass spectrometry to measure short chain fatty acids in blood. J Chromatogr B Analyt Technol Biomed Life Sci 877:719–724. doi:10.1016/j.jchromb.2009.01.039

Venkataraman C, Kuo F (2005) The G-protein coupled receptor, GPR84 regulates IL-4 production by T lymphocytes in response to CD3 crosslinking. Immunol Lett 101:144–153. doi:10.1016/j.imlet.2005.05.010

Vermeire S, Kojecký V, Knoflícek V, Reinisch W, Van Kaem T, Namour F, Beetens J, Vanhoutte F (2015) GLPG0974, an FFA2 antagonist, in ulcerative colitis: efficacy and safety in a multicenter proof of concept. J Crohn's Colitis Abstr. 10th Congress of ECCO: S39

Vettor R, Granzotto M, De Stefani D, Trevellin E, Rossato M, Farina MG, Milan G, Pilon C, Nigro A, Federspil G, Vigneri R, Vitiello L, Rizzuto R, Baratta R, Frittitta L (2008) Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization. J Clin Endocrinol Metab 93:3541–3550. doi:10.1210/jc.2007-2680

Vieira AT, Macia L, Galvão I, Martins FS, Canesso MC, Amaral FA, Garcia CC, Maslowski KM, De Leon E, Shim D, Nicoli JR, Harper JL, Teixeira MM, Mackay CR (2015) A role for gut microbiota and the metabolite-sensing receptor GPR43 in a murine model of gout. Arthritis Rheumatol 67:1646–1656. doi:10.1002/art.39107

Wang J, Wu X, Simonavicius N, Tian H, Ling L (2006) Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem 281:34457–34464. doi:10.1074/jbc.M608019200

Wang Y, Jiao X, Kayser F, Liu J, Wang Z, Wanska M, Greenberg J, Weiszmann J, Ge H, Tian H, Wong S, Schwandner R, Lee T, Li Y (2010) The first synthetic agonists of FFA2: discovery and SAR of phenylacetamides as allosteric modulators. Bioorg Med Chem Lett 20:493–498. doi:10.1016/j.bmcl.2009.11.112

Watson SJ, Brown AJ, Holliday ND (2012) Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 81:631–642. doi:10.1124/mol.111.077388

Williams IR, Parkos CA (2007) Colonic neutrophils in inflammatory bowel disease: double-edged swords of the innate immune system with protective and destructive capacity. Gastroenterology 133:2049–2052. doi:10.1053/j.gastro.2007.10.031

Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101:1045–1050. doi:10.1073/pnas.2637002100

Xiong Y, Swaminath G, Cao Q, Yang L, Guo Q, Salomonis H, Lu J, Houze JB, Dransfield PJ, Wang Y, Liu JJ, Wong S, Schwandner R, Steger F, Baribault H, Liu L, Coberly S, Miao L, Zhang J, Lin DC, Schwarz M (2013) Activation of FFA1 mediates GLP-1 secretion in mice. Evidence for allosterism at FFA1. Mol Cell Endocrinol 369:119–129. doi:10.1016/j.mce.2013.01.009

Yabuki C, Komatsu H, Tsujihata Y, Maeda R, Ito R, Matsuda-Nagasumi K, Sakuma K, Miyawaki K, Kikuchi N, Takeuchi K, Habata Y, Mori M (2013) A novel antidiabetic drug, fasiglifam/TAK-875, acts as an ago-allosteric modulator of FFAR1. PLoS One 8, e76280. doi:10.1371/journal.pone.0076280

Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJ, Smith DM, Arch JR (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett 584:2381–2386. doi:10.1016/j.febslet.2010.04.027

Zöller I, Meixner M, Hartmann D, Büssow H, Meyer R, Gieselmann V, Eckhardt M (2008) Absence of 2-hydroxylated sphingolipids is compatible with normal neural development but causes late-onset axon and myelin sheath degeneration. J Neurosci 28(39):9741–9754. doi:10.1523/JNEUROSCI.0458-08.2008

Polymorphic Variation in FFA Receptors: Functions and Consequences

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Contents

Abstract

Overfeeding of fat can cause various metabolic disorders including obesity and type 2 diabetes (T2D). Diet provided free fatty acids (FFAs) are not only essential nutrients, but they are also recognized as signaling molecules, which stimulate various important biological functions. Recently, several G protein-coupled receptors (GPCRs), including FFA1-4, have been identified as receptors of FFAs by various physiological and pharmacological studies. FFAs exert physiological functions through these FFA receptors (FFARs) depending on carbon chain length and degree of unsaturation. Functional analyses have revealed that several important metabolic processes, such as peptide hormone secretion, cell maturation and nerve activities, are regulated by FFARs and thereby FFARs contribute to the energy homeostasis through these physiological functions. Hence, FFARs are expected to be promising pharmacological targets

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for metabolic disorders since imbalances in energy homeostasis lead to metabolic disorders. In human, it is established that different responses of individuals to endogenous ligands and chemical drugs may be due to differences in the ability of such ligands to activate nucleotide polymorphic variants of receptors. However, the clear links between genetic variations that are involved in metabolic disorders and polymorphisms receptors have been relatively difficult to assess. In this review, I summarize current literature describing physiological functions of FFARs and genetic variations of those receptors to discuss the potential of FFARs as drug targets for metabolic disorders.

Keywords
Diabetes • Energy homeostasis • FFARs • Free fatty acids • Genetic variations • Genetics • Glucose • GPCRs • Insulin • Mutation • Polymorphic variations • Polymorphism • SNPs

1 Introduction

A lot of recent studies have revealed that various biomolecules derived from dietary foods, such as carbohydrate, lipid, and fiber, previously considered simply as nutrients and components of the body also act as essential signaling molecules to sense metabolic state and regulate whole body energy metabolism through, at least in part, members of the family of G protein-coupled receptors (GPCRs) (Priyadarshini et al. 2016; Watterson et al. 2014; Ulven and Christiansen 2015). Hence, from these aspects, dietary components have been considered as hormones (Ryan and Seeley 2013). Nutritional status is transmitted by the catabolites of food, which provides various simple biomolecules including free fatty acids (FFAs) in order to form a feedback loop. For example, short-chain fatty acids (SCFAs), which are generated by intestinal microbiota through fermentation of dietary fiber in the gut, not only act directly in the gut in which they are generated, but also stimulate various cell types such as immune cells and adipocytes after transport throughout the systemic circulation. Because of these characteristics, SCFAs are defined as hormone like biomolecules (Kimura et al. 2013; Maslowski et al. 2009; Macia et al. 2015; Masui et al. 2013; Trompette et al. 2014). Generally, mammals use not only glucose but also FFAs as key energy sources (Offermanns 2014; Spector and Kim 2014). On the other hand, a number of recent studies revealed that FFAs also act as signaling molecules to regulate a lot of physiological functions via their corresponding receptors. Several GPCRs have been successfully identified as receptors of FFAs (Brown et al. 2003; Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003a; Le Poul et al. 2003; Nilsson et al. 2003). SCFAs activate FFA2 and FFA3 whilst medium-chain and long-chain FFAs (MCFAs/LCFAs) activate FFA1 and FFA4 (Hirasawa et al. 2005; Itoh et al. 2003; Steneberg et al. 2005). FFAs regulate energy homeostasis via activation of these various FFAs receptors (FFARs) (Sekiguchi et al. 2015; Oh et al. 2010; Kimura et al. 2011; Ichimura

et al. 2012; Oh and Olefsky 2012; Kimura et al. 2013). FFARs are expressed in various tissues and cell types. Therefore, these receptors mediate essential physiological and pathological functions of FFAs in these tissues and cells. Hence, FFARs have potential as therapeutic targets of diseases, in particular metabolic disorders.

The genetic variation in the sequence of the same GPCR, resulting from non-synonymous single nucleotide polymorphisms (SNPs), can result in substantial differences in pharmacology and function of the receptor (Balasubramanian et al. 2005; Taneera et al. 2012). However, the functional changes and capacity of enzymes involved in drug metabolism are routinely considered as the main challenges of stratified medicine and individual use of therapeutics (Zhou et al. 2009). Recent genome-wide association studies showed that GPCRs including FFARs are also linked to disease via such SNPs (Insel et al. 2007; Taneera et al. 2012). Hence, in this review, I summarize and discuss the recent advances in research regarding FFARs and their polymorphisms.

2 Free Fatty Acid Receptors

More than 800 GPCRs are encoded in the human genomic DNA. Among them, approximately 350 GPCRs have been demonstrated to be activated by endogenously produced ligands (Fredriksson et al. 2003). The development of synthetic agonists and antagonists of specific GPCRs is essential to understand the physiological and pathological functions of individual receptors, as GPCRs have essential roles in the regulation of major physiological and pathological responses. Among the numbers of GPCRs which are stimulated and activated by metabolic biomolecules at least four receptors are identified as FFARs (Stoddart et al. 2008b). The GPR40 family genes, including GPR40, GPR41, GPR42, and GPR43 were identified in the course of a search for novel human galanin receptor subtypes in 1997 (Sawzdargo et al. 1997). The efforts to deorphanize these receptors successfully revealed that of these receptors, GPR40, GPR41 and GPR43 were activated by FFAs (Briscoe et al. 2003; Brown et al. 2003; Kotarsky et al. 2003a, b; Le Poul et al. 2003; Nilsson et al. 2003), and therefor these receptors were recognized as FFARs. They were renamed as FFA1, FFA3, and FFA2, respectively. GPR42 was first reported as inactive and a potential pseudogene. However, GPR42 was recently reported to be a functional polymorphism of GPR41. Overall identity between sequences of GPR42 and GPR41 is 98%. Furthermore, the transmembrane domain regions of GPR42 are 100% identical to that of GPR41 (Liaw and Connolly 2009). Subsequently, the phylogenetically distinct receptor for FFAs, GPR120, which was also renamed as FFA4 was identified (Hirasawa et al. 2005). FFA1, FFA2, and FFA3 genes are encoded closely at chromosome 19q13.1 and form a highly related gene family group in human (Stoddart et al. 2008b). Hence, these genes presumably developed from a single common ancestor via gene duplication. In contrast, FFA4 is not closely related in sequence to the other FFARs despite being activated by an overlapping group of MCFAs and LCFAs as FFA1 (Ichimura et al. 2014; Hirasawa et al. 2005). In

addition to the current FFA family members, there are several additional GPCRs, which appear to be activated by FFAs. GPR84 is an orphan receptor that recognizes MCFAs (Nagasaki et al. 2012; Suzuki et al. 2013), while the mouse olfactory receptor Olf78 (OR51E2 in human) appears to be activated by the SCFAs (Pluznick et al. 2013; Pluznick 2014). The following sections of this review will summarize recent advances in research regarding FFARs and their polymorphisms.

3 Polymorphic Variation of Receptors for SCFAs

SCFAs consist of carbon chain length from C2 to C6 and have various roles in physiological and pathological conditions. Generally, mammals use glucose as the main metabolic fuel under normal feeding conditions. SCFAs are produced by gut microbial fermentation of indigestible polysaccharides, including dietary fiber. Intestinal microbiota-derived SCFAs contribute a significant proportion of the daily energy requirement (Bergman 1990; Flint et al. 2008). On the other hand, the identification and deorphanization of FFA2 and FFA3 resulted in SCFAs being considered as signaling molecules through these receptors (Brown et al. 2003; Briscoe et al. 2003; Le Poul et al. 2003). FFA2 and FFA3 share 38% sequence identity with selectivity toward different fatty acid carbon chain length. Signaling through these receptors mediates numerous effects. Early studies identified various immune cells as expressing both of the SCFA receptors. In particular, FFA2 is highly expressed in monocytes and polymorphonuclear cells (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003). Both FFA2 and FFA3 are expressed in the gut, primarily by various enteroendocrine cells (Karaki et al. 2006, 2008; Tazoe et al. 2008, 2009). This may not be surprising, since the SCFAs are generated primarily in the gut through fermentation of dietary fiber. Both FFA2 and FFA3 have also been reported to be expressed in pancreatic β-cells (Kebede et al. 2008). Currently, the role of these receptors in regulating insulin secretion are attracting increasing attention (McNelis et al. 2015; Priyadarshini and Layden 2015; Priyadarshini et al. 2015; Tang et al. 2015). Expression of both FFA2 and FFA3 in adipose tissue has also been reported (Xiong et al. 2004; Han et al. 2014). Although the expression of FFA3 has been controversial, many studies have reported only FFA2 expression in adipose tissue (Hong et al. 2005; Hudson et al. 2013a; Zaibi et al. 2010). Many reports have revealed that FFA3 is expressed in the neurons present in both sympathetic ganglia and the enteric nervous system (Inoue et al. 2012; Kimura et al. 2011; Nohr et al. 2015). Furthermore, expression of both FFA2 and FFA3 has been found in various cancers, including breast, colon and liver cancer (Bindels et al. 2012; Tang et al. 2011; Yonezawa et al. 2007). Given these important roles of FFA2 and FFA3, genetic variation of these receptors could have various physiological and pathological effects.

3.1 FFA3 Functions and Polymorphisms

The deorphanization of FFA3 was reported in 2003. FFA3 was identified as a receptor for SCFAs (Brown et al. 2003; Le Poul et al. 2003). FFA3 is activated by SCFAs such as propionate (C3), butyrate (C4), and valerate (C5), which are produced by gut microbial fermentation of dietary fiber, with a rank order potency of $C3 = C4 = C5 > C6 > C2 > C1$ (Brown et al. 2003; Le Poul et al. 2003). Although FFA3 and FFA2 share the same SCFAs as ligands, the activation of FFA3 stimulates signaling pathways distinct from those of FFA2. FFA2 couples to both $G_{i/o}$ and G_q pathways, but FFA3 couples only to $G_{i/o}$ signaling (Stoddart et al. 2008b). Hence, ligand stimulation of FFA3 activates intracellular calcium ($[Ca^{2+}]_i$) responses and the phosphorylation of ERK1/2, but inhibits cAMP production. FFA3 is reported to be expressed in adipose tissue, intestinal tract, and the peripheral nervous system (Brown et al. 2003; Le Poul et al. 2003; Samuel et al. 2008; Kimura et al. 2011). Early FFA3 studies reported that FFA3 is highly expressed in adipose tissue and in adipocyte cell lines (Brown et al. 2003). FFA3 was reported to stimulate the secretion of leptin from adipocytes (Xiong et al. 2004). FFA3 knockdown decreased leptin secretion, whereas overexpression of FFA3 increased it (Xiong et al. 2004). Furthermore, propionate-stimulated leptin secretion was inhibited by pretreatment with pertussis toxin, which inhibits $G_{i/o}$ G proteins (Al-Lahham et al. 2010). However, these reports have been controversial because of many conflicting reports as to whether FFA3 and/or FFA2 is expressed in adipose tissue and adipocytes (Hong et al. 2005; Bellahcene et al. 2013; Zaibi et al. 2010). Many other groups failed to detect mRNA and/or protein expression of FFA3 in mouse adipose tissue (Kimura et al. 2011, 2013; Hong et al. 2005). In contrast, FFA2 expression has clearly been detected in mouse adipose tissues. Our study using FFA2-KO mice demonstrated that blood leptin levels were not affected by deficiency of FFA2, however, they were decreased by adipocyte-specific overexpression of FFA2 (Kimura et al. 2013). Since the effect of SCFAs on leptin secretion was eliminated by pertussis toxin treatment, this indicates that SCFA-mediated leptin secretion is linked with $G_{i/o}$-mediated signaling pathways (Xiong et al. 2004). Hence, either FFA2 and/or FFA3 could be involved in leptin secretion. Our study demonstrated that in vivo C2 administration suppressed insulin signaling in adipocytes of wild-type but not FFA2-KO mice (Kimura et al. 2013). By using adipocytes from FFA2-KO mice, it was clearly demonstrated that C2 inhibited insulin-stimulated glucose uptake in wild-type but not FFA2-KO mice and that this was blocked by pertussis toxin treatment but not by a G_q-targeted siRNA (Kimura et al. 2013). Hence, SCFA-stimulated leptin secretion appears to be mediated via FFA2, not FFA3 (Kimura et al. 2013). However, FFA3 knockdown or knockout can be associated with concomitant down-regulation of FFA2 expression (Zaibi et al. 2010). These controversial results made data interpretation difficult (Hudson et al. 2013b). Our recent studies have revealed functional roles of FFA3 expressed in the sympathetic ganglion in controlling energy expenditure in both fed and fasting states (Kimura et al. 2011). Adult FFA3-KO mice showed reduced noradrenaline. In addition, heart rate of FFA3-KO mice was decreased. Propionate

increased energy expenditure and heart rate in adult WT mice, whereas these effects were abolished in FFA3-KO mice. Moreover, the effect of propionate on the heart rate was suppressed by pretreatment with a β-adrenergic receptor blocker. These observations suggested that propionate activated the sympathetic nervous system via FFA3 (Kimura et al. 2013). Our study further demonstrated that FFA3 mediates propionate-stimulated release of noradrenaline from sympathetic neurons (Inoue et al. 2012; Kimura et al. 2013). In addition, β-hydroxybutyrate (β-HB) inhibited FFA3 activity (Kimura et al. 2013). β-HB, which is biosynthesized in liver under low-carbohydrate fed conditions, suppressed propionate-induced sympathetic activation in both primary cultured sympathetic neurons and intact mice (Inoue et al. 2012; Kimura et al. 2013). These findings indicate that biological functions of FFA3, such as sympathetic nervous system (SNS) activation by propionate and SNS inhibition by ketone bodies, might be involved in recognition of the energy condition in the body and thereby contribute to the maintenance of energy homeostasis. FFA3 is reported to be expressed in pancreatic β-cells, certain insulin-producing β-cell lines, such as MIN6, NIT-1, and βTC-6 and several types of cells in the gut (Samuel et al. 2008; Tazoe et al. 2008, 2009; Kebede et al. 2009; Zaibi et al. 2010). For example, FFA3 is expressed in peptide YY (PYY) containing enteroendocrine L cells (Tazoe et al. 2009). Samuel et al. showed that glucagon-like peptide (GLP-1) and PYY secretion is reduced in FFA3-KO mice. Tolhurst et al. reported that both C2 and C3 stimulated GLP-1 release from primary murine colonic cultures and that this was not blocked by treatment of the cells with pertussis toxin, indicating a non-$G_{i/o}$-mediated pathway. This effect was lost in cultures derived from FFA2-KO but not FFA3-KO mice. Since C2 and C3 increased $[Ca^{2+}]_i$, it was concluded that the SCFAs increased GLP-1 via a FFA2-$G_{q/11}$-mediated pathway (Tolhurst et al. 2012). Recently, Psichas et al. demonstrated that intracolonic infusion of C3 increased secretion of both GLP-1 and PYY in vivo and that these effects were abolished in FFA2-KO mice. Hence, these studies confirmed that SCFAs stimulate gut hormone secretion via FFA2 in vivo. In addition to enteroendocrine cells, FFA2 and FFA3 are also expressed in the stomach, and in particular, these receptors appear to be expressed in ghrelin-containing cells (Engelstoft et al. 2015). Hence, both C2 and C3 appear to inhibit ghrelin secretion.

SCFAs are produced in the colon through bacterial fermentation of mainly dietary fiber. Many studies have established strong links between the gut microbiota, health, and disease (Ley et al. 2006; Karlsson et al. 2013; Flint et al. 2012, 2015). Hence, many studies have aimed to assess whether FFA2 and/or FFA3 utilise bacterial microbial fermentation produced SCFAs in order to demonstrate links between the gut microbiota and health. Samuel et al. found that conventionally raised FFA3-KO mice had decreased adiposity compared to wild-type controls, but this was not apparent in germ-free (GF) mice (Samuel et al. 2008). In particular, this appeared to relate to PYY levels, which were decreased in FFA3-KO mice when gut microbiota were present but were not in GF mice. One study has also linked dietary fiber, the microbiota, SCFAs, and FFA3 to allergic airway disease (Trompette et al. 2014). In the study, a high-fiber

diet altered microbiota composition in both the gut and the lung, increasing SCFA levels, and protected against allergic airway disease. Exogenous C3 administration was found to produce similar effects to fiber, but such effects of C3 were absent in FFA3-KO but not FFA2-KO mice.

Sawzdargo et al. showed that GPR42 is encoded in tandem with FFA1, FFA2, and FFA3 on human chromosome 19q13.1 (Sawzdargo et al. 1997). GPR42 is considered to be generated by tandem duplication of FFA3 (GPR41). FFA3 and GPR42 share 98% sequence identity. The differences are only in six amino acid positions (Fig. 1). Mutation of FFA3 residue 174th Arg to Trp, which is found in 174th position of GPR42, makes FFA3 non-responsive to SCFAs. Conversely, the reciprocal mutation in GPR42 restored partial functional responsiveness. Hence, GPR42 was firstly reported as an inactive, potential pseudogene (Brown et al. 2003). However, the six amino acid differences between FFA3 and GPR42 are currently considered as polymorphic variants (Liaw and Connolly 2009). The

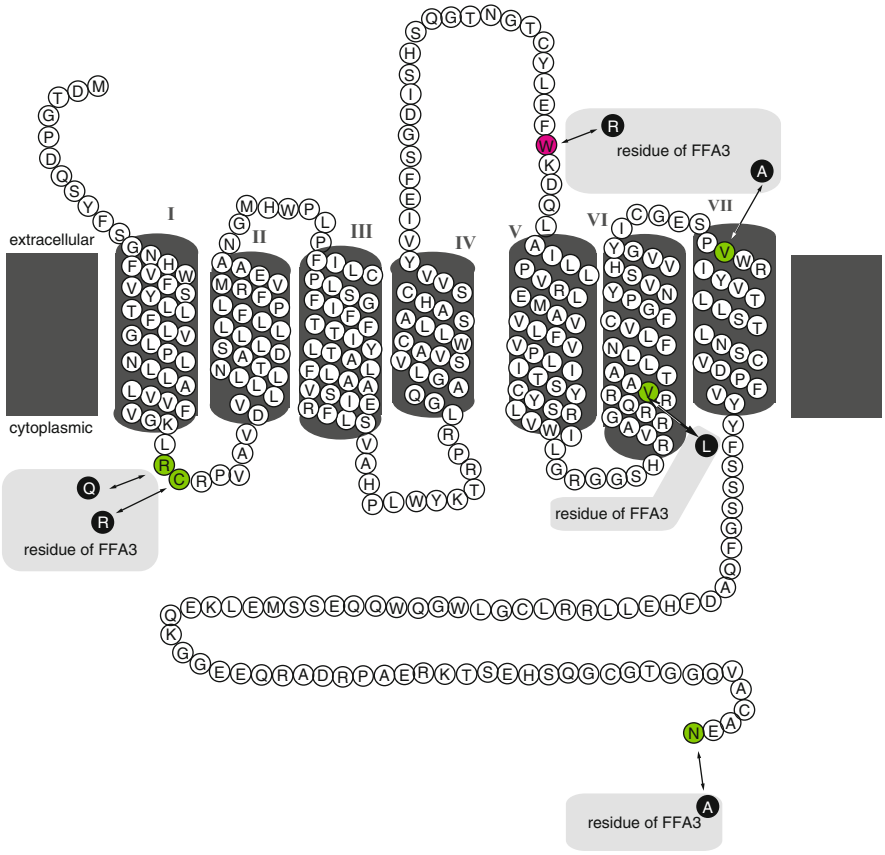

Fig. 1 Snake plot of the human GPR42. Residues colored are those that differ between GPR42 and FFA3. Arg174Trp is shown in *red*. Corresponding residue of FFA3 are shown by *white-colored* character into the *black circle*

functional Arg174 allele was detected in 61% of those subjects at the GPR42 locus based on a genotyping study of the corresponding alleles from a population of more than 100 subjects (Liaw and Connolly 2009). Recently, Puhl et al. sequenced the open reading frames of FFA3 and GPR42 from 56 individuals and found a high frequency of polymorphisms, contributing to several complex haplotypes (Puhl et al. 2015). They also identified a structural variation that results in GPR42 copy number polymorphism with a frequency of 18.8%. Sequencing analysis further demonstrated that 50.6% of GPR42 haplotypes differed from FFA3 by only a single non-synonymous substitution and that the GPR42 reference sequence matched only 4.4% of the alleles. These data suggested that GPR42 should be reclassified as a functional gene, and thereby copy number polymorphism of FFA3/GPR42 might be considered during genetic and pharmacological investigation of these receptors. Techniques for determining mRNA expression, such as Northern blot analysis, TaqMan, and RT-PCR, would be unable to distinguish FFA3 mRNA from GPR42 mRNA. Hence, previously reported FFA3 mRNA expression profiles are likely to includ both FFA3 and GPR42 expression.

169 variants of FFA3 are listed in a genetic variation database based on the 1000 genomes project (Genomes Project et al. 2015). 45th position of the amino acid sequence is one of the non-consensus residues between FFA3 and GPR42. The most common genetic variant reported is Arg45His (MAF score of 18.5%). the original FFA3/GPR42 genetic study also reported the His45 minor allele of FFA3 in one subject (Liaw and Connolly 2009). However, interchange with the GPR42 allele in 45th position, Arg45Cys, did not affect FFA3 function (Brown et al. 2003). Therefore, this position may have relatively little effect on receptor function. On the other hand, Asp158Asn and Arg185Gln, which are rarely described as missense variants, would be expected to have significant effects on FFA3 function. Hudson et al. have previously reported that FFA3 residue Asp158 is involved in forming an ionic lock interaction between arginine residues in the SCFA binding pocket that limit ligand-independent activation of the receptor (Hudson et al. 2012b). They also reported that the mouse orthologue, which displays marked constitutive activity, has an Asn residue at this position. Hence, humans carrying the Asp158Asn allele would yield an increased constitutively active FFA3 that may be relatively sensitive to endogenous ligands. Arg185 of FFA3 is conserved between FFA1 and FFA3. This residue is critical for ligand recognition and anchoring of the carboxylate moiety of the SCFAs to the binding pocket (Sum et al. 2007; Stoddart et al. 2008a). The mutation at this position has been reported to abolish SCFA interactions. Hence, Arg185Gln variant carriers are likely to be nonresponsive to SCFAs.

3.2 FFA2 Functions and Polymorphisms

FFA2 was reported to be activated by acetate (C2) through ligand screening studies for bioactive compounds by using $[Ca^{2+}]_i$ analyses (Brown et al. 2003; Nilsson et al. 2003). Although both FFA2 and FFA3 are activated by SCFAs, potency of the SCFAs for these receptors is clearly different (Brown et al. 2003; Hudson

et al. 2012a). Previous structure-activity relationship studies demonstrated that FFA2 exhibited a preference for smaller SCFAs compared to FFR3. FFR2 is activated by high micromolar or millimolar concentrations of SCFAs, such as propionate (C3), butyrate (C4) and substantially less so by caproate (C6) and formate (C1) with a rank order potency of C2 = C3 > C4 > C6 > C5 > C1 (Brown et al. 2003; Le Poul et al. 2003). As described above, FFA2 couples to both pertussis toxin-sensitive $G_{i/o}$ and G_q pathways (Andoh et al. 2003; Le Poul et al. 2003). The activation of FFA2 by endogenous agonists not only inhibits cAMP production through interactions with $G_{i/o}$ G proteins but also causes $[Ca^{2+}]_i$ elevation and promotes activation of the mitogen-activated protein kinase (MAPK) cascade via interactions with G_q family G proteins. FFA2 is expressed in immune cells, including neutrophils, monocytes and polymorphonuclear cells (Brown et al. 2003). In these cell types, FFA2 has an important role in immune cell recruitment in inflammatory responses (Le Poul et al. 2003; Sina et al. 2009; Maslowski et al. 2009; Vinolo et al. 2011). FFA2-chemotactic response was inhibited by pertussis toxin treatment, indicating that this response is mediated via $G_{i/o}$ (Sina et al. 2009). Moreover, Masui et al. reported that production of tumor necrosis factor alpha from mononuclear cells was suppressed by acetate and that the effect was inhibited by an anti-FFA2 antibody (Masui et al. 2013). Kim et al. demonstrated that SCFAs activate FFA2 and FFA3 on intestinal epithelial cells, leading to mitogen-activated protein kinase signaling and rapid production of chemokines and cytokines (Kim et al. 2013). FFA2 expression was also detected in adipose tissue, intestines, and islet cells of the pancreas (Hong et al. 2005; Regard et al. 2008; Maslowski et al. 2009). In the adipose tissue, FFA2 activation promotes adipogenesis by increasing lipid accumulation (Hong et al. 2005) and inhibiting lipolysis (Ge et al. 2008). In a series of in vitro and in vivo studies, we found that whole-body deficiency of FFA2 induced obesity in mice, whereas mice with adipose tissue-specific overexpression of FFA2 are lean under normal conditions (Kimura et al. 2013). However, one study has reported conflicting results with FFA2-KO mice. Bjursell et al. reported that FFA2-KO mice have reduced body fat mass, improved glucose control, and increased insulin sensitivity (Bjursell et al. 2011). In primary human adipocytes, an absence of relationship between FFA2 and adipocyte differentiation was found, unlike what was observed in mice (Dewulf et al. 2013). Furthermore, in an acute model of colitis, two separate studies have reported conflicting results with FFA2-KO mice. One group showed reduced (Maslowski et al. 2009) and another group showed heightened (Sina et al. 2009) inflammatory responses. In the intestines, FFA2 is associated with the regulation of appetite and insulin signaling. The intestine contributes to energy homeostasis not only by nutrient absorption but also the secretion of incretin hormones (Turton et al. 1996; Batterham et al. 2002; Chelikani et al. 2005; Koda et al. 2005). FFA2-expressing cells are co-localized with PYY-containing enteroendocrine L-cells of the rat gastrointestinal tract (Karaki et al. 2006). Furthermore, FFA2 expression in the enteroendocrine L cells mediates SCFA-induced GLP-1 release in vitro and in vivo (Tolhurst et al. 2012). FFA2 is also present in pancreatic islets, specifically the β-cells, although its role in β-cell function remains unclear (Kebede et al. 2009).

Two studies have found that SCFAs activate FFA2 to enhance glucose stimulated insulin secretion (GSIS) from murine islets both in vivo and in vitro and that this occurs through a $G_{q/11}$ and phospholipase C mediated pathway (McNelis et al. 2015; Priyadarshini et al. 2015). Another study indicated that SCFAs inhibit GSIS through both FFA2 and FFA3 and that this occurs through a $G_{i/o}$-mediated pathway. In contrast, SCFAs have no effect on GSIS in human islets, despite the fact that SCFAs activated both G_q and G_i signaling (Priyadarshini et al. 2015).

As described above, many researchers have made efforts to assess whether FFA2 and/or FFA3 mediate the functions of bacterial microbial fermentation produced SCFAs. The studies on FFA2 have demonstrated relationships between this receptor, SCFAs produced by gut microbiota and inflammatory responses. Maslowski et al. demonstrated that FFA2-KO mice showed exacerbated or unresolving inflammation in models of colitis, arthritis and asthma, indicating that stimulation of FFA2 by SCFAs was necessary for the normal inflammatory responses (Maslowski et al. 2009). GF mice, which were devoid of bacteria and generate little or no SCFAs, showed a similar dysregulation of inflammatory responses. Smith et al. showed that SCFAs produced by gut microbiota-derived bacterial fermentation regulated the size and function of the colonic regulatory T cell (TRegs) pool and protect against colitis in a FFA2-dependent manner in mice (Smith et al. 2013). It was shown that GF conditions reduced the population of TRegs in the intestine and that SCFAs restored these populations. Smith et al. demonstrated that the effects of SCFAs on GF mice were lacking in FFA2-KO mice. These data indicated that FFA2 mediated biological functions of SCFAs from gut microbiota.

The 1000 genomes project database lists 169 FFA2 variants (Genomes Project et al. 2015). The most common verified variant is Leu211His with a MAF value of 3.6%. However, at the current stage, no group has reported any relationship between this polymorphism and physiological function of FFA2 or the association with any disease/clinical phenotype, although this change in amino acid residue could possibly have impact on receptor function. The Leu211His variation could have effects on G protein coupling because the position is located in the receptor's third intracellular loop. Arg255Gln, which is a relatively rare missense variant of FFA2, also affects the receptor function (Hudson et al. 2013b). Arg residue at this position is a conserved polar residue at the upper face of transmembrane domain VII, which facilitate binding of SCFAs to the receptor. Hence, carriers of this minor allelic variant would be anticipated to be unresponsive to endogenous ligands.

4 Polymorphic Variation of Receptors for LCFAs

MCFAs and LCFAs are defined as 6–12 carbon-chain fatty acids and >12 carbon-chain fatty acids, respectively. MCFAs and LCFAs are generally considered as key energy sources for the whole body (Offermanns 2014). However, the identification of FFA1 and FFA4 as receptors for MCFAs and LCFAs showed that these FFAs also have important roles in signaling transduction as signaling molecules (Itoh et al. 2003; Hirasawa et al. 2005; Ichimura et al. 2014). These FFAs, which are

endogenous ligands of FFA1 and FFA4, are supplied by both food intake and *de novo* biosynthesis. However, FFAs, which are so called essential fatty acids, containing more than two double bonds, such as linoleic acid and docosahexaenoic acid (DHA), are not obtained by biosynthesis in humans. Hence, these essential fatty acids have to be supplied within food. Recent studies clearly demonstrated that FFA1 and FFA4 contribute to maintenance of systemic energy homeostasis (Itoh et al. 2003; Oh et al. 2010; Ichimura et al. 2012; Hara et al. 2014; Hudson et al. 2013b; Ichimura et al. 2014). Hence, FFA1 and FFA4 are prospective therapeutic targets for metabolic diseases.

4.1 FFA1 Functions and Polymorphisms

FFA1 is expressed predominantly in insulin-producing pancreatic β-cells and enhances GSIS (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003b; Latour et al. 2007). Because of these characteristics, great attention has been paid to FFA1 as a therapeutic target of metabolic diseases. Loss-of-functional analyses of FFA1 by using RNA interference, chemical compounds, or genetic deletion in mice consistently result in a significant decrease in FFA-induced GSIS (Itoh and Hinuma 2005; Salehi et al. 2005; Steneberg et al. 2005; Briscoe et al. 2006; Latour et al. 2007; Schnell et al. 2007; Kebede et al. 2008; Lan et al. 2008). Several studies have demonstrated that the FFA1-mediated effect on GSIS is mediated through $G_{q/11}$-phospholipase C and L-type Ca^{2+} pathways (Fujiwara et al. 2005; Latour et al. 2007), leading to phosphorylation of protein kinase D1 and filamentous actin remodeling (Ferdaoussi et al. 2012). Furthermore, FFA1 expression is also detected in enteroendocrine cells and FFA1 agonists have been reported to stimulate release of the incretin hormone GLP-1 and PYY from L cells (Edfalk et al. 2008), cholecystokinin (CCK) from I cells (Liou et al. 2011) and gastric inhibitory peptide (GIP) from K cells (Sykaras et al. 2012). Hence, FFA1 decreases blood glucose not only by direct stimulation of insulin secretion from pancreatic β-cells, but also by GLP-1-mediated indirect stimulation of insulin secretion (Luo et al. 2012). FFA1 expression has been also reported in taste buds and cells of the central nervous system (Ma et al. 2007; Edfalk et al. 2008; Hirasawa et al. 2008; Cartoni et al. 2010; Liou et al. 2011; Sykaras et al. 2012). Altogether, FFA1 plays important roles in regulation of systemic glucose homeostasis. Given the physiological roles of FFA1 in glucose homeostasis, FFA1 agonists are expected to be potential medicines for the treatment of type 2 diabetes (T2D). Many compounds have been reported as FFA1 agonists, and some of have entered clinical trials (Briscoe et al. 2006; Tsujihata et al. 2011; Takano et al. 2014; Defossa and Wagner 2014; Christiansen et al. 2011, 2013a, b). In particular, TAK-875 was developed as an orally available FFA1 partial agonist. A phase III clinical trial employed TAK-875 as a glucose regulating therapeutic agent. However, TAK-875 was removed from these trials due to potential liver toxicity (Ichimura et al. 2014). TAK-875 enhances GSIS and improves both postprandial and hyperglycemia with a low risk of hypoglycemia (Tsujihata et al. 2011; Defossa and Wagner 2014),

whereas it has no effect on glucagon secretion or insulin resistance (Araki et al. 2012; Bailey 2012; Burant et al. 2012; Leifke et al. 2012; Naik et al. 2012; Mancini and Poitout 2013; Poitout and Lin 2013). TAK-875 is reported to be an ago-allosteric modulator of FFA1 (Yabuki et al. 2013). Collectively, FFA1 agonists show promise as novel medicines for the treatment for T2D. However, there are a many complicating factors, such as genetic polymorphisms of human FFA1, conflicting results from animal studies, and differential biological effects of FFA1 agonists between species. Hence, physiological and pharmacological studies of FFA1 by using animal models appear to be difficult to compare.

151 variants of human FFA1 were listed in the 1000 genomes project database (Genomes Project et al. 2015). Among them, Arg211His has been identified as a major variation of FFA1 with a MAF value of 18.2%. The effect of the Arg211His polymorphism in a Japanese male population was previously reported (Ogawa et al. 2005). Ogawa et al. demonstrated that individuals homozygous for the minor Arg variant displayed reduced serum insulin and β-cell function. These results suggested that the Arg211His polymorphism might contribute to insulin secretion via FFA1. On the other hand, Hamid et al. reported distinct results of this Arg211His polymorphism (Hamid et al. 2005). They employedd oral glucose tolerance tests and concluded that this polymorphism did not contribute to insulin secretion (Hamid et al. 2005). Moreover, no differences in allelic frequency between healthy and diabetic individuals have been found in either of these studies. No effect of Arg211His variation on FFA1 function has been detected by in vitro studies (Smith et al. 2009; Hamid et al. 2005). Hence this variant should be more carefully explored. Besides Arg211His polymorphism, some relatively rare variants of FFA1 have been assessed. MAF of Gly180Ser polymorphism is 0.42% in non-obese individuals. However, MAF of the variant is 1.07% in obese individuals and 2.60% in severely obese individuals (Vettor et al. 2008). Oral glucose tolerance testing of carriers of the polymorphism demonstrated that insulin secretion was significantly reduced in carriers of the minor Ser allele (Vettor et al. 2008). Furthermore, in vitro $[Ca^{2+}]_i$ mobilization analysis indicated that the FFA1 variant reduced receptor function. However, Smith et al. failed to reproduce this in vitro result (Smith et al. 2009). Asp175Asn, which is another rare variation of FFA1, has also been reported to inhibit receptor function of FFA1 in vitro (Hamid et al. 2005). However, distinct results indicating that the receptor function of this variant is similar to the wild type have been also described (Smith et al. 2009). Since synthetic selective agonists have not been used on these variants, such analyses might be of considerable interest to examine. Not only missense polymorphisms, but also two kinds of SNPs upstream of the FFA1 gene associated with β-cell function has been reported (Kalis et al. 2007). The effect of three common SNPs of FFAR1 (rs2301151; rs16970264; rs1573611) on pancreatic function, body mass index (BMI), body composition and plasma lipids have been investigated. Although rs1573611 and rs2301151 were associated with some of these parameters, the authors concluded that differences in body composition and lipids associated with common SNPs in the FFA1 gene were apparently not mediated by changes in insulin sensitivity or β-cell function. Wagner

et al. examined the interaction of genetic variation in FFA1 with FFAs and insulin secretion and revealed that the inverse association of FFAs and secretion of insulin was modulated by FFA1 SNP rs1573611 and became steeper for carriers of the minor allele (Wagner et al. 2013). Previously, it was reported that carriers of the minor allele of the Pro12Ala polymorphism (rs1805192) had lower insulin secretion during hyperglycemic clamp studies conducted with a concomitant intravenous lipid infusion, but no difference was seen between the genotypes without increasing blood FFAs (Stumvoll and Haring 2002). Based on this report, Wagner et al. demonstrated that two FFA1 SNPs (rs12462800 and rs10422744) were associated with reduced insulin secretion in participants concomitantly carrying a PPARγ minor allele and having high fasting FFAs (Wagner et al. 2014). These SNPs (rs12462800 and rs10422744) are located 0.8 kb apart in an intergenic regulatory area between the FFA1 and FFA3 genes, 3.5 and 3.8 kb from the 3′ end of the single FFA1 exon. These SNPs are more distal from the gene than the previously described FFA1 SNP rs1573611 which directly interacts with fasting FFAs in association with insulin secretion (Wagner et al. 2013). Collectively, although some kinds of studies established the relationships between polymorphisms of FFA1 and FFA1 receptor functions, further detailed analysis to elucidate the genetic variations, which effect FFA1 function or increase risk of metabolic diseases, is required.

4.2 FFA4 Functions and Polymorphisms

In 2005, FFA4 was deorphanized and identified as a FFA receptor (Hirasawa et al. 2005). FFA4 lacked substantial homology with GPR40 FFARs family members. Although the amino acid homology between FFA1 and FFA4 is only 10%, both of these receptors share similar endogenous ligands. FFA4 is activated by carbon-chain length 14–18 saturated FFAs C6 to C22 unsaturated FFAs (Fukunaga et al. 2006; Ichimura et al. 2014). Ligand stimulation of FFA4 increases $[Ca^{2+}]_i$ but does not increase or decrease cAMP production in human or mouse FFA4-expresseing cells. Based on these and other results, FFA4 is coupled to G_q protein family (Hirasawa et al. 2005; Hara et al. 2009; Hudson et al. 2013c; Moore et al. 2009; Watson et al. 2012). FFA4-mediated increase of $[Ca^{2+}]_i$ is blocked by the $G_{q/11}$-selective inhibitor YM-254890 (Hudson et al. 2013c). Recent studies indicated that some physiological function of FFA4 might be mediated by G_i-family G proteins. For example, pertussis toxin treatment blocked FFA4-mediated inhibition of ghrelin secretion from mouse gastric cells (Engelstoft et al. 2013) as well as somatostatin release from pancreatic islet delta cells (Stone et al. 2014). Stimulation of FFA4 also activates ERK1/2 and PI3-kinase signaling cascades (Katsuma et al. 2005; Hara et al. 2009). Furthermore, Oh et al. showed that stimulation of FFA4 by ω-3 FFAs contributes to anti-inflammatory responses (Oh et al. 2010). Such anti-inflammatory effects are exerted by the suppression of Toll-like receptor via a β-arrestin2 signaling pathway and transforming growth factor-β activated kinase 1 (TAK1) associated with TNF-α inflammation signaling

pathway. FFA4 expression has also been detected in adipose tissue and adipocyte cell lines (Gotoh et al. 2007; Ichimura et al. 2012; Oh et al. 2010). In adipose tissue, FFA4 has been reported to contribute to adipocyte maturation (Gotoh et al. 2007; Ichimura et al. 2012) and glucose uptake through the induction of translocation of glucose transporter 4 (Oh et al. 2010). We have reported increased FFA4 expression in obese human subjects (Ichimura et al. 2012). On the other hand, there are conflicting results; Rodriguez-Pacheco et al. have reported decreased FFA4 expression in obese human subjects (Rodriguez-Pacheco et al. 2014). Although gene expression of FFA4 has been detected in pancreatic islet and cultured β-cell line, FFA4 does not appear to directly stimulate insulin secretion in pancreatic β-cells (Kebede et al. 2009; Taneera et al. 2012). Rather, FFA4 indirectly enhances insulin secretion by GLP-1 secretion from gut enteroendocrine cells (Hirasawa et al. 2005) and protects against cell death in pancreatic islet β-cells (Taneera et al. 2012). To date, limited reports indicated FFA4 expression in pancreatic α cells (Suckow et al. 2014) and δ cells (Stone et al. 2014). Moreover, FFA4 is also reported to be expressed in airway smooth muscle (Mizuta et al. 2015) and taste buds (Galindo et al. 2012; Matsumura et al. 2009). However, biological functions of FFA4 in these tissues are unclear or controversial (Martin et al. 2012; Ancel et al. 2015). FFA4 is expressed in the enteroendocrine cells on each of the L, K, and I cells (Hirasawa et al. 2005; Iwasaki et al. 2015; Sykaras et al. 2012). In addition, a direct correlation between BMI and FFA4 transcript levels have been observed in human duodenum (Little et al. 2014). Taken together, FFA4 is believed to be a potential therapeutic target for metabolic diseases, including T2D and obesity.

FFA4 was placed in the top 16 of the ranked list of the risk genes identified in whole genome studies to identify genes for T2D (Taneera et al. 2012). Furthermore, at least 18 missense polymorphisms of FFA4 have been listed in publically available databases (Genomes Project et al. 2015). Among these variants, the Arg67Cys polymorphism was identified as the only high frequency gene variation with a MAF value of 14.9% (Fig. 2). We have shown that this polymorphism has little effect however on pharmacological functions of FFA4 (Ichimura et al. 2012). Consistent with this result, the Arg67Cys variant has only a weak tendency toward an association between the polymorphism and obesity in humans with an odds ratio of 1.16. We have also identified the Arg270His polymorphism as a less common variant with a MAF value of 1.3–3%. We also showed that the Arg270His polymorphism is significantly associated with obesity with an odds ratio of 1.62 (Fig. 2). In vitro analyses successfully indicated that this polymorphism not only significantly reduced receptor function, but also acted as a dominant-negative like receptor. FFA4-KO mice fed a high-fat diet develop obesity, glucose intolerance and fatty liver with decreased adipocyte differentiation and lipogenesis and enhanced hepatic lipogenesis. These human and mice studies consistently showed that FFA4 has a key role in sensing dietary fat and, therefore, controlling energy balance. In addition to the gene polymorphisms, Moore et al. previously reported two splice variant isoforms of FFA4 (Moore et al. 2009). These splice variant isoforms are distinguished by the presence or absence of a 16-amino acid insertion, which is positioned in the third intracellular loop (Fig. 2). Studies of longer isoform function in

mice or other rodents are not possible because the longer isoform of FFA4 exists only in human. Furthermore, the shorter isoform of FFA4 is coupled with both G protein-dependent and β-arrestin pathways, whereas the longer isoform is coupled only with the β-arrestin pathway (Watson et al. 2012). Additionally, the longer isoform of human FFA4 has been detected in only a few tissues, particularly in the colon (Galindo et al. 2012). Based on these reports, the function of human FFA4 may be at least to some extent different from FFA4 of other species. Hence, the functional and physiological importance of human long isoform remains uncertain. Recently, other groups also reported the relationship between this variant of FFA4 and human metabolism. Waguri et al. reported that genetic variation of FFA4 including this variant and dietary fat intake could be a possible determinant of BMI (Waguri et al. 2013). Bonnefond et al. also reported that this variant associated with increased fasting plasma glucose levels independent of BMI but not with measures of insulin resistance or T2D (Bonnefond et al. 2015). Furthermore, Marruzillo et al. reported that heterozygous child carriers of this FFA4 variant showed significantly higher alanine transaminase (ALT) levels than wild-type subjects, and also showed an odds ratio to have pathologic ALT (Marzuillo et al. 2014), indicating the relationship between liver injury in children and this variant. Recently, Vestmar et al. showed that this variant of FFA4 inhibited ligand-depending activation of G_q and G_i signalling in vitro (Vestmar et al. 2016). Since Vestmar et al. also used the shorter isoform for in vitro studies, these results also indicated that Arg254His variant reduced function in both $G_{q/11}$- and $G_{i/o}$-FFA4 signaling, although some function remained. Further, in this study, FFA4-arrestin interactions were not altered for this variant (Vestmar et al. 2016). Vestimar et al. failed to show the association of this variant with either increased risk of obesity or increased fasting plasma glucose levels in a Danish study population (Vestmar et al. 2016). It is not clear at present of the basis of these complicated results, however, as there is a very low frequency of this variant in the analyzed population, especially the age of subjects might affect the results. Our group, Waguri et al. and Bonnefond et al. included children, adolescent and adult individuals in the analyses. On the other hand, Vestmar et al. analyzed only adults. Based on these differences, further detailed analyses using more extreme cases or obesity among the young age subjects are needed to clarify the relationship between FFA4 variant and metabolic diseases.

5 Conclusions

FFAs, which are provided within the diet, via intestinal bacterial fermentation of dietary fiber, and de novo synthesis, act as important signaling molecules through FFARs. Hence, FFARs are seen as new potential therapeutic targets for metabolic disorders. To date, there is more attention on the effects of genetic variants of the FFARs on the pharmacology and functions of these receptors as these are one of causes of metabolic disorders. More detailed information of the relationship between genetic variations of FFARs and receptor function with selective ligands

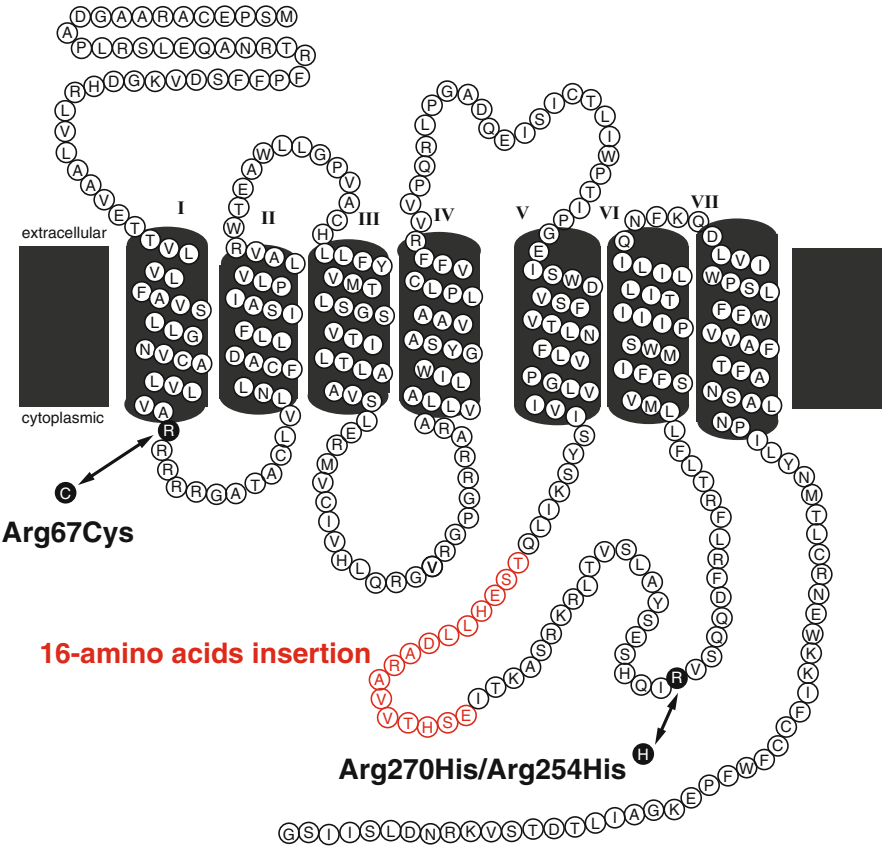

Fig. 2 Snake plot of the human free fatty acid receptor 4 (FFA4). The position of a 16-amino acid insertion (shown in *red*), Arg67Cys and Arg270His/Arg254His variants are shown

for each receptor might be required for the development of therapeutic agents targeting FFARs. A deeper understanding of the genetic variations of each member of FFAR family might open a new avenue of research in the development of therapeutic agents targeting FFARs.

Conflict of Interest The authors declare no conflict of interest.

References

Al-Lahham SH, Roelofsen H, Priebe M, Weening D, Dijkstra M, Hoek A, Rezaee F, Venema K, Vonk RJ (2010) Regulation of adipokine production in human adipose tissue by propionic acid. Eur J Clin Invest 40(5):401–407. doi:10.1111/j.1365-2362.2010.02278.x

Ancel D, Bernard A, Subramaniam S, Hirasawa A, Tsujimoto G, Hashimoto T, Passilly-Degrace P, Khan NA, Besnard P (2015) The oral lipid sensor GPR120 is not indispensable for the

orosensory detection of dietary lipids in mice. J Lipid Res 56(2):369–378. doi:10.1194/jlr.M055202

Andoh A, Tsujikawa T, Fujiyama Y (2003) Role of dietary fiber and short-chain fatty acids in the colon. Curr Pharm Des 9(4):347–358

Araki T, Hirayama M, Hiroi S, Kaku K (2012) GPR40-induced insulin secretion by the novel agonist TAK-875: first clinical findings in patients with type 2 diabetes. Diabetes Obes Metab 14(3):271–278. doi:10.1111/j.1463-1326.2011.01525.x

Bailey CJ (2012) Could FFAR1 assist insulin secretion in type 2 diabetes? Lancet 379 (9824):1370–1371. doi:10.1016/S0140-6736(12)60165-2

Balasubramanian S, Xia Y, Freinkman E, Gerstein M (2005) Sequence variation in G-protein-coupled receptors: analysis of single nucleotide polymorphisms. Nucleic Acids Res 33 (5):1710–1721. doi:10.1093/nar/gki311

Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR (2002) Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 418(6898):650–654. doi:10.1038/nature02666

Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, Smith DM, Cawthorne MA, Stocker CJ, Arch JR (2013) Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. Br J Nutr 109(10):1755–1764. doi:10.1017/S0007114512003923

Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70(2):567–590

Bindels LB, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, Scott KP, Buc Calderon P, Feron O, Muccioli GG, Sonveaux P, Cani PD, Delzenne NM (2012) Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. Br J Cancer 107 (8):1337–1344. doi:10.1038/bjc.2012.409

Bjursell M, Admyre T, Goransson M, Marley AE, Smith DM, Oscarsson J, Bohlooly YM (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. Am J Physiol Endocrinol Metab 300(1):E211–E220. doi:10.1152/ajpendo.00229.2010

Bonnefond A, Lamri A, Leloire A, Vaillant E, Roussel R, Levy-Marchal C, Weill J, Galan P, Hercberg S, Ragot S, Hadjadj S, Charpentier G, Balkau B, Marre M, Fumeron F, Froguel P (2015) Contribution of the low-frequency, loss-of-function p.R270H mutation in FFAR4 (GPR120) to increased fasting plasma glucose levels. J Med Genet 52(9):595–598. doi:10.1136/jmedgenet-2015-103065

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR Jr, Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278(13):11303–11311. doi:10.1074/jbc.M211495200

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 148(5):619–628. doi:10.1038/sj.bjp.0706770

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM, Wise A, Dowell SJ (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278(13):11312–11319. doi:10.1074/jbc.M211609200

Burant CF, Viswanathan P, Marcinak J, Cao C, Vakilynejad M, Xie B, Leifke E (2012) TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial. Lancet 379(9824):1403–1411. doi:10.1016/S0140-6736(11)61879-5

Cartoni C, Yasumatsu K, Ohkuri T, Shigemura N, Yoshida R, Godinot N, le Coutre J, Ninomiya Y, Damak S (2010) Taste preference for fatty acids is mediated by GPR40 and GPR120. J Neurosci 30(25):8376–8382. doi:10.1523/JNEUROSCI.0496-10.2010

Chelikani PK, Haver AC, Reidelberger RD (2005) Intravenous infusion of peptide YY(3-36) potently inhibits food intake in rats. Endocrinology 146(2):879–888. doi:10.1210/en.2004-1138

Christiansen E, Urban C, Grundmann M, Due-Hansen ME, Hagesaether E, Schmidt J, Pardo L, Ullrich S, Kostenis E, Kassack M, Ulven T (2011) Identification of a potent and selective free fatty acid receptor 1 (FFA1/GPR40) agonist with favorable physicochemical and in vitro ADME properties. J Med Chem 54(19):6691–6703. doi:10.1021/jm2005699

Christiansen E, Due-Hansen ME, Urban C, Grundmann M, Schmidt J, Hansen SV, Hudson BD, Zaibi M, Markussen SB, Hagesaether E, Milligan G, Cawthorne MA, Kostenis E, Kassack MU, Ulven T (2013a) Discovery of a potent and selective free fatty acid receptor 1 agonist with low lipophilicity and high oral bioavailability. J Med Chem 56(3):982–992. doi:10.1021/jm301470a

Christiansen E, Hansen SV, Urban C, Hudson BD, Wargent ET, Grundmann M, Jenkins L, Zaibi M, Stocker CJ, Ullrich S, Kostenis E, Kassack MU, Milligan G, Cawthorne MA, Ulven T (2013b) Discovery of TUG-770: a highly potent free fatty acid receptor 1 (FFA1/GPR40) agonist for treatment of type 2 diabetes. ACS Med Chem Lett 4(5):441–445. doi:10.1021/ml4000673

Defossa E, Wagner M (2014) Recent developments in the discovery of FFA1 receptor agonists as novel oral treatment for type 2 diabetes mellitus. Bioorg Med Chem Lett 24(14):2991–3000. doi:10.1016/j.bmcl.2014.05.019

Dewulf EM, Ge Q, Bindels LB, Sohet FM, Cani PD, Brichard SM, Delzenne NM (2013) Evaluation of the relationship between GPR43 and adiposity in human. Nutr Metab 10 (1):11. doi:10.1186/1743-7075-10-11

Edfalk S, Steneberg P, Edlund H (2008) Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. Diabetes 57(9):2280–2287. doi:10.2337/db08-0307

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2 (4):376–392. doi:10.1016/j.molmet.2013.08.006

Engelstoft MS, Lund ML, Grunddal KV, Egerod KL, Osborne-Lawrence S, Poulsen SS, Zigman JM, Schwartz TW (2015) Research resource: a chromogranin a reporter for serotonin and histamine secreting enteroendocrine cells. Mol Endocrinol 29(11):1658–1671. doi:10.1210/me.2015-1106

Ferdaoussi M, Bergeron V, Zarrouki B, Kolic J, Cantley J, Fielitz J, Olson EN, Prentki M, Biden T, MacDonald PE, Poitout V (2012) G protein-coupled receptor (GPR)40-dependent potentiation of insulin secretion in mouse islets is mediated by protein kinase D1. Diabetologia 55 (10):2682–2692. doi:10.1007/s00125-012-2650-x

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 6(2):121–131. doi:10.1038/nrmicro1817

Flint HJ, Scott KP, Louis P, Duncan SH (2012) The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol 9(10):577–589. doi:10.1038/nrgastro.2012.156

Flint HJ, Duncan SH, Scott KP, Louis P (2015) Links between diet, gut microbiota composition and gut metabolism. Proc Nutr Soc 74(1):13–22. doi:10.1017/S0029665114001463

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63(6):1256–1272. doi:10.1124/mol.63.6.1256

Fujiwara K, Maekawa F, Yada T (2005) Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release. Am J Physiol Endocrinol Metab 289(4):E670–E677. doi:10.1152/ajpendo.00035.2005

Fukunaga S, Setoguchi S, Hirasawa A, Tsujimoto G (2006) Monitoring ligand-mediated internalization of G protein-coupled receptor as a novel pharmacological approach. Life Sci 80 (1):17–23

Galindo MM, Voigt N, Stein J, van Lengerich J, Raguse JD, Hofmann T, Meyerhof W, Behrens M (2012) G protein-coupled receptors in human fat taste perception. Chem Senses 37 (2):123–139. doi:10.1093/chemse/bjr069

Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, Tian H, Li Y (2008) Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 149(9):4519–4526. doi:10.1210/en.2008-0059

Genomes Project Consortium, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR (2015) A global reference for human genetic variation. Nature 526(7571):68–74. doi:10.1038/nature15393

Gotoh C, Hong YH, Iga T, Hishikawa D, Suzuki Y, Song SH, Choi KC, Adachi T, Hirasawa A, Tsujimoto G, Sasaki S, Roh SG (2007) The regulation of adipogenesis through GPR120. Biochem Biophys Res Commun 354(2):591–597

Hamid YH, Vissing H, Holst B, Urhammer SA, Pyke C, Hansen SK, Glumer C, Borch-Johnsen K, Jorgensen T, Schwartz TW, Pedersen O, Hansen T (2005) Studies of relationships between variation of the human G protein-coupled receptor 40 Gene and Type 2 diabetes and insulin release. Diabet Med 22(1):74–80. doi:10.1111/j.1464-5491.2005.01505.x

Han JH, Kim IS, Jung SH, Lee SG, Son HY, Myung CS (2014) The effects of propionate and valerate on insulin responsiveness for glucose uptake in 3T3-L1 adipocytes and C2C12 myotubes via G protein-coupled receptor 41. PLoS One 9(4):e95268. doi:10.1371/journal.pone.0095268

Hara T, Hirasawa A, Sun Q, Sadakane K, Itsubo C, Iga T, Adachi T, Koshimizu TA, Hashimoto T, Asakawa Y, Tsujimoto G (2009) Novel selective ligands for free fatty acid receptors GPR120 and GPR40. Naunyn Schmiedebergs Arch Pharmacol 380(3):247–255. doi:10.1007/s00210-009-0425-9

Hara T, Kashihara D, Ichimura A, Kimura I, Tsujimoto G, Hirasawa A (2014) Role of free fatty acid receptors in the regulation of energy metabolism. Biochim Biophys Acta 1841 (9):1292–1300. doi:10.1016/j.bbalip.2014.06.002

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11(1):90–94. doi:10.1038/nm1168

Hirasawa A, Hara T, Katsuma S, Adachi T, Tsujimoto G (2008) Free fatty acid receptors and drug discovery. Biol Pharm Bull 31(10):1847–1851

Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, Lee HG, Katoh K, Roh SG, Sasaki S (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinology 146(12):5092–5099. doi:10.1210/en.2005-0545

Hudson BD, Christiansen E, Tikhonova IG, Grundmann M, Kostenis E, Adams DR, Ulven T, Milligan G (2012a) Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. FASEB J 26(12):4951–4965. doi:10.1096/fj.12-213314

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G (2012b) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. J Biol Chem 287(49):41195–41209. doi:10.1074/jbc.M112.396259

Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, Pandey SK, Ward RJ, Marquez R, Tikhonova IG, Ulven T, Milligan G (2013a) Defining the molecular

basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288(24):17296–17312. doi:10.1074/jbc.M113.455337

Hudson BD, Murdoch H, Milligan G (2013b) Minireview: the effects of species ortholog and SNP variation on receptors for free fatty acids. Mol Endocrinol 27(8):1177–1187. doi:10.1210/me.2013-1085

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, Heathcote H, Tobin AB, Ulven T, Milligan G (2013c) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 84(5):710–725. doi:10.1124/mol.113.087783

Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, Choquet H, Besnard P, Lecoeur C, Vivequin S, Ayukawa K, Takeuchi M, Ozawa K, Tauber M, Maffeis C, Morandi A, Buzzetti R, Elliott P, Pouta A, Jarvelin MR, Korner A, Kiess W, Pigeyre M, Caiazzo R, Van Hul W, Van Gaal L, Horber F, Balkau B, Levy-Marchal C, Rouskas K, Kouvatsi A, Hebebrand J, Hinney A, Scherag A, Pattou F, Meyre D, Koshimizu TA, Wolowczuk I, Tsujimoto G, Froguel P (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 483(7389):350–354. doi:10.1038/nature10798

Ichimura A, Hasegawa S, Kasubuchi M, Kimura I (2014) Free fatty acid receptors as therapeutic targets for the treatment of diabetes. Front Pharmacol 5:236. doi:10.3389/fphar.2014.00236

Inoue D, Kimura I, Wakabayashi M, Tsumoto H, Ozawa K, Hara T, Takei Y, Hirasawa A, Ishihama Y, Tsujimoto G (2012) Short-chain fatty acid receptor GPR41-mediated activation of sympathetic neurons involves synapsin 2b phosphorylation. FEBS Lett 586(10):1547–1554. doi:10.1016/j.febslet.2012.04.021

Insel PA, Tang CM, Hahntow I, Michel MC (2007) Impact of GPCRs in clinical medicine: monogenic diseases, genetic variants and drug targets. Biochim Biophys Acta 1768 (4):994–1005. doi:10.1016/j.bbamem.2006.09.029

Itoh Y, Hinuma S (2005) GPR40, a free fatty acid receptor on pancreatic beta cells, regulates insulin secretion. Hepatol Res 33(2):171–173

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422(6928):173–176. doi:10.1038/nature01478

Iwasaki K, Harada N, Sasaki K, Yamane S, Iida K, Suzuki K, Hamasaki A, Nasteska D, Shibue K, Joo E, Harada T, Hashimoto T, Asakawa Y, Hirasawa A, Inagaki N (2015) Free fatty acid receptor GPR120 is highly expressed in enteroendocrine K cells of the upper small intestine and has a critical role in GIP secretion after fat ingestion. Endocrinology 156(3):837–846. doi:10.1210/en.2014-1653

Kalis M, Leveen P, Lyssenko V, Almgren P, Groop L, Cilio CM (2007) Variants in the FFAR1 gene are associated with beta cell function. PLoS One 2(11):e1090. doi:10.1371/journal.pone.0001090

Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, Kuwahara A (2006) Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 324(3):353–360. doi:10.1007/s00441-005-0140-x

Karaki S, Tazoe H, Hayashi H, Kashiwabara H, Tooyama K, Suzuki Y, Kuwahara A (2008) Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Histol 39 (2):135–142. doi:10.1007/s10735-007-9145-y

Karlsson F, Tremaroli V, Nielsen J, Backhed F (2013) Assessing the human gut microbiota in metabolic diseases. Diabetes 62(10):3341–3349. doi:10.2337/db13-0844

Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G (2005) Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine

enteroendocrine cell line STC-1. J Biol Chem 280(20):19507–19515. doi:10.1074/jbc.M412385200

Kebede M, Alquier T, Latour MG, Semache M, Tremblay C, Poitout V (2008) The fatty acid receptor GPR40 plays a role in insulin secretion in vivo after high-fat feeding. Diabetes 57 (9):2432–2437. doi:10.2337/db08-0553

Kebede MA, Alquier T, Latour MG, Poitout V (2009) Lipid receptors and islet function: therapeutic implications? Diabetes Obes Metab 11(Suppl 4):10–20. doi:10.1111/j.1463-1326.2009.01114.x

Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH (2013) Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 145(2):396–406.e1–10. doi:10.1053/j.gastro.2013.04.056

Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, Tsujimoto G (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc Natl Acad Sci U S A 108 (19):8030–8035. doi:10.1073/pnas.1016088108

Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, Tani T, Takahashi T, Miyauchi S, Shioi G, Inoue H, Tsujimoto G (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829. doi:10.1038/ncomms2852

Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, Niijima A, Furuya M, Inomata N, Osuye K, Nakazato M (2005) The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. Endocrinology 146(5):2369–2375. doi:10.1210/en.2004-1266

Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B (2003a) A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem Biophys Res Commun 301 (2):406–410. doi:10.1016/s0006-291x(02)03064-4

Kotarsky K, Nilsson NE, Olde B, Owman C (2003b) Progress in methodology. Improved reporter gene assays used to identify ligands acting on orphan seven-transmembrane receptors. Pharmacol Toxicol 93(6):249–258

Lan H, Hoos LM, Liu L, Tetzloff G, Hu W, Abbondanzo SJ, Vassileva G, Gustafson EL, Hedrick JA, Davis HR (2008) Lack of FFAR1/GPR40 does not protect mice from high-fat diet-induced metabolic disease. Diabetes 57(11):2999–3006. doi:10.2337/db08-0596

Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V (2007) GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. Diabetes 56 (4):1087–1094. doi:10.2337/db06-1532

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278(28):25481–25489. doi:10.1074/jbc.M301403200

Leifke E, Naik H, Wu J, Viswanathan P, Demanno D, Kipnes M, Vakilynejad M (2012) A multiple-ascending-dose study to evaluate safety, pharmacokinetics, and pharmacodynamics of a novel GPR40 agonist, TAK-875, in subjects with type 2 diabetes. Clin Pharmacol Ther 92 (1):29–39. doi:10.1038/clpt.2012.43

Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. Nature 444(7122):1022–1023. doi:10.1038/4441022a

Liaw CW, Connolly DT (2009) Sequence polymorphisms provide a common consensus sequence for GPR41 and GPR42. DNA Cell Biol 28(11):555–560. doi:10.1089/dna.2009.0916

Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ, Raybould HE, Wank S (2011) The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. Gastroenterology 140(3):903–912. doi:10.1053/j.gastro.2010.10.012

Little TJ, Isaacs NJ, Young RL, Ott R, Nguyen NQ, Rayner CK, Horowitz M, Feinle-Bisset C (2014) Characterization of duodenal expression and localization of fatty acid-sensing receptors

in humans: relationships with body mass index. Am J Physiol Gastrointest Liver Physiol 307 (10):G958–G967. doi:10.1152/ajpgi.00134.2014

Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, Vimolratana M, Houze JB, Wong S, Toteva M, Shan B, Li F, Zhuang R, Lin DC (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7(10):e46300. doi:10.1371/journal.pone.0046300

Ma D, Tao B, Warashina S, Kotani S, Lu L, Kaplamadzhiev DB, Mori Y, Tonchev AB, Yamashima T (2007) Expression of free fatty acid receptor GPR40 in the central nervous system of adult monkeys. Neurosci Res 58(4):394–401

Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR (2015) Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun 6:6734. doi:10.1038/ncomms7734

Mancini AD, Poitout V (2013) The fatty acid receptor FFA1/GPR40 a decade later: how much do we know? Trends Endocrinol Metab 24(8):398–407. doi:10.1016/j.tem.2013.03.003; S1043-2760(13)00050-7 [pii]

Martin C, Passilly-Degrace P, Chevrot M, Ancel D, Sparks SM, Drucker DJ, Besnard P (2012) Lipid-mediated release of GLP-1 by mouse taste buds from circumvallate papillae: putative involvement of GPR120 and impact on taste sensitivity. J Lipid Res 53(11):2256–2265. doi:10.1194/jlr.M025874

Marzuillo P, Grandone A, Conte M, Capuano F, Cirillo G, Di Sessa A, Umano GR, Romano R, Perrone L, del Giudice EM (2014) Novel association between a nonsynonymous variant (R270H) of the G-protein-coupled receptor 120 and liver injury in children and adolescents with obesity. J Pediatr Gastroenterol Nutr 59(4):472–475. doi:10.1097/MPG.0000000000000463

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461(7268):1282–1286. doi:10.1038/nature08530

Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, Izawa S, Kondo Y, Ito Y, Tamura Y, Yanamoto K, Noda H, Tanabe A, Okaniwa N, Yamaguchi Y, Iwamoto T, Kasugai K (2013) G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflamm Bowel Dis 19(13):2848–2856. doi:10.1097/01.MIB.0000435444.14860.ea

Matsumura S, Eguchi A, Mizushige T, Kitabayashi N, Tsuzuki S, Inoue K, Fushiki T (2009) Colocalization of GPR120 with phospholipase-Cbeta2 and alpha-gustducin in the taste bud cells in mice. Neurosci Lett 450(2):186–190. doi:10.1016/j.neulet.2008.11.056

McNelis JC, Lee YS, Mayoral R, van der Kant R, Johnson AM, Wollam J, Olefsky JM (2015) GPR43 potentiates beta-cell function in obesity. Diabetes 64(9):3203–3217. doi:10.2337/db14-1938

Mizuta K, Zhang Y, Mizuta F, Hoshijima H, Shiga T, Masaki E, Emala CW Sr (2015) Novel identification of the free fatty acid receptor FFAR1 that promotes contraction in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 309(9):L970–L982. doi:10.1152/ajplung.00041.2015

Moore K, Zhang Q, Murgolo N, Hosted T, Duffy R (2009) Cloning, expression, and pharmacological characterization of the GPR120 free fatty acid receptor from cynomolgus monkey: comparison with human GPR120 splice variants. Comp Biochem Physiol B Biochem Mol Biol 154(4):419–426. doi:10.1016/j.cbpb.2009.08.005

Nagasaki H, Kondo T, Fuchigami M, Hashimoto H, Sugimura Y, Ozaki N, Arima H, Ota A, Oiso Y, Hamada Y (2012) Inflammatory changes in adipose tissue enhance expression of GPR84, a medium-chain fatty acid receptor: TNFalpha enhances GPR84 expression in adipocytes. FEBS Lett 586(4):368–372. doi:10.1016/j.febslet.2012.01.001

Naik H, Vakilynejad M, Wu J, Viswanathan P, Dote N, Higuchi T, Leifke E (2012) Safety, tolerability, pharmacokinetics, and pharmacodynamic properties of the GPR40 agonist TAK-875: results from a double-blind, placebo-controlled single oral dose rising study in healthy volunteers. J Clin Pharmacol 52(7):1007–1016. doi:10.1177/0091270011409230; 0091270011409230 [pii]

Nilsson NE, Kotarsky K, Owman C, Olde B (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 303(4):1047–1052. doi:10.1016/s0006-291x(03)00488-1

Nohr MK, Egerod KL, Christiansen SH, Gille A, Offermanns S, Schwartz TW, Moller M (2015) Expression of the short chain fatty acid receptor GPR41/FFAR3 in autonomic and somatic sensory ganglia. Neuroscience 290:126–137. doi:10.1016/j.neuroscience.2015.01.040

Offermanns S (2014) Free fatty acid (FFA) and hydroxy carboxylic acid (HCA) receptors. Annu Rev Pharmacol Toxicol 54:407–434. doi:10.1146/annurev-pharmtox-011613-135945

Ogawa T, Hirose H, Miyashita K, Saito I, Saruta T (2005) GPR40 gene Arg211His polymorphism may contribute to the variation of insulin secretory capacity in Japanese men. Metab Clin Exp 54(3):296–299. doi:10.1016/j.metabol.2004.09.008

Oh DY, Olefsky JM (2012) Omega 3 fatty acids and GPR120. Cell Metab 15(5):564–565. doi:10.1016/j.cmet.2012.04.009

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142(5):687–698. doi:10.1016/j.cell.2010.07.041

Pluznick J (2014) A novel SCFA receptor, the microbiota, and blood pressure regulation. Gut Microbes 5(2):202–207. doi:10.4161/gmic.27492

Pluznick JL, Protzko RJ, Gevorgyan H, Peterlin Z, Sipos A, Han J, Brunet I, Wan LX, Rey F, Wang T, Firestein SJ, Yanagisawa M, Gordon JI, Eichmann A, Peti-Peterdi J, Caplan MJ (2013) Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. Proc Natl Acad Sci U S A 110(11):4410–4415. doi:10.1073/pnas.1215927110

Poitout V, Lin DC (2013) Modulating GPR40: therapeutic promise and potential in diabetes. Drug Discov Today 18(23–24):1301–1308. doi:10.1016/j.drudis.2013.09.003

Priyadarshini M, Layden BT (2015) FFAR3 modulates insulin secretion and global gene expression in mouse islets. Islets 7(2):e1045182. doi:10.1080/19382014.2015.1045182

Priyadarshini M, Villa SR, Fuller M, Wicksteed B, Mackay CR, Alquier T, Poitout V, Mancebo H, Mirmira RG, Gilchrist A, Layden BT (2015) An acetate-specific GPCR, FFAR2, regulates insulin secretion. Mol Endocrinol 29(7):1055–1066. doi:10.1210/me.2015-1007

Priyadarshini M, Wicksteed B, Schiltz GE, Gilchrist A, Layden BT (2016) SCFA receptors in pancreatic beta cells: novel diabetes targets? Trends Endocrinol Metab. doi:10.1016/j.tem.2016.03.011

Puhl HL 3rd, Won YJ, Lu VB, Ikeda SR (2015) Human GPR42 is a transcribed multisite variant that exhibits copy number polymorphism and is functional when heterologously expressed. Sci Rep 5:12880. doi:10.1038/srep12880

Regard JB, Sato IT, Coughlin SR (2008) Anatomical profiling of G protein-coupled receptor expression. Cell 135(3):561–571. doi:10.1016/j.cell.2008.08.040

Rodriguez-Pacheco F, Garcia-Serrano S, Garcia-Escobar E, Gutierrez-Repiso C, Garcia-Arnes J, Valdes S, Gonzalo M, Soriguer F, Moreno-Ruiz FJ, Rodriguez-Canete A, Gallego-Perales JL, Martinez-Ferriz A, Rojo-Martinez G, Garcia-Fuentes E (2014) Effects of obesity/fatty acids on the expression of GPR120. Mol Nutr Food Res 58(9):1852–1860. doi:10.1002/mnfr.201300666

Ryan KK, Seeley RJ (2013) Physiology. Food as a hormone. Science 339(6122):918–919. doi:10.1126/science.1234062

Salehi A, Flodgren E, Nilsson NE, Jimenez-Feltstrom J, Miyazaki J, Owman C, Olde B (2005) Free fatty acid receptor 1 (FFA(1)R/GPR40) and its involvement in fatty-acid-stimulated insulin secretion. Cell Tissue Res 322(2):207–215

Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 105(43):16767–16772. doi:10.1073/pnas.0808567105

Sawzdargo M, George SR, Nguyen T, Xu S, Kolakowski LF, O'Dowd BF (1997) A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. Biochem Biophys Res Commun 239(2):543–547

Schnell S, Schaefer M, Schofl C (2007) Free fatty acids increase cytosolic free calcium and stimulate insulin secretion from beta-cells through activation of GPR40. Mol Cell Endocrinol 263(1–2):173–180. doi:10.1016/j.mce.2006.09.013

Sekiguchi H, Kasubuchi M, Hasegawa S, Pelisch N, Kimura I, Ichimura A (2015) A novel antidiabetic therapy: free fatty acid receptors as potential drug target. Curr Diabetes Rev 11 (2):107–115

Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, Raabe B, Chalaris A, Scheller J, Rehmann A, Franke A, Ott S, Hasler R, Nikolaus S, Folsch UR, Rose-John S, Jiang HP, Li J, Schreiber S, Rosenstiel P (2009) G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 183(11):7514–7522. doi:10.4049/jimmunol.0900063

Smith NJ, Stoddart LA, Devine NM, Jenkins L, Milligan G (2009) The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1. J Biol Chem 284(26):17527–17539. doi:10.1074/jbc.M109.012849

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341(6145):569–573. doi:10.1126/science.1241165

Spector AA, Kim HY (2014) Discovery of essential fatty acids. J Lipid Res. doi:10.1194/jlr.R055095

Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H (2005) The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab 1(4):245–258. doi:10.1016/j.cmet.2005.03.007

Stoddart LA, Smith NJ, Jenkins L, Brown AJ, Milligan G (2008a) Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. J Biol Chem 283 (47):32913–32924. doi:10.1074/jbc.M805601200

Stoddart LA, Smith NJ, Milligan G (2008b) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev 60(4):405–417. doi:10.1124/pr.108.00802

Stone VM, Dhayal S, Brocklehurst KJ, Lenaghan C, Sorhede Winzell M, Hammar M, Xu X, Smith DM, Morgan NG (2014) GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans. Diabetologia 57 (6):1182–1191. doi:10.1007/s00125-014-3213-0

Stumvoll M, Haring H (2002) The peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism. Diabetes 51(8):2341–2347

Suckow AT, Polidori D, Yan W, Chon S, Ma JY, Leonard J, Briscoe CP (2014) Alteration of the glucagon axis in GPR120 (FFAR4) knockout mice: a role for GPR120 in glucagon secretion. J Biol Chem 289(22):15751–15763. doi:10.1074/jbc.M114.568683

Sum CS, Tikhonova IG, Neumann S, Engel S, Raaka BM, Costanzi S, Gershengorn MC (2007) Identification of residues important for agonist recognition and activation in GPR40. J Biol Chem 282(40):29248–29255. doi:10.1074/jbc.M705077200

Suzuki M, Takaishi S, Nagasaki M, Onozawa Y, Iino I, Maeda H, Komai T, Oda T (2013) Medium-chain fatty acid-sensing receptor, GPR84, is a proinflammatory receptor. J Biol Chem 288(15):10684–10691. doi:10.1074/jbc.M112.420042

Sykaras AG, Demenis C, Case RM, McLaughlin JT, Smith CP (2012) Duodenal enteroendocrine I-cells contain mRNA transcripts encoding key endocannabinoid and fatty acid receptors. PLoS One 7(8):e42373. doi:10.1371/journal.pone.0042373

Takano R, Yoshida M, Inoue M, Honda T, Nakashima R, Matsumoto K, Yano T, Ogata T, Watanabe N, Toda N (2014) Discovery of 3-aryl-3-ethoxypropanoic acids as orally active GPR40 agonists. Bioorg Med Chem Lett 24(13):2949–2953. doi:10.1016/j.bmcl.2014.04.065

Taneera J, Lang S, Sharma A, Fadista J, Zhou Y, Ahlqvist E, Jonsson A, Lyssenko V, Vikman P, Hansson O, Parikh H, Korsgren O, Soni A, Krus U, Zhang E, Jing XJ, Esguerra JL, Wollheim CB, Salehi A, Rosengren A, Renstrom E, Groop L (2012) A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. Cell Metab 16 (1):122–134. doi:10.1016/j.cmet.2012.06.006

Tang Y, Chen Y, Jiang H, Robbins GT, Nie D (2011) G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. Int J Cancer 128(4):847–856. doi:10.1002/ijc.25638

Tang C, Ahmed K, Gille A, Lu S, Grone HJ, Tunaru S, Offermanns S (2015) Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nat Med 21(2):173–177. doi:10.1038/nm.3779

Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki SI, Kuwahara A (2008) Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol 59(Suppl 2):251–262

Tazoe H, Otomo Y, Karaki S, Kato I, Fukami Y, Terasaki M, Kuwahara A (2009) Expression of short-chain fatty acid receptor GPR41 in the human colon. Biomed Res 30(3):149–156

Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM (2012) Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 61(2):364–371. doi:10.2337/db11-1019

Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, Marsland BJ (2014) Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20(2):159–166. doi:10.1038/nm.3444

Tsujihata Y, Ito R, Suzuki M, Harada A, Negoro N, Yasuma T, Momose Y, Takeuchi K (2011) TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats. J Pharmacol Exp Ther 339(1):228–237. doi:10.1124/jpet.111.183772; jpet.111.183772 [pii]

Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR (1996) A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379(6560):69–72. doi:10.1038/379069a0

Ulven T, Christiansen E (2015) Dietary fatty acids and their potential for controlling metabolic diseases through activation of FFA4/GPR120. Annu Rev Nutr 35:239–263. doi:10.1146/annurev-nutr-071714-034410

Vestmar MA, Andersson EA, Christensen CR, Hauge M, Glumer C, Linneberg A, Witte DR, Jorgensen ME, Christensen C, Brandslund I, Lauritzen T, Pedersen O, Holst B, Grarup N, Schwartz TW, Hansen T (2016) Functional and genetic epidemiological characterisation of the FFAR4 (GPR120) p.R270H variant in the Danish population. J Med Genet. doi:10.1136/jmedgenet-2015-103728

Vettor R, Granzotto M, De Stefani D, Trevellin E, Rossato M, Farina MG, Milan G, Pilon C, Nigro A, Federspil G, Vigneri R, Vitiello L, Rizzuto R, Baratta R, Frittitta L (2008) Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization. J Clin Endocrinol Metab 93(9):3541–3550. doi:10.1210/jc.2007-2680

Vinolo MA, Ferguson GJ, Kulkarni S, Damoulakis G, Anderson K, Bohlooly YM, Stephens L, Hawkins PT, Curi R (2011) SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. PLoS One 6(6):e21205. doi:10.1371/journal.pone.0021205

Wagner R, Kaiser G, Gerst F, Christiansen E, Due-Hansen ME, Grundmann M, Machicao F, Peter A, Kostenis E, Ulven T, Fritsche A, Haring HU, Ullrich S (2013) Reevaluation of fatty acid receptor 1 as a drug target for the stimulation of insulin secretion in humans. Diabetes 62 (6):2106–2111. doi:10.2337/db12-1249

Wagner R, Hieronimus A, Lamprinou A, Heni M, Hatziagelaki E, Ullrich S, Stefan N, Staiger H, Haring HU, Fritsche A (2014) Peroxisome proliferator-activated receptor gamma (PPARG) modulates free fatty acid receptor 1 (FFAR1) dependent insulin secretion in humans. Mol Metab 3(6):676–680. doi:10.1016/j.molmet.2014.07.001

Waguri T, Goda T, Kasezawa N, Yamakawa-Kobayashi K (2013) The combined effects of genetic variations in the GPR120 gene and dietary fat intake on obesity risk. Biomed Res 34(2):69–74

Watson SJ, Brown AJ, Holliday ND (2012) Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 81(5):631–642. doi:10.1124/mol.111.077388

Watterson KR, Hudson BD, Ulven T, Milligan G (2014) Treatment of type 2 diabetes by free fatty acid receptor agonists. Front Endocrinol 5:137. doi:10.3389/fendo.2014.00137

Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101(4):1045–1050. doi:10.1073/pnas.2637002100

Yabuki C, Komatsu H, Tsujihata Y, Maeda R, Ito R, Matsuda-Nagasumi K, Sakuma K, Miyawaki K, Kikuchi N, Takeuchi K, Habata Y, Mori M (2013) A novel antidiabetic drug, fasiglifam/TAK-875, acts as an ago-allosteric modulator of FFAR1. PLoS One 8(10):e76280. doi:10.1371/journal.pone.0076280

Yonezawa T, Kobayashi Y, Obara Y (2007) Short-chain fatty acids induce acute phosphorylation of the p38 mitogen-activated protein kinase/heat shock protein 27 pathway via GPR43 in the MCF-7 human breast cancer cell line. Cell Signal 19(1):185–193. doi:10.1016/j.cellsig.2006.06.004

Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJ, Smith DM, Arch JR (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett 584(11):2381–2386. doi:10.1016/j.febslet.2010.04.027

Zhou SF, Liu JP, Chowbay B (2009) Polymorphism of human cytochrome P450 enzymes and its clinical impact. Drug Metab Rev 41(2):89–295. doi:10.1080/03602530902843483

The Role and Future of FFA1 as a Therapeutic Target

Julien Ghislain and Vincent Poitout

Contents

Abstract

Of the 415 million people suffering from diabetes worldwide, 90% have type 2 diabetes. Type 2 diabetes is characterized by hyperglycemia and occurs in obese individuals as a result of insulin resistance and inadequate insulin levels. Accordingly, diabetes drugs are tailored to enhance glucose disposal or target

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the pancreatic islet β cell to increase insulin secretion. The majority of the present-day insulin secretagogues, however, increase the risk of iatrogenic hypoglycemia, and hence alternatives are actively sought. The long-chain fatty acid, G protein-coupled receptor FFA1/Gpr40, is expressed in β cells, and its activation potentiates insulin secretion in a glucose-dependent manner. Preclinical data indicate that FFA1 agonism is an effective treatment to restore glucose homeostasis in rodent models of diabetes. This initial success prompted clinical trials in type 2 diabetes patients, the results of which were promising; however, the field suffered a significant setback when the lead compound TAK-875/fasiglifam was withdrawn from clinical development due to liver safety concerns. Nevertheless, recent developments have brought to light a surprising complexity of FFA1 agonist action, signaling diversity, and biological outcomes, raising hopes that with a greater understanding of the mechanisms at play the second round will be more successful.

Keywords
β cell • Allosteric agonist • Biased agonism • FFA1/Gpr40 • Functional selectivity • Insulin secretion • Pancreatic islet • Type 2 diabetes

1 Introduction

The International Diabetes Federation predicts that by 2040, one in ten adults will be affected by diabetes. The most prevalent form, type 2 diabetes (T2D), makes up about 90% of the cases; the other 10% are due to type 1 and gestational diabetes. T2D is a chronic disorder of glucose homeostasis that is characterized by hyperglycemia and if left unchecked leads to long-term complications such as cardiovascular disease, stroke, diabetic retinopathy, and kidney failure, dramatically increasing the risk of death. Besides the grave consequences to an individual's well-being, economically T2D is a major burden, and costs are expected to escalate in the near term. The global T2D epidemic is closely linked to obesity rates that have increased dramatically as a result of overeating and sedentary lifestyle. Obesity augments circulating nonesterified fatty acids (NEFAs), glycerol, inflammatory cytokines, hormones, and other factors that increase insulin resistance in the peripheral tissue, such as the liver, muscle, and fat, compromising glucose disposal and increasing hepatic glucose output (gluconeogenesis and glycogenolysis). In general, however, insulin resistance alone will not precipitate diabetes as the body responds to insulin resistance with enhanced function of β cells in the pancreatic islets of Langerhans increasing insulin levels, thus maintaining normoglycemia. Over time, however, genetic and environmental factors contribute to islet β-cell failure and T2D ensues (Kahn et al. 2006; Prentki and Nolan 2006).

Beyond lifestyle intervention, a number of drugs have been developed for the management of T2D, many of which act to enhance glucose disposal. Metformin, a first-line treatment for T2D, primarily acts by suppressing hepatic gluconeogenesis,

whereas thiazolidinediones are peroxisome proliferator-activated receptor (PPAR) agonists that improve insulin sensitivity primarily in adipocytes, and gliflozins are SGLT2 inhibitors which reduce reabsorption of glucose in the kidney, thus lowering glycemia. A number of drugs that increase insulin secretion by the β cell have also been developed. One such class, the sulfonylureas (e.g., glimepiride), blocks ATP-sensitive K^+ (K_{ATP}) channels in the β-cell membrane leading to membrane depolarization and insulin secretion. Unfortunately, these drugs raise the risk of life-threatening hypoglycemia. The GLP-1 analogs (e.g., exenatide) and DPP inhibitors (e.g., sitagliptin) which prolong the half-life of endogenous GLP-1 and other gut peptides in the circulation stimulate insulin secretion in a glucose-dependent manner and hence offer an advantage over sulfonylureas by reducing the risk of iatrogenic hypoglycemia. Although it has long been known that lipids also potentiate insulin secretion in a glucose-dependent manner, the possibility that this might be exploited for diabetes therapy was realized only following the discovery that free fatty acids (FFA) act on the β cell, in part, by their binding to the G protein-coupled receptor (GPCR), FFA1 (Briscoe et al. 2003; Itoh et al. 2003). In this review, we present an overview of the biology and pharmacobiology of FFA1 in the control of insulin secretion and glucose homeostasis, and then in light of the withdrawal of a first-in-class FFA1 agonist, TAK-875 (fasiglifam), after a phase 3 clinical trial (Kaku et al. 2015), we discuss emerging themes in a reviving effort to exploit FFA1 for antidiabetic therapeutics.

2 Stimulus-Secretion Coupling in the β Cell

Given that the major site of FFA1 action is the β cell, we will begin with a review of the mechanisms controlling insulin secretion. In the β cell, insulin is contained in secretory vesicles that fuse with the plasma membrane in response to secretagogue stimuli to release insulin to the extracellular environment. Insulin secretion is biphasic; the first, rapid phase is characterized by the fusion of a small pool of pre-docked vesicles, the so-called readily releasable pool, whereas the second, prolonged phase involves the recruitment of insulin granules from intracellular stores to the membrane and their subsequent docking and fusion. Glucose is the primary regulator of insulin release (Fig. 1). Glucose enters the β cell by facilitated diffusion via GLUT2 transporters (GLUT1 in humans) in the membrane, at which point it is phosphorylated by glucokinase to glucose-6-phosphate. Glucokinase acts as a glucose sensor; the low affinity of this enzyme for glucose allows for significant variation in activity within physiological glucose concentrations. Once in the cell, glucose enters glycolysis and the Krebs cycle, where multiple ATP molecules are produced by oxidation. The resulting rise in intracellular ATP:ADP ratio closes the ATP-sensitive SUR1/Kir6.2 potassium channels preventing potassium ions (K^+) from escaping the β cell. The accumulation of K^+ results in membrane depolarization and opening of voltage-gated L-type calcium (Ca^{2+}) channels, allowing calcium ions to enter the cell. An increase in intracellular Ca^{2+} activates the exocytotic machinery causing the fusion of insulin-containing vesicles to the plasma

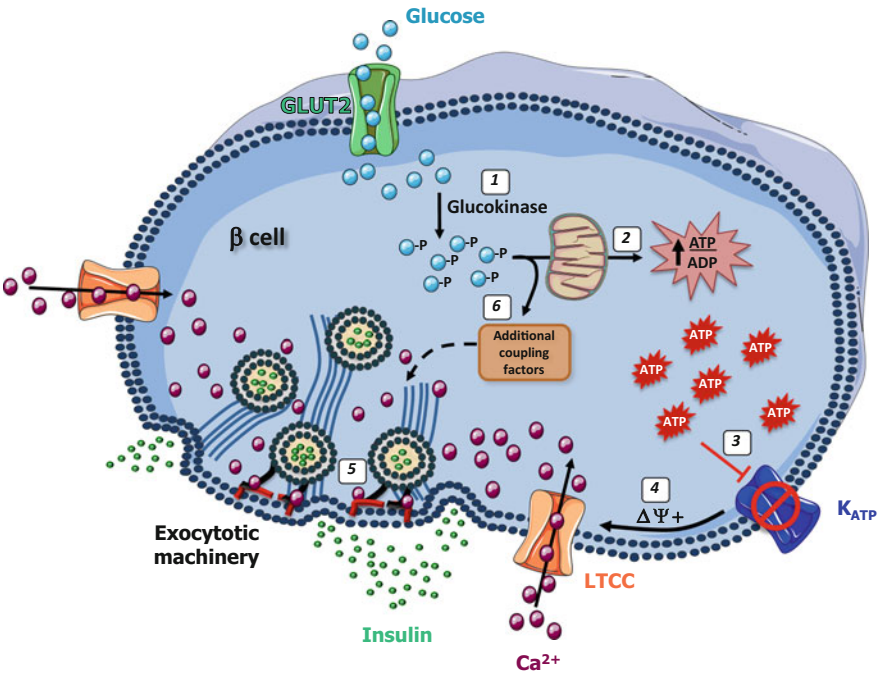

Fig. 1 Mechanisms of glucose-stimulated insulin secretion. Glucose enters the β cell by facilitated diffusion via the glucose transporter GLUT2 (GLUT1 in humans). Intracellular glucose is phosphorylated by glucokinase generating glucose-6-phosphate (G6P) which enters glycolysis to produce pyruvate (*1*). Pyruvate then enters the Krebs cycle leading to a rise in the ATP:ADP ratio (*2*) and closure of ATP-gated potassium channels (*3*). The accumulation of K^+ results in plasma membrane depolarization ($\Delta\psi$) and opening of voltage-gated Ca^{2+} channels (L-type Ca^{2+} channels, LTCC) (*4*). Finally, influx of extracellular Ca^{2+} through LTCC leads to an increase in cytosolic Ca^{2+} levels, which activates the exocytotic machinery controlling the fusion of insulin-containing vesicles with the plasma membrane (*5*). In parallel, additional metabolic coupling factors resulting from glucose metabolism amplify insulin secretion by regulating insulin vesicle trafficking, cytoskeletal proteins, membrane channels, and the exocytotic machinery (*6*). Reprinted from Mancini and Poitout (2013) with permission from Elsevier

membrane. In parallel to this triggering pathway, glucose metabolism generates additional metabolic coupling factors and second messenger signals that amplify insulin secretion by regulating insulin vesicle trafficking, cytoskeletal proteins, membrane channels, and the exocytotic machinery (Prentki et al. 2013; Wang and Thurmond 2009) (Fig. 1).

The β cell acts as a rheostat integrating diverse signals that modulate the insulin secretory response. Indeed, a number of metabolic, hormonal, and neuronal factors, many of which act through GPCRs (Ahren 2009; Amisten et al. 2013), are responsible for the fine-tuning of insulin secretion. Among these, stimulators of insulin secretion such as GLP-1 signal via a heterotrimeric G protein, Gs-coupled GPCR, which activates adenylate cyclases (AC) and increase intracellular cAMP levels,

whereas inhibitors of insulin secretion such as somatostatin bind to a Gi/o-coupled GPCR which inhibits AC activity. Another class of stimulatory GPCRs couple to Gq, which triggers phospholipase C-mediated hydrolysis of membrane phospholipids generating inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). In the β cell, the M3 muscarinic (acetylcholine) receptor and FFA1 are the predominant Gq-coupled receptors described to date.

3 FFA1 in the Control of Insulin Secretion

FFA1, a member of the class A (rhodopsin-like) GPCRs, was deorphanized in 2003 by screening compounds for changes in intracellular Ca^{2+} levels in transiently transfected cells. FFA1 binds saturated and mono- and polyunsaturated FFA with carbon chain lengths greater than six, the medium- to long-chain fatty acids (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003). As FFA1 is expressed in pancreatic islets and β-cell lines from both rodents (Itoh et al. 2003) and humans (Briscoe et al. 2003; Tomita et al. 2006) at relatively high levels, the possibility that FFA1 contributes to FFA potentiation of insulin secretion was investigated. Itoh et al. showed that FFA potentiation of insulin secretion in MIN6 mouse insulinoma cells is dependent on FFA1 (Itoh et al. 2003). Subsequently, using FFA1 knockout mice, we showed that FFA1 mediates FFA-induced insulin secretion in vivo and in islets ex vivo controlling about 50% of the response (Kebede et al. 2008; Latour et al. 2007). In light of the important contribution of FFA metabolism in insulin secretion, whether FFA1 signaling influences intracellular fuel metabolism was investigated. Although lipid profiling in mouse islets ex vivo did not reveal any change in nutrient metabolism in the absence of FFA1 (Alquier et al. 2009), more recently, using β-cell lines, glucose and lipid metabolism was affected by FFA1 signaling and found to contribute to the potentiation of insulin secretion (El-Azzouny et al. 2014; Kristinsson et al. 2015) pointing to a possible synergy between these two pathways in the control of insulin secretion.

FFA1 G protein coupling was first studied by Briscoe et al. (2003) and later substantiated by other labs (Latour et al. 2007; Stoddart et al. 2007). Together, these studies highlight a pathway whereby FFA1 couples predominantly to $G_q/_{11}$ which promotes phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate into DAG and IP3 (Fig. 2). Ferdaoussi et al. (2012) then went on to show that the protein kinase C-related kinase, PKD1, is phosphorylated/activated downstream of FFA1 in a DAG-dependent manner and is responsible for cytoskeletal F-actin remodeling and the potentiation of the second phase of insulin secretion. Although IP3 mobilizes Ca^{2+} from endoplasmic reticulum stores, extracellular sources may be responsible for the increase in intracellular Ca^{2+} in response to FFA1 agonism (Fujiwara et al. 2005; Itoh et al. 2003; Schnell et al. 2007). However, in parallel to the G protein-dependent events, GPCRs also couple with multifunctional adaptor proteins, the β-arrestins. This so-called G protein-independent signaling was first presented in a study by Mancini et al. showing that FFA1 recruits β-arrestins in a ligand-dependent manner (Mancini et al. 2015). Interestingly, early

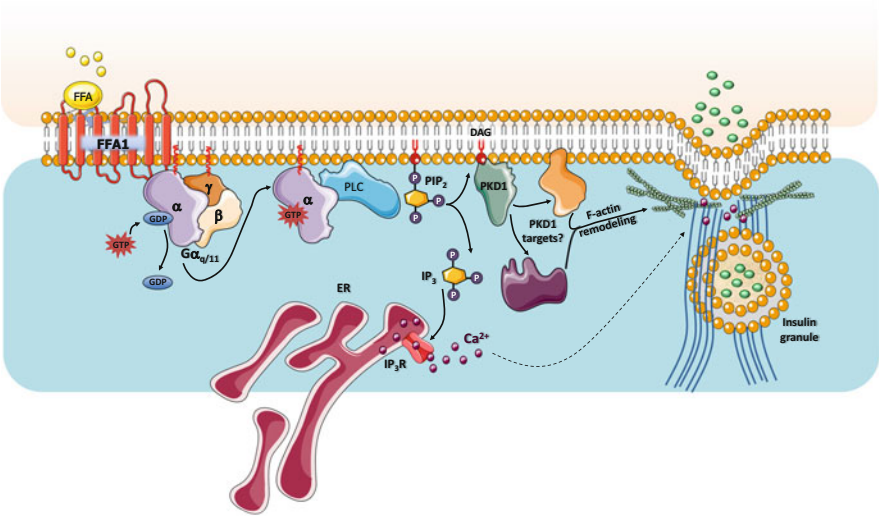

Fig. 2 FFA potentiation of glucose-stimulated insulin secretion via FFA1. Following FFA binding to FFA1 and activating GDP-for-GTP exchange on the α subunit of receptor-associated heterotrimeric G protein Gq/11 lead to its dissociation from the β/γ subunit. The active (GTP bound) α subunit triggers phospholipase C (PLC)-dependent cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG promotes the phosphorylation and activation of the protein kinase C isozyme, PKD1, which in turn phosphorylates and activates effectors implicated in filamentous (F)-actin remodeling, potentiating the second phase of glucose-stimulated insulin secretion. Although IP3 stimulates Ca^{2+} efflux from the endoplasmic reticulum (ER) increasing intracellular Ca^{2+} levels, its role in insulin secretion in response to FFA activation of FFA1 is unclear at present. Reprinted from Mancini and Poitout (2013) with permission from Elsevier

studies into FFA1 signaling suggest the involvement of the MAPK (ERK1/2) pathway (Itoh et al. 2003), a known effector of β-arrestin signaling. However, the significance of this pathway downstream of FFA1 remains unresolved as inhibition of MAPK does not affect FFA-dependent insulin secretion (Itoh et al. 2003).

4 FFA1 Signaling in Other Tissues

The potential benefits of FFA1 agonism for the treatment of T2D are not limited to the direct control of insulin secretion. In fact FFA1 acts in several tissues important in energy homeostasis. In the intestine, FFA1 is expressed in endocrine L, K, and I cells which secrete GLP-1, GIP, and CCK, respectively (Edfalk et al. 2008; Liou et al. 2011; Parker et al. 2009; Sykaras et al. 2012; Xiong et al. 2013). These so-called incretin hormones have a positive impact on glucose homeostasis not only by stimulating insulin secretion from the β cell but also by reducing glucagon secretion and gastric emptying and increasing satiety. In primary cultures of murine fetal intestinal cells and purified populations of murine intestinal cells expressing

incretin hormones, FFA1 mediates FFA stimulation of GLP-1, GIP, and CCK secretion (Liou et al. 2011; Xiong et al. 2013). In vivo, oral gavage with oil or fats induces an increase in circulating incretin hormone levels in control but not in FFA1 KO mice (Edfalk et al. 2008; Liou et al. 2011; Xiong et al. 2013).

Glucagon is secreted from islet α cells and acts in a manner opposite to insulin protecting from hypoglycemia. Glucagon is released into the bloodstream in response to low blood glucose levels and primarily acts on the liver to promote gluconeogenesis and glycogenolysis to increase blood glucose levels. The capacity of FFA to modulate glucagon secretion has been known for some time; however, the importance of FFA1 as a transducer of FFA-induced effects on glucagon secretion was only recently established. By comparing islets isolated from wild-type and FFA1 KO mice or by antisense-specific targeting of FFA1, FFA potentiation of glucagon secretion was found to be FFA1 dependent (Flodgren et al. 2007; Suckow et al. 2014; Wang et al. 2011). Interestingly, Pax6, a transcription factor important in α-cell development and function that is essential for the proper control of glucagon secretion, targets FFA1 transcriptional activation (Gosmain et al. 2012). Although the FFA1-dependent regulation of glucagon secretion can be viewed as potentially protective against hypoglycemia, the significance of this observation from a therapeutic perspective remains to be determined as the role of FFA1 signaling in α cells in vivo and its impact on glucose homeostasis have not been demonstrated.

FFA1 is expressed in several regions of the central nervous system (CNS) in humans, primates, and rodents (Briscoe et al. 2003; Itoh et al. 2003; Nakamoto et al. 2012) and is increased following cerebral ischemia in neurogenic niches (Ma et al. 2008). In the CNS, FFA1 has been proposed to act as a receptor for polyunsaturated fatty acids mediating their beneficial effects in antinociception (Nakamoto et al. 2012, 2013) and adult neurogenesis (Boneva and Yamashima 2012) and has been implicated in fatty acid taste preference (Cartoni et al. 2010) and neurovascular degeneration (Honore et al. 2013). Interestingly, agonist stimulation of FFA1 improves cognitive performance in an Alzheimer's disease mouse model (Khan et al. 2016). Although these studies are still in an early stage, the data suggest a potential of FFA1 as a therapeutic target for the treatment of inflammatory pain and possibly degenerative diseases including Alzheimer's and Parkinson's (Khan and He 2015).

In the bone, FFA1 signaling contributes to the control of bone density by regulating osteoclastogenesis (Cornish et al. 2008) and prevents ovariectomy-induced bone loss by inhibiting osteoclast differentiation (Wauquier et al. 2013). However, as FFA1 activation following treatment with thiazolidinediones may contribute to an increased risk of bone fracture by promoting osteocyte apoptosis (Mieczkowska et al. 2012), the potential of FFA1 as a therapeutic target for the control of bone turnover will require further investigation.

5 Role of FFA1 in Metabolic Disorders

Etiological studies of T2D progression suggest that chronic hyperglycemia and hyperlipidemia aggravate β-cell dysfunction, a phenomenon known as glucolipotoxicity (Poitout and Robertson 2008; Prentki and Nolan 2006). This concept is supported by numerous in vitro studies showing that whereas FFA acutely potentiates glucose-stimulated insulin secretion, chronic exposure of islets to FFA, particularly in the presence of high glucose concentrations, is detrimental to β-cell function and survival (Poitout 2008). Hence, whether FFA1 in the β cell transduces the negative effects of lipids to precipitate T2D onset was an important question that would greatly impact any drug development strategy. Indeed, early in vivo FFA1 functional studies raised the alarm. In 2005, Steneberg et al. presented a compelling in vivo functional analysis of FFA1 using FFA1 KO mice and transgenic mice overexpressing FFA1 under control of the Pdx-1 promoter (Steneberg et al. 2005). Consistent with previous findings, FFA1 was necessary for the acute effects of FFA on insulin secretion; however, surprisingly, the FFA chronic effects on β-cell function were also mediated by FFA1. Whereas loss of FFA1-protected mice from high-fat diet induced hyperinsulinemia, hyperlipidemia, hyperglycemia, and glucose intolerance, overexpression of FFA1 in the β cell negatively affected β-cell function and accelerated the onset of diabetes-like symptoms. Although these initial studies supported a pathophysiological role of FFA1 in glucose homeostasis, subsequent studies led to a major reversal of this view.

To test this question further, three independent mouse FFA1 null alleles were investigated. In these mice, FFA potentiation of insulin secretion was consistently reduced in the absence of FFA1 (Lan et al. 2008; Latour et al. 2007; Matsuda-Nagasumi et al. 2013). However, in contrast to the study by Steneberg et al., when islets were exposed ex vivo to glucolipotoxic stress conditions, loss of FFA1 did not lead to an improvement in β-cell function (Latour et al. 2007; Tan et al. 2008). Importantly, in vivo no significant differences in weight, adiposity, glucose tolerance, insulin sensitivity, and insulin secretion or liver steatosis were noted in FFA1 KO mice either under normal physiological conditions or following chronic metabolic stress induced by high-fat feeding, by genetically induced diabetes, or by exposure to a β3 adrenergic receptor agonist to elevate blood FFA levels (Kebede et al. 2008; Lan et al. 2008; Matsuda-Nagasumi et al. 2013; Pang et al. 2010). These studies clearly indicate that FFA1 is not detrimental to energy metabolism under normal and diabetogenic conditions. Interestingly, in some studies, the data point to a possible beneficial role of FFA1. Kebede et al. (2008) found that FFA1 KO mice develop fasting hyperglycemia earlier than their control littermates. Furthermore, using a transgenic mouse overexpressing human FFA1 under the control of the mouse insulin 2 gene promoter, Nagasumi et al. (2009) showed that these mice had improved islet function and did not develop glucose intolerance as their wild-type counterparts when exposed to chronic metabolic stress.

Given the importance FFA1 in glucose homeostasis in rodents, a number of genetic studies were undertaken in humans to identify FFA1 polymorphisms in

diabetes. Non-synonymous variants Arg104Pro, Asp175Asn, Arg211His, and Gly180Ser and a number of single nucleotide polymorphisms located in the intergenic FFA1 gene region were identified in T2D patients and control groups (Guo et al. 2015; Hamid et al. 2005; Kalis et al. 2007; Vettor et al. 2008). Despite the effect of some of these variants on FFA1 cell surface expression and signaling activity, no link to increased T2D risk has been demonstrated for any of these variants. Similarly, genome-wide association studies have not revealed any variants in the FFA1 gene region that co-segregate with the prediabetic/diabetic phenotype (Ramos et al. 2014). Interestingly, however, Arg211His and Gly180Ser and non-coding SNPs rs1978013 and rs1978014, located upstream of the FFA1 gene, are associated with reduced insulinogenic index (HOMA-β), and insulin levels are considerably lower in carriers in response to a nutrient load (Kalis et al. 2007; Ogawa et al. 2005; Vettor et al. 2008). Furthermore, analysis of FFA1 expression levels in islets showed a positive correlation with the insulinogenic index (Tomita et al. 2006) as well as the insulin secretory response in isolated islets (Del Guerra et al. 2010). Together, these studies support a positive role of FFA1 in insulin secretion in humans. However, variants in FFA1 have been associated with increasing BMI and blood lipid levels, and these parameters are themselves associated with poor β-cell function (Walker et al. 2011). One of these variants, rs1573611, also affects the relationship between circulating FFA and β-cell function (Wagner et al. 2013) and has been proposed to contribute to an increased risk of β-cell failure (Wagner et al. 2014), but this remains to be directly demonstrated.

6 Preclinical FFA1 Pharmacology

A comprehensive description of FFA1 synthetic ligands is beyond the scope of this review. Here we review some of the most important developments in the therapeutic targeting of FFA1 and highlight how this has helped to dissect the pharmacological properties of FFA1.

As discussed above, early functional studies in rodents led to the concept that antagonism of FFA1 may be beneficial for the treatment of T2D (Steneberg et al. 2005). It was reasoned that FFA1 antagonists might protect against pancreatic β-cell dysfunction by reducing β-cell exhaustion and improve insulin sensitivity by alleviating hyperinsulinemia. Accordingly, several synthetic antagonists were developed that inhibit the FFA1-dependent insulinotropic effects (Hu et al. 2009; Kristinsson et al. 2013). In ex vivo human islet studies, the FFA1 antagonist ANT203 protects against β-cell dysfunction and apoptosis induced by chronic FFA exposure (Kristinsson et al. 2013). However, in vivo the benefits of FFA1 antagonism were less convincing; DC260126, a FFA1 antagonist, decreased insulin levels and improve insulin tolerance when chronically administered to obese Zucker rats and obese diabetic db/db mice (Sun et al. 2013; Zhang et al. 2010). However, although β-cell apoptosis was reduced, there was no evidence for

improvement in glycemia, lipidemia, glucose tolerance, food intake, or body weight.

In contrast to FFA1 antagonism, agonism robustly alleviates diabetes symptoms in rodent models. Numerous selective, orally available FFA1 synthetic agonists mimicking the endogenous FFA with an acidic head group and hydrophobic tail have been developed. A first-in-class FFA1 agonist TAK-875, fasiglifam, is a partial agonist compared to the natural ligand, γ-linoleic acid, based on Ca^{2+} mobilization in FFA1-transfected heterologous cells and exerts insulinotropic activity in rodent islets and β-cell lines (Tsujihata et al. 2011; Yabuki et al. 2013). Acute administration of TAK-875 to male Zucker diabetic fatty rats and female Wistar fatty rats with impaired glucose tolerance augments plasma insulin levels and reduces fasting hyperglycemia (Negoro et al. 2010; Tsujihata et al. 2011). In contrast, in fasted normal rats, TAK-875 neither enhances insulin secretion nor causes hypoglycemia, confirming the glucose-dependent insulinotropic effect of FFA1 agonism. Although prolonged exposure of β-cell lines or islets to TAK-875 neither disrupts β-cell function nor increases apoptosis (Tsujihata et al. 2011), in another study, TAK-875 amplified lipotoxicity (Kristinsson et al. 2013). However, in vivo 6-week treatment with TAK-875 alone or in combination with metformin improves glycemic control and insulin levels as well as islet morphology in Zucker diabetic fatty rats (Ito et al. 2013). Importantly, similar improvements in islet function in vitro, and glycemic control in vivo, have been documented in diabetic rodents exposed to different FFA1 synthetic agonists, including GW-9508 (Ou et al. 2013), CNX-011-67 (Gowda et al. 2013; Sunil et al. 2014; Verma et al. 2014a), Cpd-B/C (Tan et al. 2008), and AS2034178 (Tanaka et al. 2013), both acutely and chronically. Together, these studies clearly illustrate that long-term exposure to synthetic FFA1 agonists has a positive effect on the control of diabetes symptoms in rodent models.

Meanwhile, a family of orally bioavailable FFA1 agonists was developed by researchers at Amgen that highlights an important aspect of in vivo efficacy. In Ca^{2+} mobilization assays, the parent compound, AMG837, is a partial agonist that potentiates insulin secretion from isolated islets ex vivo in a FFA1-dependent manner (Houze et al. 2012; Lin et al. 2011). In vivo, AMG837 increases insulin secretion and improves glucose levels in normal and Zucker fatty rats following both acute and chronic 21-day exposure. The initial success of AMG837 encouraged, through high-throughput screening and structure-activity relationship studies, the development of a series of FFA1 agonists. One such compound, AM-1638, is a full agonist that shows improved in vivo efficacy compared to AMG837 following an oral glucose tolerance test (OGTT) in high-fat-fed, streptozotocin-treated, and NONcNZO10/LtJ diabetic mice (Brown et al. 2012; Luo et al. 2012). Interestingly, AM-1638 not only potentiates insulin secretion from pancreatic islets with greater efficacy than AMG837, but it also stimulates GLP-1 and GIP secretion from intestinal enteroendocrine cells, a property that AMG837 only weakly possesses (Luo et al. 2012). Importantly, the authors showed that the improved glucose tolerance and insulin secretion in mice treated with AM-1638 were not only FFA1 dependent but also GLP-1 dependent; when mice were

simultaneously treated with a GLP-1 antagonist, glucose tolerance and insulin levels decreased. To begin to understand why AMG837 and AM-1638 differ in their capacity to stimulate GLP-1 secretion from intestinal cells yet both possess insulinotropic activity on β cells, the authors compared the efficacy of these compounds in a Ca^{2+} mobilization assay in CHO cells transfected with decreasing amounts of FFA1 plasmid. Whereas AM-1638 and the natural ligand docosahexaenoic acid (DHA) were largely equi-efficacious irrespective of the level of receptor expression, AMG837 activity was rapidly lost as the level of receptor expression decreased. Accordingly, the authors suggest enteroendocrine L cells may express inadequate cell surface FFA1 compared to the pancreatic β cell to garner sufficient AMG837 activity for GLP-1 secretion.

The discovery that the recruitment of the enteroinsular axis leads to heightened FFA1 agonist efficacy will have implications for the development of FFA1 agonists. Hence, although FFA1 agonists GW-9508, TUG-424, and TAK-875 exert beneficial effects on glucose homeostasis in diabetic animal models, their partial agonist properties likely provide limited benefits to incretin levels and overall efficacy (Luo et al. 2012). Interestingly, the FFA1 agonist AS2575959 alone does not increase plasma GLP-1 levels in mice; however, when combined with the DPP inhibitor, sitagliptin leads to a synergistic increase in GLP-1 and insulin levels and enhanced glucose tolerance (Tanaka et al. 2014).

Although the preclinical studies indicate that FFA1 agonist exposure improves glucose homeostasis in diabetes, a number of studies suggest that their beneficial effects may not be fully realized due to downregulation of β-cell FFA1 expression in diabetes. Whereas short-term exposure of mouse and human islets ex vivo to supraphysiological glucose concentrations augments FFA1 mRNA and protein expression, via increased binding of pancreas-duodenum homeobox-1 (Pdx-1) to the FFA1 promoter, and correlates with increased insulinotropic activity of FFA (Kebede et al. 2012), long-term exposure of islets to high glucose reduces FFA1 expression (Meidute Abaraviciene et al. 2013). In vivo, although FFA1 is increased in islets of prediabetic Zucker rats, hyperglycemic Goto-Kakizaki rats displayed dramatically reduced FFA1 expression in islets (Meidute Abaraviciene et al. 2013). Similarly, in a rat model of nutrient excess, infusion of high glucose and lipids over 3 days downregulates FFA1 expression in islets (Fontes et al. 2010). FFA1 mRNA expression is reduced in islets from humans with T2D (Del Guerra et al. 2010). Rosiglitazone a thiazolidinedione activates PPARγ in β cells to promote FFA1 expression and insulin secretion (Kim et al. 2013; Meidute Abaraviciene et al. 2013), suggesting that these drugs might increase FFA1 agonist efficacy.

7 Allosterism, Ligand Cooperativity, and Functional Selectivity at FFA1

In radioligand-binding interaction assays, Lin et al. (2012) showed that partial agonists such as AMG837 and full agonists such as AM-1638 exert their effects by binding to distinct sites on FFA1. Another ligand class, represented by

AM-8182, competes poorly with AMG837 and AM-1638 indicative of binding to a third site. Interestingly, only AM-8182 but not AMG837 or AM-1638 interacts competitively with the natural ligand DHA, suggesting that the binding site for AM-8182 is shared with the orthosteric site. This study highlights the presence of multiple allosteric ligand-binding sites on FFA1 and is in agreement with the FFA1 crystal structure data that suggest the existence of several potential, topographically distinct, ligand-binding pockets (Srivastava et al. 2014). As binding of allosteric ligands often modulates the potency and/or efficacy of ligand binding to the orthosteric site, binding and functional cooperativity of these FFA1 agonists was investigated (Lin et al. 2012; Xiong et al. 2013). The authors reported that when ligands for distinct allosteric sites were combined or paired with the orthosteric ligands α-linoleic acid or DHA, binding cooperativity and functional cooperativity in the form of enhanced signaling and insulin secretion in MIN6 cells and mouse islets ex vivo were observed. Importantly, the in vitro cooperativity translated into enhanced in vivo efficacy whereby acute administration of agonists alone had little effect; however, when coadministered, glucose tolerance and insulin and GLP-1 secretion were dramatically increased. Such allosterism and ligand cooperativity were also described in studies of TAK-875. Mutational analyses indicated that TAK-875 binds to a distinct site on FFA1 relative to γ-linoleic acid (Yabuki et al. 2013), data that was also supported by the analysis of the crystal structure of FFA1 in complex with TAK-875 (Srivastava et al. 2014). Then, in functional studies, TAK-875 was found to behave as an ago-allosteric modulator; alone TAK-875 potentiates glucose-stimulated insulin secretion albeit weakly; however, when combined with γ-linoleic acid, insulin secretion is synergistically increased (Yabuki et al. 2013). Finally, in diabetic rats, depletion of NEFAs reduces the insulinotropic effect of TAK-875 supporting in vivo functional cooperativity.

Another important property of FFA1 agonism that has recently come to light is that of functional selectivity. Functional selectivity, or biased agonism, is based on the notion that different ligands may favor activation of distinct signaling arms by stabilizing distinct receptor conformations (Rankovic et al. 2016). Hauge et al. (2015) showed that AM-1638 (and the related AM-5262 (Wang et al. 2013)) signals not only via Gq but also Gs, whereas linoleic acid, TAK-875, and AMG837 signal exclusively via Gq, in FFA1-transfected COS cells. The authors then showed that the Gq-only agonists weakly activate incretin secretion from L cells, whereas the Gq + Gs agonists potently stimulate GLP-1 secretion. Hence, rather than differences in the level of receptor expression between cell types (Luo et al. 2012), the distinct biological effects of these allosteric FFA1 agonists may be explained by functional selectivity. Interestingly, FFA1 downstream signaling is not restricted to Gq- and Gs-dependent events as several studies have revealed that FFA1 also couples with the inhibitory G protein, Gi (Fujita et al. 2011; Schroder et al. 2010), and β-arrestins (Mancini et al. 2015; Qian et al. 2014) (Fig. 3).

β-arrestins are multifunctional adaptor proteins classically known for their role in the downregulation of GPCR signaling via GPCR desensitization and internalization. Second messenger-dependent protein kinase phosphorylation of GPCRs leads to recruitment of β-arrestins to the GPCR and steric hindrance of further G

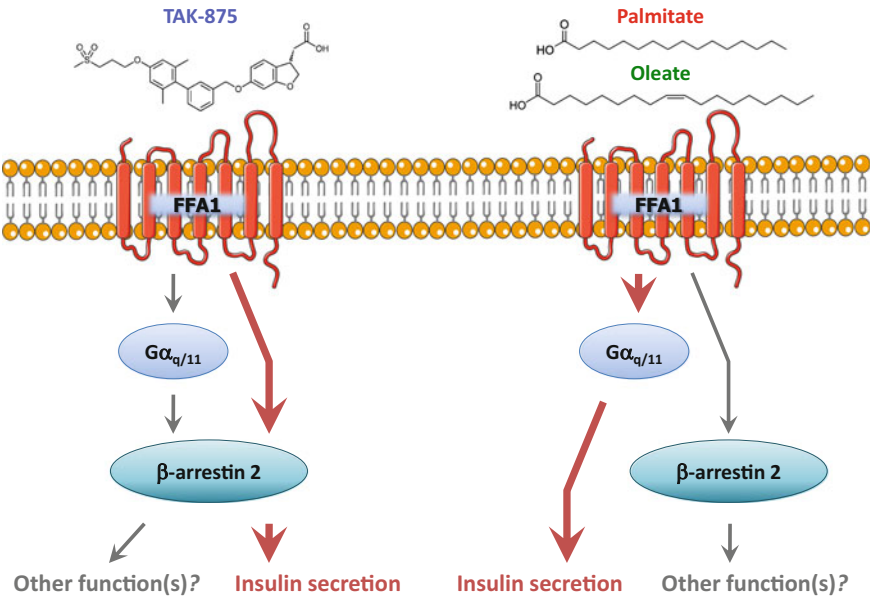

Fig. 3 Model for biased agonism at FFA1. Activation of FFA1 potentiates glucose-stimulated insulin secretion via G protein (Gq/11)- and β-arrestin-dependent mechanisms. The natural agonists, palmitate (PA) and oleate (OA), and the synthetic agonist TAK-875 display inverse relative efficacies for Gq/11 and β-arrestin pathway activation (as depicted by *arrow thickness*). TAK-875 recruits β-arrestin via Gq/11-dependent and Gq/11-independent mechanisms to potentiate insulin secretion (*left*). Although PA and OA also engage β-arrestin, the Gq/11 pathway serves as the main signal responsible for the insulinotropic activity of FFA (*right*). β-arrestin signaling is postulated to exert beneficial effects in addition to insulin secretion. This figure was originally published in Mancini et al. (2015) © the American Society for Biochemistry and Molecular Biology

protein activation (desensitization) and recruitment of key players controlling receptor endocytosis (internalization) (Zhang et al. 1997). Qian et al. (2014) showed that β-arrestin-2 is recruited to human FFA1 in response to FFA stimulation, and both β-arrestin-2 and GRK2 contribute to ligand-dependent receptor internalization and recycling. More recently, however, nonclassical functions of β-arrestins have been described whereby β-arrestins link GPCRs to downstream signaling events via Src family tyrosine kinases, c-jun terminal kinase 3, MAPK cascade, and Akt, in what is known as G protein-independent signaling (McDonald and Lefkowitz 2001). In a recent study (Mancini et al. 2015), we demonstrated, using bioluminescence-based biosensors in transfected HEK cells, FFA1-β-arrestin coupling and discovered that whereas TAK875 recruits β-arrestins with higher efficacy compared to FFA, the reverse is true for Gq recruitment to FFA1 (Fig. 3). Importantly, the insulinotropic activity of TAK-875, but not FFA, is dependent on β-arrestin, as FFA-induced insulin secretion is not affected in β-arrestin-2 mutant mouse islets, whereas the TAK-875 response is dramatically

reduced. Hence, this study illustrates biased signaling at FFA1 whereby the allosteric ligand TAK-875 is β-arrestin biased and the orthosteric natural ligands are Gq biased.

A detailed understanding of functional selectivity at FFA1 may help to pave the way for the development of safer, more efficacious agonists. For example, FFA1 signaling in β cells exerts not only insulinotropic activity but also cytoprotective promotion of anti-inflammatory and antiapoptotic effects (Verma et al. 2014b; Wagner et al. 2013; Zhang et al. 2007), and the evidence indicates that the MAPK pathway, a known effector of β-arrestin signaling, may be involved (Panse et al. 2015; Zhang et al. 2007). Hence, FFA1-biased agonists favoring β-arrestin signaling might provide additional benefits to their insulinotropic activity.

8 Clinical Benefits of FFA1 Agonism

The compelling preclinical data indicating a beneficial effect of FFA1 synthetic agonists on glucose homeostasis in metabolically compromised rodents has prompted a number of pharmaceutical companies to initiate clinical trials. Takeda has pioneered this field with TAK-875 undertaking extensive trials in North America and Japan. T2D patients not responding to diet modification or exercise (Araki et al. 2012; Kaku et al. 2013) or metformin (Burant et al. 2012) were enrolled. In phase 2 trials, both dose (6.25–200 mg orally once daily) and duration of exposure (2–12 weeks) were investigated and compared to participants receiving placebo or a frontline T2D drug, glimepiride, a sulfonylurea which stimulates insulin secretion by activating the K^+ channels in the β-cell membrane. Overall, the data were encouraging; those receiving TAK-875 exhibited significantly lower Hba1c levels without hypoglycemic episodes. A phase 3 study was then initiated in Japan to test the benefits of TAK-875 (25 or 50 mg daily) treatment compared to placebo over a longer period of time (24 weeks) (Kaku et al. 2015). In line with the previous phase 2 studies, recipients of TAK-875 showed a dose-dependent improvement in HbA1c and fasting glucose levels at 24 weeks. Unfortunately, the number of trial participants was too low to reach statistical significance. Furthermore, 24-week exposure to TAK-875 is too short given that T2D patients would be expected to receive lifelong treatment. Finally, concerns about liver safety were subsequently raised, which led to abrupt termination of the trial and withdrawal of TAK-875 from clinical development.

The disappointing results from the phase 3 trial of a first-in-class FFA1 agonist were a major setback for the field. The prevailing view is that liver toxicity is likely to be FFA1 independent; unfortunately, the majority of FFA1 agonists share a similar structural feature, i.e., a lipophilic pharmacophore which may cause these toxic effects (Defossa and Wagner 2014). Eli Lilly and Amgen among others also initiated phase 1/2 clinical trials with lead compounds LY2881835 and AMG837, respectively. Although the details are shrouded, Eli Lilly reported that many participants exhibited clinically significant side effect, and trials for both

compounds were discontinued for undisclosed reasons (Defossa and Wagner 2014; Luo et al. 2012).

9 Concluding Remarks

Functional studies in rodents have revealed an important role of FFA1 in the potentiation of insulin secretion and the control of glucose homeostasis and highlighted both direct effects, via action on the β cell, and indirect effects, by engaging the enteroinsular axis. In depth, preclinical and clinical studies underscore the potential of FFA1 as a drug target to treat T2D with reduced risk of iatrogenic hypoglycemia compared to conventional approaches. The detailed dissection of the mechanism of FFA1 agonist action, including identification of allosteric binding sites, agonist structure-activity relationships, and the resolution of the FFA1 crystal structure in complex with TAK-875, has provided a solid foundation for rational drug design and paved the way for the development of dualsteric ligands (that bind simultaneously to both the orthosteric and allosteric sites) and functionally selective agonists with preferred activity profiles. Although evidence supports the engagement of multiple signaling pathways and biological outcomes downstream of FFA1, more studies will be required to determine the potential therapeutic benefits of functionally selective FFA1 agonists. Furthermore, given the failure of TAK-875 in clinical trials, the design of novel, potent FFA1 agonist classes with reduced risk of liver toxicity will be required. Along these lines, Li et al. generated FFA1 agonists with reduced lipophilicity that show promising preclinical results (Li et al. 2016). Armed with a deeper understanding and our hindsight to guide us, the future for the development of FFA1 agonists to improve T2D treatment is starting to look brighter.

References

Ahren B (2009) Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. Nat Rev Drug Discov 8:369–385. doi:10.1038/nrd2782

Alquier T, Peyot ML, Latour MG, Kebede M, Sorensen CM, Gesta S, Ronald Kahn C, Smith RD, Jetton TL, Metz TO, Prentki M, Poitout V (2009) Deletion of GPR40 impairs glucose-induced insulin secretion in vivo in mice without affecting intracellular fuel metabolism in islets. Diabetes 58:2607–2615. doi:10.2337/db09-0362

Amisten S, Salehi A, Rorsman P, Jones PM, Persaud SJ (2013) An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. Pharmacol Ther 139:359–391. doi:10.1016/j.pharmthera.2013.05.004

Araki T, Hirayama M, Hiroi S, Kaku K (2012) GPR40-induced insulin secretion by the novel agonist TAK-875: first clinical findings in patients with type 2 diabetes. Diabetes Obes Metab 14:271–278. doi:10.1111/j.1463-1326.2011.01525.x

Boneva NB, Yamashima T (2012) New insights into "GPR40-CREB interaction in adult neurogenesis" specific for primates. Hippocampus 22:896–905. doi:10.1002/hipo.20951

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR Jr, Shabon U, Spinage

LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311. doi:10.1074/jbc.M211495200

Brown SP, Dransfield PJ, Vimolratana M, Jiao X, Zhu L, Pattaropong V, Sun Y, Liu J, Luo J, Zhang J, Wong S, Zhuang R, Guo Q, Li F, Medina JC, Swaminath G, Lin DC, Houze JB (2012) Discovery of AM-1638: a potent and orally bioavailable GPR40/FFA1 full agonist. ACS Med Chem Lett 3:726–730. doi:10.1021/ml300133f

Burant CF, Viswanathan P, Marcinak J, Cao C, Vakilynejad M, Xie B, Leifke E (2012) TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial. Lancet 379:1403–1411. doi:10.1016/s0140-6736(11)61879-5

Cartoni C, Yasumatsu K, Ohkuri T, Shigemura N, Yoshida R, Godinot N, le Coutre J, Ninomiya Y, Damak S (2010) Taste preference for fatty acids is mediated by GPR40 and GPR120. J Neurosci 30:8376–8382. doi:10.1523/jneurosci.0496-10.2010

Cornish J, MacGibbon A, Lin JM, Watson M, Callon KE, Tong PC, Dunford JE, van der Does Y, Williams GA, Grey AB, Naot D, Reid IR (2008) Modulation of osteoclastogenesis by fatty acids. Endocrinology 149:5688–5695. doi:10.1210/en.2008-0111

Defossa E, Wagner M (2014) Recent developments in the discovery of FFA1 receptor agonists as novel oral treatment for type 2 diabetes mellitus. Bioorg Med Chem Lett 24:2991–3000. doi:10.1016/j.bmcl.2014.05.019

Del Guerra S, Bugliani M, D'Aleo V, Del Prato S, Boggi U, Mosca F, Filipponi F, Lupi R (2010) G-protein-coupled receptor 40 (GPR40) expression and its regulation in human pancreatic islets: the role of type 2 diabetes and fatty acids. Nutr Metab Cardiovasc Dis 20:22–25. doi:10.1016/j.numecd.2009.02.008

Edfalk S, Steneberg P, Edlund H (2008) Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. Diabetes 57:2280–2287. doi:10.2337/db08-0307

El-Azzouny M, Evans CR, Treutelaar MK, Kennedy RT, Burant CF (2014) Increased glucose metabolism and glycerolipid formation by fatty acids and GPR40 receptor signaling underlies the fatty acid potentiation of insulin secretion. J Biol Chem 289:13575–13588. doi:10.1074/jbc.M113.531970

Ferdaoussi M, Bergeron V, Zarrouki B, Kolic J, Cantley J, Fielitz J, Olson EN, Prentki M, Biden T, MacDonald PE, Poitout V (2012) G protein-coupled receptor (GPR)40-dependent potentiation of insulin secretion in mouse islets is mediated by protein kinase D1. Diabetologia 55:2682–2692. doi:10.1007/s00125-012-2650-x

Flodgren E, Olde B, Meidute-Abaraviciene S, Winzell MS, Ahren B, Salehi A (2007) GPR40 is expressed in glucagon producing cells and affects glucagon secretion. Biochem Biophys Res Commun 354:240–245. doi:10.1016/j.bbrc.2006.12.193

Fontes G, Zarrouki B, Hagman DK, Latour MG, Semache M, Roskens V, Moore PC, Prentki M, Rhodes CJ, Jetton TL, Poitout V (2010) Glucolipotoxicity age-dependently impairs beta cell function in rats despite a marked increase in beta cell mass. Diabetologia 53:2369–2379. doi:10.1007/s00125-010-1850-5

Fujita T, Matsuoka T, Honda T, Kabashima K, Hirata T, Narumiya S (2011) A GPR40 agonist GW9508 suppresses CCL5, CCL17, and CXCL10 induction in keratinocytes and attenuates cutaneous immune inflammation. J Invest Dermatol 131:1660–1667. doi:10.1038/jid.2011.123

Fujiwara K, Maekawa F, Yada T (2005) Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release. Am J Physiol Endocrinol Metab 289:E670–E677. doi:10.1152/ajpendo.00035.2005

Gosmain Y, Cheyssac C, Masson MH, Guerardel A, Poisson C, Philippe J (2012) Pax6 is a key component of regulated glucagon secretion. Endocrinology 153:4204–4215. doi:10.1210/en.2012-1425

Gowda N, Dandu A, Singh J, Biswas S, Raghav V, Lakshmi MN, Shilpa PC, Sunil V, Reddy A, Sadasivuni M, Aparna K, Verma MK, Moolemath Y, Anup MO, Venkataranganna MV, Somesh BP, Jagannath MR (2013) Treatment with CNX-011-67, a novel GPR40 agonist,

delays onset and progression of diabetes and improves beta cell preservation and function in male ZDF rats. BMC Pharmacol Toxicol 14:28. doi:10.1186/2050-6511-14-28

Guo S, Zhang J, Zhang S, Li J (2015) A single amino acid mutation (R104P) in the E/DRY motif of GPR40 impairs receptor function. PLoS One 10, e0141303. doi:10.1371/journal.pone.0141303

Hamid YH, Vissing H, Holst B, Urhammer SA, Pyke C, Hansen SK, Glumer C, Borch-Johnsen K, Jorgensen T, Schwartz TW, Pedersen O, Hansen T (2005) Studies of relationships between variation of the human G protein-coupled receptor 40 gene and type 2 diabetes and insulin release. Diabet Med 22:74–80. doi:10.1111/j.1464-5491.2005.01505.x

Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di Salvo J, Weinglass AB, Engelstoft MS, Madsen AN, Luckmann M, Miller MW, Trujillo ME, Frimurer TM, Holst B, Howard AD, Schwartz TW (2015) GPR40 (FFAR1) – combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo. Mol Metab 4:3–14. doi:10.1016/j.molmet.2014.10.002

Honore JC, Kooli A, Hamel D, Alquier T, Rivera JC, Quiniou C, Hou X, Kermorvant-Duchemin E, Hardy P, Poitout V, Chemtob S (2013) Fatty acid receptor Gpr40 mediates neuromicrovascular degeneration induced by transarachidonic acids in rodents. Arterioscler Thromb Vasc Biol 33:954–961. doi:10.1161/atvbaha.112.300943

Houze JB, Zhu L, Sun Y, Akerman M, Qiu W, Zhang AJ, Sharma R, Schmitt M, Wang Y, Liu J, Liu J, Medina JC, Reagan JD, Luo J, Tonn G, Zhang J, Lu JY, Chen M, Lopez E, Nguyen K, Yang L, Tang L, Tian H, Shuttleworth SJ, Lin DC (2012) AMG 837: a potent, orally bioavailable GPR40 agonist. Bioorg Med Chem Lett 22:1267–1270. doi:10.1016/j.bmcl.2011.10.118

Hu H, He LY, Gong Z, Li N, Lu YN, Zhai QW, Liu H, Jiang HL, Zhu WL, Wang HY (2009) A novel class of antagonists for the FFAs receptor GPR40. Biochem Biophys Res Commun 390:557–563. doi:10.1016/j.bbrc.2009.10.004

Ito R, Tsujihata Y, Matsuda-Nagasumi K, Mori I, Negoro N, Takeuchi K (2013) TAK-875, a GPR40/FFAR1 agonist, in combination with metformin prevents progression of diabetes and beta-cell dysfunction in Zucker diabetic fatty rats. Br J Pharmacol 170:568–580. doi:10.1111/bph.12297

Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422:173–176. doi:10.1038/nature01478

Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444:840–846. doi:10.1038/nature05482

Kaku K, Araki T, Yoshinaka R (2013) Randomized, double-blind, dose-ranging study of TAK-875, a novel GPR40 agonist, in Japanese patients with inadequately controlled type 2 diabetes. Diabetes Care 36:245–250. doi:10.2337/dc12-0872

Kaku K, Enya K, Nakaya R, Ohira T, Matsuno R (2015) Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial. Diabetes Obes Metab 17:675–681. doi:10.1111/dom.12467

Kalis M, Leveen P, Lyssenko V, Almgren P, Groop L, Cilio CM (2007) Variants in the FFAR1 gene are associated with beta cell function. PLoS One 2, e1090. doi:10.1371/journal.pone.0001090

Kebede M, Alquier T, Latour MG, Semache M, Tremblay C, Poitout V (2008) The fatty acid receptor GPR40 plays a role in insulin secretion in vivo after high-fat feeding. Diabetes 57:2432–2437. doi:10.2337/db08-0553

Kebede M, Ferdaoussi M, Mancini A, Alquier T, Kulkarni RN, Walker MD, Poitout V (2012) Glucose activates free fatty acid receptor 1 gene transcription via phosphatidylinositol-3-kinase-dependent O-GlcNAcylation of pancreas-duodenum homeobox-1. Proc Natl Acad Sci U S A 109:2376–2381. doi:10.1073/pnas.1114350109

Khan MZ, He L (2015) The role of polyunsaturated fatty acids and GPR40 receptor in brain. Neuropharmacology. doi:10.1016/j.neuropharm.2015.05.013

Khan MZ, Zhuang X, He L (2016) GPR40 receptor activation leads to CREB phosphorylation and improves cognitive performance in an Alzheimer's disease mouse model. Neurobiol Learn Mem 131:46–55. doi:10.1016/j.nlm.2016.03.006

Kim HS, Hwang YC, Koo SH, Park KS, Lee MS, Kim KW, Lee MK (2013) PPAR-gamma activation increases insulin secretion through the up-regulation of the free fatty acid receptor GPR40 in pancreatic beta-cells. PLoS One 8, e50128. doi:10.1371/journal.pone.0050128

Kotarsky K, Nilsson NE, Flodgren E, Owman C, Olde B (2003) A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem Biophys Res Commun 301:406–410

Kristinsson H, Smith DM, Bergsten P, Sargsyan E (2013) FFAR1 is involved in both the acute and chronic effects of palmitate on insulin secretion. Endocrinology 154:4078–4088. doi:10.1210/en.2013-1352

Kristinsson H, Bergsten P, Sargsyan E (2015) Free fatty acid receptor 1 (FFAR1/GPR40) signaling affects insulin secretion by enhancing mitochondrial respiration during palmitate exposure. Biochim Biophys Acta 1853:3248–3257. doi:10.1016/j.bbamcr.2015.09.022

Lan H, Hoos LM, Liu L, Tetzloff G, Hu W, Abbondanzo SJ, Vassileva G, Gustafson EL, Hedrick JA, Davis HR (2008) Lack of FFAR1/GPR40 does not protect mice from high-fat diet-induced metabolic disease. Diabetes 57:2999–3006. doi:10.2337/db08-0596

Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V (2007) GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. Diabetes 56:1087–1094. doi:10.2337/db06-1532

Li Z, Qiu Q, Xu X, Wang X, Jiao L, Su X, Pan M, Huang W, Qian H (2016) Design, synthesis and structure-activity relationship studies of new thiazole-based free fatty acid receptor 1 agonists for the treatment of type 2 diabetes. Eur J Med Chem 113:246–257. doi:10.1016/j.ejmech.2016.02.040

Lin DC, Zhang J, Zhuang R, Li F, Nguyen K, Chen M, Tran T, Lopez E, Lu JY, Li XN, Tang L, Tonn GR, Swaminath G, Reagan JD, Chen JL, Tian H, Lin YJ, Houze JB, Luo J (2011) AMG 837: a novel GPR40/FFA1 agonist that enhances insulin secretion and lowers glucose levels in rodents. PLoS One 6, e27270. doi:10.1371/journal.pone.0027270

Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J, Vimolratana M, Jiao XY, Wang Y, Birdsall NJ, Swaminath G (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859. doi:10.1124/mol.112.079640

Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ, Raybould HE, Wank S (2011) The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. Gastroenterology 140:903–912. doi:10.1053/j.gastro.2010.10.012

Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, Vimolratana M, Houze JB, Wong S, Toteva M, Shan B, Li F, Zhuang R, Lin DC (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7, e46300. doi:10.1371/journal.pone.0046300

Ma D, Lu L, Boneva NB, Warashina S, Kaplamadzhiev DB, Mori Y, Nakaya MA, Kikuchi M, Tonchev AB, Okano H, Yamashima T (2008) Expression of free fatty acid receptor GPR40 in the neurogenic niche of adult monkey hippocampus. Hippocampus 18:326–333. doi:10.1002/hipo.20393

Mancini AD, Poitout V (2013) The fatty acid receptor FFA1/GPR40 a decade later: how much do we know? Trends Endocrinol Metab 24:398–407. doi:10.1016/j.tem.2013.03.003

Mancini AD, Bertrand G, Vivot K, Carpentier E, Tremblay C, Ghislain J, Bouvier M, Poitout V (2015) beta-Arrestin recruitment and biased agonism at free fatty acid receptor 1. J Biol Chem 290:21131–21140. doi:10.1074/jbc.M115.644450

Matsuda-Nagasumi K, Takami-Esaki R, Iwachidow K, Yasuhara Y, Tanaka H, Ogi K, Nakata M, Yano T, Hinuma S, Taketomi S, Odaka H, Kaisho Y (2013) Lack of GPR40/FFAR1 does not

induce diabetes even under insulin resistance condition. Diabetes Obes Metab 15:538–545. doi:10.1111/dom.12065

McDonald PH, Lefkowitz RJ (2001) Beta-Arrestins: new roles in regulating heptahelical receptors' functions. Cell Signal 13:683–689

Meidute Abaraviciene S, Muhammed SJ, Amisten S, Lundquist I, Salehi A (2013) GPR40 protein levels are crucial to the regulation of stimulated hormone secretion in pancreatic islets. Lessons from spontaneous obesity-prone and non-obese type 2 diabetes in rats. Mol Cell Endocrinol 381:150–159. doi:10.1016/j.mce.2013.07.025

Mieczkowska A, Basle MF, Chappard D, Mabilleau G (2012) Thiazolidinediones induce osteocyte apoptosis by a G protein-coupled receptor 40-dependent mechanism. J Biol Chem 287:23517–23526. doi:10.1074/jbc.M111.324814

Nagasumi K, Esaki R, Iwachidow K, Yasuhara Y, Ogi K, Tanaka H, Nakata M, Yano T, Shimakawa K, Taketomi S, Takeuchi K, Odaka H, Kaisho Y (2009) Overexpression of GPR40 in pancreatic beta-cells augments glucose-stimulated insulin secretion and improves glucose tolerance in normal and diabetic mice. Diabetes 58:1067–1076. doi:10.2337/db08-1233

Nakamoto K, Nishinaka T, Matsumoto K, Kasuya F, Mankura M, Koyama Y, Tokuyama S (2012) Involvement of the long-chain fatty acid receptor GPR40 as a novel pain regulatory system. Brain Res 1432:74–83. doi:10.1016/j.brainres.2011.11.012

Nakamoto K, Nishinaka T, Sato N, Mankura M, Koyama Y, Kasuya F, Tokuyama S (2013) Hypothalamic GPR40 signaling activated by free long chain fatty acids suppresses CFA-induced inflammatory chronic pain. PLoS One 8, e81563. doi:10.1371/journal.pone.0081563

Negoro N, Sasaki S, Mikami S, Ito M, Suzuki M, Tsujihata Y, Ito R, Harada A, Takeuchi K, Suzuki N, Miyazaki J, Santou T, Odani T, Kanzaki N, Funami M, Tanaka T, Kogame A, Matsunaga S, Yasuma T, Momose Y (2010) Discovery of TAK-875: a potent, selective, and orally bioavailable GPR40 agonist. ACS Med Chem Lett 1:290–294. doi:10.1021/ml1000855

Ogawa T, Hirose H, Miyashita K, Saito I, Saruta T (2005) GPR40 gene Arg211His polymorphism may contribute to the variation of insulin secretory capacity in Japanese men. Metabolism 54:296–299. doi:10.1016/j.metabol.2004.09.008

Ou HY, Wu HT, Hung HC, Yang YC, Wu JS, Chang CJ (2013) Multiple mechanisms of GW-9508, a selective G protein-coupled receptor 40 agonist, in the regulation of glucose homeostasis and insulin sensitivity. Am J Physiol Endocrinol Metab 304:E668–E676. doi:10.1152/ajpendo.00419.2012

Pang Z, Wu N, Zhang X, Avallone R, Croci T, Dressler H, Palejwala V, Ferrara P, Tocci MJ, Polites HG (2010) GPR40 is partially required for insulin secretion following activation of beta3-adrenergic receptors. Mol Cell Endocrinol 325:18–25. doi:10.1016/j.mce.2010.04.014

Panse M, Gerst F, Kaiser G, Teutsch CA, Dolker R, Wagner R, Haring HU, Ullrich S (2015) Activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) by free fatty acid receptor 1 (FFAR1/GPR40) protects from palmitate-induced beta cell death, but plays no role in insulin secretion. Cell Physiol Biochem 35:1537–1545. doi:10.1159/000373969

Parker HE, Habib AM, Rogers GJ, Gribble FM, Reimann F (2009) Nutrient-dependent secretion of glucose-dependent insulinotropic polypeptide from primary murine K cells. Diabetologia 52:289–298. doi:10.1007/s00125-008-1202-x

Poitout V (2008) Glucolipotoxicity of the pancreatic beta-cell: myth or reality? Biochem Soc Trans 36:901–904. doi:10.1042/bst0360901

Poitout V, Robertson RP (2008) Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 29:351–366. doi:10.1210/er.2007-0023

Prentki M, Nolan CJ (2006) Islet beta cell failure in type 2 diabetes. J Clin Invest 116:1802–1812. doi:10.1172/jci29103

Prentki M, Matschinsky FM, Madiraju SR (2013) Metabolic signaling in fuel-induced insulin secretion. Cell Metab 18:162–185. doi:10.1016/j.cmet.2013.05.018

Qian J, Wu C, Chen X, Li X, Ying G, Jin L, Ma Q, Li G, Shi Y, Zhang G, Zhou N (2014) Differential requirements of arrestin-3 and clathrin for ligand-dependent and -independent internalization of human G protein-coupled receptor 40. Cell Signal 26:2412–2423. doi:10.1016/j.cellsig.2014.07.019

Ramos EM, Hoffman D, Junkins HA, Maglott D, Phan L, Sherry ST, Feolo M, Hindorff LA (2014) Phenotype-Genotype Integrator (PheGenI): synthesizing genome-wide association study (GWAS) data with existing genomic resources. Eur J Hum Genet 22:144–147. doi:10.1038/ejhg.2013.96

Rankovic Z, Brust TF, Bohn LM (2016) Biased agonism: an emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 26:241–250. doi:10.1016/j.bmcl.2015.12.024

Schnell S, Schaefer M, Schofl C (2007) Free fatty acids increase cytosolic free calcium and stimulate insulin secretion from beta-cells through activation of GPR40. Mol Cell Endocrinol 263:173–180. doi:10.1016/j.mce.2006.09.013

Schroder R, Janssen N, Schmidt J, Kebig A, Merten N, Hennen S, Muller A, Blattermann S, Mohr-Andra M, Zahn S, Wenzel J, Smith NJ, Gomeza J, Drewke C, Milligan G, Mohr K, Kostenis E (2010) Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. Nat Biotechnol 28:943–949. doi:10.1038/nbt.1671

Srivastava A, Yano J, Hirozane Y, Kefala G, Gruswitz F, Snell G, Lane W, Ivetac A, Aertgeerts K, Nguyen J, Jennings A, Okada K (2014) High-resolution structure of the human GPR40 receptor bound to allosteric agonist TAK-875. Nature 513:124–127. doi:10.1038/nature13494

Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H (2005) The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. Cell Metab 1:245–258. doi:10.1016/j.cmet.2005.03.007

Stoddart LA, Brown AJ, Milligan G (2007) Uncovering the pharmacology of the G protein-coupled receptor GPR40: high apparent constitutive activity in guanosine 5'-O-(3-[35S]thio) triphosphate binding studies reflects binding of an endogenous agonist. Mol Pharmacol 71:994–1005. doi:10.1124/mol.106.031534

Suckow AT, Polidori D, Yan W, Chon S, Ma JY, Leonard J, Briscoe CP (2014) Alteration of the glucagon axis in GPR120 (FFAR4) knockout mice: a role for GPR120 in glucagon secretion. J Biol Chem 289:15751–15763. doi:10.1074/jbc.M114.568683

Sun P, Wang T, Zhou Y, Liu H, Jiang H, Zhu W, Wang H (2013) DC260126: a small-molecule antagonist of GPR40 that protects against pancreatic beta-Cells dysfunction in db/db mice. PLoS One 8, e66744. doi:10.1371/journal.pone.0066744

Sunil V, Verma MK, Oommen AM, Sadasivuni M, Singh J, Vijayraghav DN, Chandravanshi B, Shetty J, Biswas S, Dandu A, Moolemath Y, Venkataranganna MV, Somesh BP, Jagannath MR (2014) CNX-011-67, a novel GPR40 agonist, enhances glucose responsiveness, insulin secretion and islet insulin content in n-STZ rats and in islets from type 2 diabetic patients. BMC Pharmacol Toxicol 15:19. doi:10.1186/2050-6511-15-19

Sykaras AG, Demenis C, Case RM, McLaughlin JT, Smith CP (2012) Duodenal enteroendocrine I-cells contain mRNA transcripts encoding key endocannabinoid and fatty acid receptors. PLoS One 7, e42373. doi:10.1371/journal.pone.0042373

Tan CP, Feng Y, Zhou YP, Eiermann GJ, Petrov A, Zhou C, Lin S, Salituro G, Meinke P, Mosley R, Akiyama TE, Einstein M, Kumar S, Berger JP, Mills SG, Thornberry NA, Yang L, Howard AD (2008) Selective small-molecule agonists of G protein-coupled receptor 40 promote glucose-dependent insulin secretion and reduce blood glucose in mice. Diabetes 57:2211–2219. doi:10.2337/db08-0130

Tanaka H, Yoshida S, Oshima H, Minoura H, Negoro K, Yamazaki T, Sakuda S, Iwasaki F, Matsui T, Shibasaki M (2013) Chronic treatment with novel GPR40 agonists improve whole-body glucose metabolism based on the glucose-dependent insulin secretion. J Pharmacol Exp Ther 346:443–452. doi:10.1124/jpet.113.206466

Tanaka H, Yoshida S, Minoura H, Negoro K, Shimaya A, Shimokawa T, Shibasaki M (2014) Novel GPR40 agonist AS2575959 exhibits glucose metabolism improvement and synergistic

effect with sitagliptin on insulin and incretin secretion. Life Sci 94:115–121. doi:10.1016/j.lfs.2013.11.010

Tomita T, Masuzaki H, Iwakura H, Fujikura J, Noguchi M, Tanaka T, Ebihara K, Kawamura J, Komoto I, Kawaguchi Y, Fujimoto K, Doi R, Shimada Y, Hosoda K, Imamura M, Nakao K (2006) Expression of the gene for a membrane-bound fatty acid receptor in the pancreas and islet cell tumours in humans: evidence for GPR40 expression in pancreatic beta cells and implications for insulin secretion. Diabetologia 49:962–968. doi:10.1007/s00125-006-0193-8

Tsujihata Y, Ito R, Suzuki M, Harada A, Negoro N, Yasuma T, Momose Y, Takeuchi K (2011) TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats. J Pharmacol Exp Ther 339:228–237. doi:10.1124/jpet.111.183772

Verma MK, Biswas S, Chandravanshi B, Neelima K, Oommen AM, Jagannath MR, Somesh BP (2014a) A novel GPR40 agonist, CNX-011-67, suppresses glucagon secretion in pancreatic islets under chronic glucolipotoxic conditions in vitro. BMC Res Notes 7:595. doi:10.1186/1756-0500-7-595

Verma MK, Sadasivuni MK, Yateesh AN, Neelima K, Mrudula S, Reddy M, Smitha R, Biswas S, Chandravanshi B, Pallavi PM, Oommen AM, Jagannath MR, Somesh BB (2014b) Activation of GPR40 attenuates chronic inflammation induced impact on pancreatic beta-cells health and function. BMC Cell Biol 15:24. doi:10.1186/1471-2121-15-24

Vettor R, Granzotto M, De Stefani D, Trevellin E, Rossato M, Farina MG, Milan G, Pilon C, Nigro A, Federspil G, Vigneri R, Vitiello L, Rizzuto R, Baratta R, Frittitta L (2008) Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization. J Clin Endocrinol Metab 93:3541–3550. doi:10.1210/jc.2007-2680

Wagner R, Kaiser G, Gerst F, Christiansen E, Due-Hansen ME, Grundmann M, Machicao F, Peter A, Kostenis E, Ulven T, Fritsche A, Haring HU, Ullrich S (2013) Reevaluation of fatty acid receptor 1 as a drug target for the stimulation of insulin secretion in humans. Diabetes 62:2106–2111. doi:10.2337/db12-1249

Wagner R, Staiger H, Ullrich S, Stefan N, Fritsche A, Haring HU (2014) Untangling the interplay of genetic and metabolic influences on beta-cell function: examples of potential therapeutic implications involving TCF7L2 and FFAR1. Mol Metab 3:261–267. doi:10.1016/j.molmet.2014.01.001

Walker CG, Goff L, Bluck LJ, Griffin BA, Jebb SA, Lovegrove JA, Sanders TA, Frost GS (2011) Variation in the FFAR1 gene modifies BMI, body composition and beta-cell function in overweight subjects: an exploratory analysis. PLoS One 6, e19146. doi:10.1371/journal.pone.0019146

Wang Z, Thurmond DC (2009) Mechanisms of biphasic insulin-granule exocytosis – roles of the cytoskeleton, small GTPases and SNARE proteins. J Cell Sci 122:893–903. doi:10.1242/jcs.034355

Wang L, Zhao Y, Gui B, Fu R, Ma F, Yu J, Qu P, Dong L, Chen C (2011) Acute stimulation of glucagon secretion by linoleic acid results from GPR40 activation and [Ca2+]i increase in pancreatic islet {alpha}-cells. J Endocrinol 210:173–179. doi:10.1530/joe-11-0132

Wang Y, Liu JJ, Dransfield PJ, Zhu L, Wang Z, Du X, Jiao X, Su Y, Li AR, Brown SP, Kasparian A, Vimolratana M, Yu M, Pattaropong V, Houze JB, Swaminath G, Tran T, Nguyen K, Guo Q, Zhang J, Zhuang R, Li F, Miao L, Bartberger MD, Correll TL, Chow D, Wong S, Luo J, Lin DC, Medina JC (2013) Discovery and optimization of potent GPR40 full agonists containing tricyclic spirocycles. ACS Med Chem Lett 4:551–555. doi:10.1021/ml300427u

Wauquier F, Philippe C, Leotoing L, Mercier S, Davicco MJ, Lebecque P, Guicheux J, Pilet P, Miot-Noirault E, Poitout V, Alquier T, Coxam V, Wittrant Y (2013) The free fatty acid receptor G protein-coupled receptor 40 (GPR40) protects from bone loss through inhibition of osteoclast differentiation. J Biol Chem 288:6542–6551. doi:10.1074/jbc.M112.429084

Xiong Y, Swaminath G, Cao Q, Yang L, Guo Q, Salomonis H, Lu J, Houze JB, Dransfield PJ, Wang Y, Liu JJ, Wong S, Schwandner R, Steger F, Baribault H, Liu L, Coberly S, Miao L, Zhang J, Lin DC, Schwarz M (2013) Activation of FFA1 mediates GLP-1 secretion in mice. Evidence for allosterism at FFA1. Mol Cell Endocrinol 369:119–129. doi:10.1016/j.mce.2013.01.009

Yabuki C, Komatsu H, Tsujihata Y, Maeda R, Ito R, Matsuda-Nagasumi K, Sakuma K, Miyawaki K, Kikuchi N, Takeuchi K, Habata Y, Mori M (2013) A novel antidiabetic drug, fasiglifam/TAK-875, acts as an ago-allosteric modulator of FFAR1. PLoS One 8, e76280. doi:10.1371/journal.pone.0076280

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ, Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. Receptors Channels 5:193–199

Zhang Y, Xu M, Zhang S, Yan L, Yang C, Lu W, Li Y, Cheng H (2007) The role of G protein-coupled receptor 40 in lipoapoptosis in mouse beta-cell line NIT-1. J Mol Endocrinol 38:651–661. doi:10.1677/jme-06-0048

Zhang X, Yan G, Li Y, Zhu W, Wang H (2010) DC260126, a small-molecule antagonist of GPR40, improves insulin tolerance but not glucose tolerance in obese Zucker rats. Biomed Pharmacother 64:647–651. doi:10.1016/j.biopha.2010.06.008

Gut Hormone Regulation and Secretion via FFA1 and FFA4

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Contents

Abstract

The digestion, absorption and utilisation of dietary triglycerides are controlled by gut hormones, released from enteroendocrine cells along the length of the gastrointestinal tract. Major players in the detection of ingested lipids are the free fatty acid receptors FFA1 and FFA4, which are highly expressed on enteroendocrine cells. These receptors are activated when free fatty acids

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(FFA) are absorbed across the intestinal epithelium, and provide a dynamic hormonal signal indicating that lipids are arriving in the bloodstream from the gut. This review addresses our current knowledge of how ingested triglycerides modulate gut hormone release via FFA1 and FFA4.

Keywords
CCK • FFA1 • FFA4 • Ghrelin • GIP • GLP-1 • Incretin

1 Introduction

Animals and humans depend on efficient assimilation of nutrients from ingested food whilst minimising the loss of energy-rich components in the faeces. The gastrointestinal (GI) tract has thus evolved to continuously monitor the progress of digestion and absorption, and correspondingly to adapt rates of GI motility and secretion. This coordination of activity is, at least in part, mediated by gut hormones secreted from enteroendocrine cells, which also help to prepare the body for the arrival of additional nutrients.

Fat is an important macronutrient in the human diet, contributing on average 30–40% of the total energy in food, although the percentage can be much higher in western diets, mostly in the form of triglycerides. In addition to its role as an energy source fat also aids the absorption of lipophilic compounds, including many vitamins, and provides essential fatty acids such as linoleic and α-linolenic acid which cannot be synthesised de novo by the human body. In all cases examined to date, the detection of ingested fat by enteroendocrine cells has been linked to the generation and/or absorption of triacylglycerol digestion products rather than raised concentrations of triglycerides themselves. The availability of free fatty acids can be detected by the G-protein coupled receptors FFA1 and FFA4. This review focuses on these long chain FFA receptors, but in the context of gut endocrine cells, these receptors work in concert with other G-protein coupled receptors responsive to 2-monoglycerides (GPR119) and bile acids (GPBAR1) (Reimann et al. 2012).

Gut hormones released following fat ingestion have local and peripheral actions that control activity of the GI tract and modulate food intake, insulin secretion and adipocyte function. To understand the roles of FFA receptors in the gut endocrine system, it is important to take into account the spatial distribution of different receptors and hormones along the GI tract, the processes of triglyceride digestion and absorption, how the ligands access their receptors and the cellular effects of activating FFA receptors on enteroendocrine cells.

1.1 Gut Hormones Responsive to Fat Absorption

The intestinal epithelium secretes a number of peptides, rendering it the largest endocrine organ in the body. Its complexity arises not only from the diversity of

hormones secreted but also from the range of stimuli and signalling pathways involved in gut hormone release. Secretin was discovered by Starling and Bayliss in 1902 (Bayliss and Starling 1902) and was the first gut peptide to be characterised as a hormone. The discovery of gastrin and cholecystokinin (CCK) followed, and today, approximately 30 genes encoding gut hormones have been identified (Ahlman and Nilsson 2001). Gut hormones are loosely categorised by their structural characteristics, physiological roles, cells of origin and locations along the GI tract (Sjölund et al. 1983). In the context of fat sensing, much attention has focussed on CCK, glucose-dependent insulinotropic-polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and ghrelin. In the small intestine, all are produced by open-type endocrine cells, which lie scattered through the epithelial layer and face into the gut lumen (Gribble and Reimann 2016; Ku et al. 2003). Gastric ghrelin is released by closed-type cells that don't make contact with the luminal contents (Stengel and Taché 2009).

CCK is produced by I-cells in the small intestine, predominantly in the duodenum, where it is found colocalised in many of the same cells as GIP and GLP-1 (Habib et al. 2012; Egerod et al. 2012). Its local actions are to stimulate gall bladder contraction and increase pancreatic enzyme secretion, and it inhibits gastric emptying through a neural circuit involving the vagus nerve (Dockray 2009). These properties serve to link digestive processes to the rate of arrival of fats in the duodenal lumen. Because the bile and pancreatic enzymes that promote intestinal fat digestion only arrive in the lumen as a consequence of CCK action, the first peak of CCK secretion after fat ingestion is likely to be triggered by products of gastric triglyceride digestion. CCK action on the afferent vagus also inhibits food intake (Dockray 2009).

GIP is synthesised by K-cells, found in greatest numbers in the epithelium of the duodenum and proximal jejunum (Sjölund et al. 1983; Parker et al. 2009). GLP-1 is produced by an overlapping population of enteroendocrine cells known as L-cells, but is found in highest amounts in the distal small intestine and colon (Holst 2007). Both hormones are released into the bloodstream within minutes of food ingestion, and are rapidly inactivated in the circulation by the hydrolytic enzyme dipeptidyl-peptidase 4 (Mentlein 1999; Hansen et al. 1999). Their concentrations remain elevated for several hours, depending on the meal composition, and whereas free sugars trigger rapid and short lived GLP-1 and GIP transients, responses to fat ingestion are more sustained (Thomsen et al. 1999).

GIP and GLP-1 have effects on gastric acid secretion and gastric emptying, but are better known for their actions as incretin hormones, responsible for augmenting glucose-dependent insulin secretion (Creutzfeldt 1979; Nauck et al. 1993). The incretin effect accounts for 50–70% of normal post-prandial insulin secretion (Baggio and Drucker 2007; Holst 2007), and may be physiologically important for the insulin response to lipid ingestion (Lindgren et al. 2011). As insulin is the body's major anabolic hormone critical for promoting fat uptake and storage in adipocytes, the fat-triggered secretion of incretin hormones and insulin can be considered as a mechanism to prepare tissues for the arrival of lipids in the bloodstream from ingested food. It should be noted that FFA1 is also expressed

in the insulin-secreting pancreatic β-cell itself, whereas FFA4 in the endocrine pancreas seems to be restricted to δ-cells secreting somatostatin, an inhibitor of insulin secretion. Direct monitoring of FFA by the endocrine pancreas, however, lacks the inherent link with post-prandial absorption, whereas GIP and GLP-1 concentrations in plasma convey this information, analogous to their incretin action boosting autonomous glucose sensing by the β-cell. In this context it is interesting that GIP has been shown also to exert direct effects on adipose tissue, such as promotion of triglyceride incorporation (Weaver et al. 2008; Yip et al. 1998). GLP-1 and the cosecreted hormone peptide YY (PYY) also play a major role in the ileal brake – a mechanism that slows gastric emptying if nutrients reach too low in the GI tract, thereby restoring an appropriate balance between the rate of arrival of nutrients in the small intestine and their rates of digestion and absorption (Wen et al. 1995). GLP-1 and PYY additionally promote satiety and reduce food intake (Karra and Batterham 2010).

Ghrelin is produced by closed-type enteroendocrine cells in the stomach, as well as a smaller population of small intestinal open-type cells (Stengel and Taché 2009). Ghrelin has an interesting, although not completely established, relationship with ingested fat. Active ghrelin contains an octanoyl group, added by the enzyme ghrelin O-acyltransferase, which mostly sources the octanoyl fatty acid group from ingested lipids (Nishi et al. 2005). Secretion of ghrelin, however, is high in the fasting state and reduced following ingestion of high fat foods (Erdmann et al. 2003). Instillation of olive oil into the stomach with the pylorus ligated was sufficient to suppress plasma ghrelin concentrations (Lu et al. 2012b), suggesting the involvement of FFA released in the gastric lumen. Ghrelin is best known as a "hunger hormone" as it promotes food seeking behaviour in the fasting state (Skibicka and Dickson 2013).

A number of cell lines have been used to model these different enteroendocrine cell types (Kuhre et al. 2016). STC-1 cells are a mouse line from a small intestinal tumour and are used to study the secretion of GIP (Rindi et al. 1990), secretin, CCK and GLP-1 (Geraedts et al. 2009). GLUTag cells were derived from a murine colonic tumour and predominantly secrete GLP-1 and CCK (Drucker et al. 1994; Sidhu et al. 2000). NCI-H716 cells are often used as a human model of GLP-1 release, although they do not respond to all physiological stimuli (Reimer et al. 2001). SG-1 and MGN3-1 are murine cell lines used to study gastric ghrelin secretion (Gong et al. 2014; Koyama et al. 2016).

1.2 Generation of FFA in the Gut Lumen

Digestion of fats begins in the oral cavity and stomach through the action of lingual and gastric lipases. These are acid lipases with pH optima between 3.5 and 5 that preferentially cleave FFA from the sn-3 position of triglycerides (Carrière et al. 1997; Liao et al. 1984). Through the action of acid lipases, chyme leaving the stomach contains FFA that can target CCK-producing I-cells in the proximal duodenum. However, gastric lipase activity only releases approximately 10% of

FFA from triglycerides in the stomach leaving a mixture of intact triglycerides, FFA and 1,2 diglycerides, so CCK and GIP producing cells in the proximal duodenum predominantly encounter long chain FFA rather than 2-monoglycerides. In keeping with the idea that lipid-triggered CCK release is dependent on FFA rather than 2-monoglycerides, 1,3-dioctanoyl-2-oleoylglycerol was a much weaker stimulant of CCK release than olive oil in humans (Mandøe et al. 2015). Interestingly, diacylglycerols were also a weaker stimulus of GIP secretion than triacylglycerols when administered by gavage in mice, although the corresponding release of FFA from the two oils was not assessed in this study (Shimotoyodome et al. 2009).

In the small intestine triglycerides and their hydrolysis products form complexes with phospholipids, cholesterol and bile salts to generate structures known as lipid micelles. Pancreatic lipases hydrolyse triglycerides at both the sn-1 and sn-3 positions, producing further FFA and 2-monoglycerides (Carrière et al. 1997) and the emulsification promotes substrate delivery to the epithelial brush border for uptake by enterocytes (Lindström et al. 1988).

2 FFA1 and FFA4 in the Regulation of Gut Hormone Secretion

2.1 Expression of FFA1 in the Gut

Expression of G-protein coupled receptors (GPCRs) in intestinal endocrine cells has been investigated by a variety of methodologies. The generation in recent years of genetically modified mouse models expressing fluorescent reporters in different enteroendocrine cell populations driven by cell-specific hormonal promoters has enabled the purification and transcriptomic analysis of different endocrine cell types. By this method, *ffar1* expression has been detected in L, K and I cells (Parker et al. 2009; Reimann et al. 2008; Sykaras et al. 2012; Liou et al. 2011), but not gastric ghrelin cells (Engelstoft et al. 2013; Lu et al. 2012b) or gastric somatostatin-secreting D-cells (Adriaenssens et al. 2015; Egerod et al. 2015). Using an alternative approach, genetically modified mice were generated with the *lacZ* reporter gene inserted into the *ffar1* locus. In these mice, lacZ activity, representing sites of *ffar1* expression, was observed in scattered epithelial cells along the length of the gastrointestinal track and more specifically in the gastric pylorus, duodenum, jejunum, ileum and colon (Edfalk et al. 2008). Double immunohistochemical analysis of mouse duodenum showed that *lacZ* expressed from the *ffar1* locus was located in intestinal cells staining positive for GIP, GLP-1, gastrin, ghrelin, CCK, PYY, secretin and serotonin but not somatostatin. In the distal small intestine, *ffar1*-driven *lacZ* was largely colocalised with GLP-1 (Edfalk et al. 2008; Xiong et al. 2013).

FFA1 has also been identified in the human intestinal tract. Transcripts of the receptor were found in human duodenum, and immunostaining with an FFA1-specific antibody showed that the labelled epithelial cells had a flask-shaped

morphology typical of enteroendocrine cells (Little et al. 2014). Dual-immunolabelling established that FFA1 was located in cells expressing GLP-1 and CCK, although the percentage overlap was low, with only 2% of GLP-1 positive cells and 0.3% of CCK positive cells exhibiting staining for FFA1 (Little et al. 2014). FFA1 mRNA and protein have also been detected in human ileal mucosa (Tsukahara et al. 2015). In cell line models, *ffar1* has been found in STC-1, GLUTag and NCI-H716 cells (Katsuma et al. 2005; Hirasawa et al. 2005; Reimann et al. 2008; Lauffer et al. 2009).

2.2 Expression of FFA4 in the Gut

Similar to FFA1, FFA4 has been detected along the length of the intestinal tract, with levels being higher in human and mouse colon than in the small intestine (Hirasawa et al. 2005). In the colon, GLP-1 immunopositive cells were found to contain *ffar4* by in situ hybridisation (Hirasawa et al. 2005). Human duodenal biopsies confirmed the epithelial expression of FFA4 and localised it in "open type" intestinal endocrine cells, particularly those containing GLP-1 and CCK. Percentages of enteroendocrine cells staining positive for FFA4 were higher than observed for FFA1, accounting for 23% of GLP-1 positive cells (c.f. 3% for FFA1) and 3% of CCK positive cells (c.f. 0.3% for FFA1) (Little et al. 2014).

Transgenic mouse models with labelled enteroendocrine cell populations have shown that *ffar4* is expressed in small intestinal K-cells (Parker et al. 2009; Iwasaki et al. 2015), L-cells (Reimann et al. 2008) and I-cells (Liou et al. 2011; Sykaras et al. 2012). In purified K-cells, *ffar4* expression was found to be higher in cells from the proximal compared with the distal small intestine, whereas *ffar1* exhibited the reverse gradient, being more highly expressed in distal than proximal K-cells (Iwasaki et al. 2015). Using similar techniques, *ffar4* expression has also been detected in gastric ghrelin-secreting cells (Engelstoft et al. 2013; Lu et al. 2012b) and somatostatin-producing D-cells (Egerod et al. 2015). Using mice with targeted *lacZ* expression in the *ffar4* locus, labelled cells were found scattered in the intestinal epithelial layer (Stone et al. 2014). *Ffar4* expression was found in STC-1 (Katsuma et al. 2005), GLUTag (Reimann et al. 2008) and NCI-H716 (Lauffer et al. 2009) cells, as well as the ghrelin-secreting cell lines SG-1 and MGN3-1 (Koyama et al. 2016; Gong et al. 2014).

2.3 Natural and Small Molecule Ligands for FFA1 and FFA4

Deorphanisation of FFA1 (GPR40) revealed it to be a receptor responsive to a broad range of medium and long chain FFA with chain lengths as low as C6 (pEC_{50} 4.3), exhibiting the highest potency for 5,8,11-Eicosatriynoic acid (C20; pEC_{50} 5.7) (Briscoe et al. 2003). Responses of heterologously expressed FFA1 in HEK293 cells to FFA were predominantly attributable to $G_{\alpha q/11}$ activation (Briscoe et al. 2003). Despite the similarities between the fatty acid responsiveness of FFA1 and

enteroendocrine hormone secretion, however, FFA1 was not immediately linked to intestinal fat sensing.

Cloned, heterologously expressed FFA4 similarly exhibited relatively broad fatty acid specificity, responding to FFA with chain lengths of C14 (pEC_{50} 4.5) and above, with a particularly high potency for α-linolenic acid (pEC_{50} 6.4) (Hirasawa et al. 2005). A more recent head to head comparison of FFA1 and FFA4, however, concluded that the two receptors exhibit very similar affinities across a range of fatty acids (Christiansen et al. 2015).

The overlapping profiles of natural ligands for FFA1 and FFA4, and the finding that both receptors are predominantly coupled to $G_{\alpha q}$ signalling pathways in HEK293 and STC-1 cells, have made it difficult to distinguish the relative functional importance of each receptor for gut hormone secretion. Although α-linolenic acid is frequently used as a "specific" agonist of FFA4, it is also active on FFA1 at similar concentrations (Christiansen et al. 2015). The first widely used small molecule agonist of FFA receptors, GW9508, was also only of limited use in this context, as it exhibited limited selectivity for FFA1 over FFA4 (Hudson et al. 2013). More recently, however, a number of small molecules have been generated, that exhibit good receptor specificity and high potency, including TAK-875, AMG837, AM1638 and AM8182. Several binding sites have been postulated for these FFA1 specific agonists, based on the reciprocal enhancement of AMG837- and AM1638-binding and negative cooperativity between AMG837 and AM8182 (Lin et al. 2012). Binding to either site seems to promote $G_{\alpha q}$-signalling, but positive functional cooperativity was observed when both sides were occupied simultaneously. In support of this idea, AMG837 and AM1638 exhibited different behaviour when compared in functional assays. In one study, AMG837 was reported as a partial agonist due to its lower efficiency in IP_3 generation and Ca^{2+} signalling (Lin et al. 2012), whereas in another study, AM1638 was found additionally to trigger $G_{\alpha s}$-dependent cAMP elevation (Hauge et al. 2015). Molecular docking simulation based on the published crystal structure of FFA1 (after removal of the co-crystallised TAK-875 from the model), however, failed to identify different sites for AM1638 and AMG837, as all the investigated agonists were found to dock into the TAK-875 site (Hauge et al. 2015).

A number of small molecules have been developed specifically to target FFA4, including grifolic acid, TUG-891, NCG21, CpdA and GSK137647A. Grifolic acid is a partial agonist of GPR120, which promoted ERK phosphorylation and Ca^{2+} elevation in FFA4- but not FFA1-expressing cells (Hara et al. 2009). TUG-891 exhibited 50–300 fold greater potency on human FFA4 compared with human FFA1 in β-arrestin 2 and Ca^{2+} assays, but was only weakly FFA4-specific on the corresponding murine receptors in Ca^{2+} mobilisation assays (Hudson et al. 2013). In parallel experiments, α-linolenic acid exhibited only threefold higher affinity for FFA4 over FFA1, and NCG21 exhibited ~10-fold greater affinity for human FFA4 over FFA1 (Hudson et al. 2013; Sun et al. 2010). CpdA is an orally available ligand that exhibited at least 100-fold higher affinity for FFA4 over FFA1, and was equally effective on murine and human receptors (Oh et al. 2014). GSK137647A was generated as an FFA4 selective agonist, exhibiting >100-fold selectivity for

FFA4 over FFA1 and retaining activity and selectivity for murine and human receptors (Sparks et al. 2014). Interestingly FFA4-selective agonists have been linked to the inhibition of ghrelin (Engelstoft et al. 2013) and somatostatin (Egerod et al. 2015; Stone et al. 2014) secretion. Rather than reflecting biased agonism of the drugs at the receptor level, a general "tuning" of signalling to $G_{\alpha i}$-dependent inhibition in ghrelin-secreting cells was suggested, based on high expression of $G_{\alpha i/o}$ subunits (although high $G_{\alpha q}$ expression was also observed). In support of this hypothesis, ghrelin secretion was also inhibited downstream of the calcium sensing receptor (CaSR), which in other cell types tends to preferentially couple to $G_{\alpha q}$ (Engelstoft et al. 2013). However, a cell-type specific $G_{\alpha i}$-"tuning" does not hold for somatostatin-secreting D-cells, in which CaSR-stimulation elevated cytosolic Ca^{2+} and promoted secretion, whereas an FFA4 selective agonist inhibited secretion (Egerod et al. 2015).

2.4 Studies on FFA1 and FFA4 in Enteroendocrine Cell Lines

Even before the identification of FFA1 and FFA4, FFA with chain lengths greater than C12 had been identified as stimulants of GLP-1, CCK and secretin secretion from enteroendocrine cell lines (McLaughlin et al. 1998; Sidhu et al. 2000; Chang et al. 2000). In fetal rat intestinal cultures and GLUTag cells, the response was found to be specific for monounsaturated FFA with chain length of C14 or above (Rocca and Brubaker 1995; Brubaker et al. 1998). A number of studies proposed that fatty acid triggered hormone secretion from STC-1 and GLUTag cells was linked to the elevation of intracellular Ca^{2+} (Sidhu et al. 2000; McLaughlin et al. 1998), and in STC-1 cells it was shown that secretion triggered by sodium oleate involved L-type voltage-gated Ca^{2+} channels and protein kinase C (Chang et al. 2000). Treatment of STC-1 cells with α-linolenic acid or an alternative omega-3 fatty acid docosahexaenoic acid, as well as the omega-7 palmitoleic acid, promoted Ca^{2+} elevation, ERK phosphorylation and the secretion of GLP-1, suggesting the involvement of $G_{\alpha q}$-dependent signalling pathways (Hirasawa et al. 2005). In the latter study, α-linolenic acid triggered GLP-1 release was abolished by extracellular Ca^{2+} removal, but was not impaired by inhibitors of L-type Ca^{2+} channels, phospholipase C or ERK kinase.

Knockdown and overexpression studies have been performed in cell lines to evaluate the relative importance of FFA1 and FFA4 for gut hormone secretion. In STC-1 cells, Ca^{2+} and secretory responses to α-linolenic acid were abrogated by siRNA-mediated *ffar4* but not *ffar1* knockdown, leading the authors to conclude that only FFA4 is important for fatty acid-triggered hormone secretion from this cell line (Hirasawa et al. 2005). It should be noted, however, that the concentration of α-linolenic acid used for Ca^{2+} imaging was relatively low (10 μM), and might at this level have preferentially activated FFA4 rather than FFA1. Confirming the importance of FFA4, the human cell line NCI-H716 secreted GLP-1 in response to α-linolenic acid when transfected with *ffar4* but not *ffar1* (Hirasawa et al. 2005).

Knockdown of *ffar4* but not *ffar1* in STC-1 cells was also found to impair GLP-1 release triggered by 100 μM α-linolenic acid (Tanaka et al. 2008).

Pharmacological studies support the importance of FFA4 for hormone secretion from enteroendocrine cell lines. GLP-1 secretion from GLUTag and STC-1 cells was strongly stimulated by the FFA4 agonist TUG-891, although it should be remembered that this compound had only weak selectivity for FFA4 over FFA1 in the mouse (Hudson et al. 2013). In STC-1 cells, NCG21 increased Ca^{2+}, ERK phosphorylation and GLP-1 secretion (Sun et al. 2010), and grifolic acid enhanced GLP-1 release (Hara et al. 2009). In the human cell line NCI-H716, the FFA4 agonist GSK137647A increased GLP-1 secretion (Sparks et al. 2014).

It is surprising that activation of $G_{\alpha q}$-coupled FFA receptors in STC-1 and GLUTag cells by natural fatty acid ligands triggers a Ca^{2+} signal that is dependent on extracellular Ca^{2+} and in some cases on L-type voltage-gated Ca^{2+} channels. Bombesin-triggered Ca^{2+} signals in GLUTag cells also occur downstream of $G_{\alpha q}$-coupled receptors, but are attributable to IP_3-mediated Ca^{2+} release from intracellular stores (Reimann et al. 2006). The results suggest that downstream signalling from FFA receptors in enteroendocrine cells activates an atypical pathway leading to plasma membrane Ca^{2+} entry, possibly involving membrane depolarisation. In keeping with this idea, linoleic acid was found to elevate Ca^{2+} in STC-1 cells through a mechanism that involved both FFA4 and the transient receptor potential channel TRPM5. It was proposed that FFA4-dependent Ca^{2+}-release from intracellular stores activated TRPM5 on the plasma membrane, resulting in membrane depolarisation, opening of voltage-gated calcium channels and Ca^{2+} influx from the extracellular medium (Fig. 1) (Shah et al. 2012).

Contrary to the stimulatory action of FFA4 ligands on CCK and GLP-1 secretion, α-linolenic acid and GW9508 suppressed ghrelin secretion from SG-1 cells (Gong et al. 2014), and the effect was lost after siRNA mediated knockdown of *ffar4* but not *ffar1*. In MGN3-1 cells, palmitate suppressed cAMP concentrations, suggesting the involvement of a $G_{\alpha i}$-coupled signalling pathway in ghrelin-secreting cells (Koyama et al. 2016).

2.5 FFA1-Dependence of Gut Hormone Secretion in Primary Enteroendocrine Cells and Rodent Models

Despite the conclusions from cell line studies that FFA1 is not involved in CCK or GLP-1 secretion (Tanaka et al. 2008), a number of studies in rodents have nonetheless demonstrated roles for FFA1 in gut hormone release. Plasma levels of CCK after gavage with olive oil were diminished in mice with knockout of *ffar1* (*ffar1*$^{-/-}$) compared with wild type controls (Liou et al. 2011). *Ffar1*$^{-/-}$ mice also exhibited significantly reduced levels of GLP-1 and GIP after consuming a high fat diet compared with wild type controls (Edfalk et al. 2008), and reduced GLP-1 secretory responses following an acute corn oil gavage (Xiong et al. 2013). Primary mixed intestinal cultures have mimicked these in vivo effects, revealing impaired GLP-1 secretory responses to α-linolenic acid in preparations from *ffar1*$^{-/-}$ mice

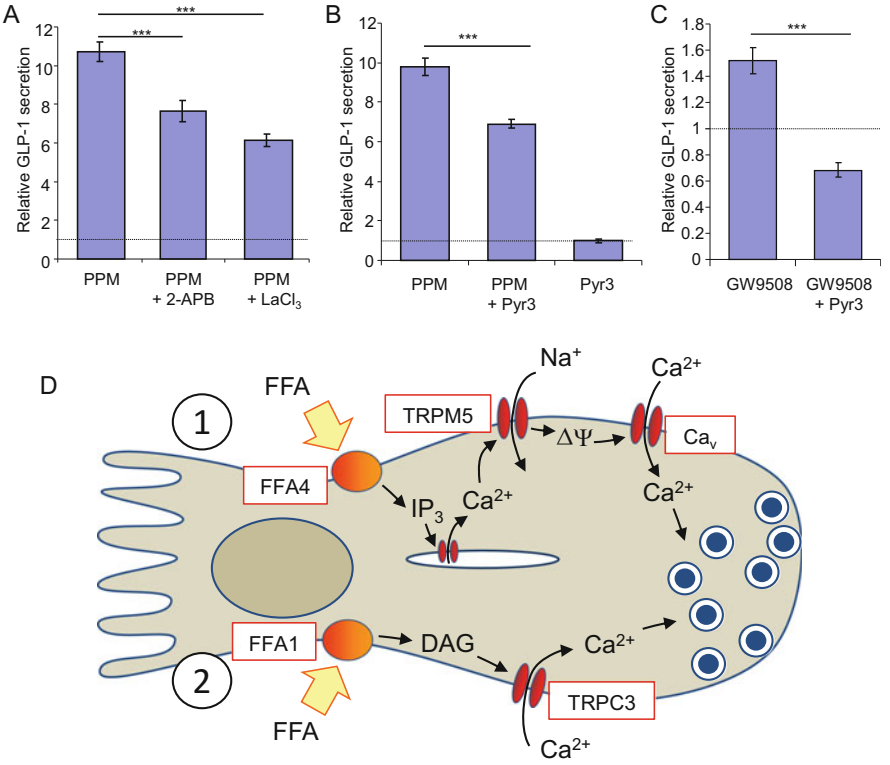

Fig. 1 TRP channel dependent signalling pathways underlie Ca^{2+} signalling downstream of FFA receptors in enteroendocrine cells. (**a**–**c**) GLP-1 secretion from primary mixed murine duodenal cultures triggered by either post-prandial micelles (PPM) containing oleic acid (200 μM), 2-monooleoyl-glycerol (70 μmol/l), L-α-lysophosphatidylcholine (70 μM), cholesterol (17 μM) and taurocholic acid (700 μM), or GW9508 (100 μM). Stimuli were applied in the absence or presence of 2-aminoethoxydiphenyl borate (2-APB, 100 μM), La^{3+} (50 μM) or Pyr 3 (10 μM), as indicated. Secretion assays were performed and analysed as described previously (Reimann et al. 2008). (**d**) Signalling pathways shown to be activated downstream of FFA4 in STC-1 cells (*1*), or FFA1 in primary L-cells (*2*). In STC-1 cells (*1*), FFA4 activation triggers IP_3-dependent stored Ca^{2+} release, which activates TRPM5 channels causing membrane depolarisation and opening of voltage-gated Ca^{2+} channels (Ca_v). In primary L-cells (*2*), FFA1 activation likely opens TRPC3 channels via the second messenger diacylglycerol (DAG), which directly allow Ca^{2+} entry

(Hauge et al. 2015). Primary FACS-purified I-cells from *ffar1*$^{-/-}$ mice similarly exhibited attenuated CCK secretion in response to linolenic acid (Liou et al. 2011).

GW9508 increased GLP-1 secretion from primary human colonic cultures (Habib et al. 2013), but as it is a ligand for FFA4 as well as FFA1, these findings did not distinguish the relative importance of FFA1 and FFA4. More recent studies have employed newer agonists of FFA1 with increased receptor specificity. Two full agonists, AM-1638 and AM-6226, significantly increased GLP-1 and GIP secretion from cultured fetal rat intestinal cells, and enhanced glucose stimulated

insulin secretion (Luo et al. 2012). AM-1638 and AM-5262, which activated $G_{\alpha s}$ as well as $G_{\alpha q}$-dependent signalling in heterologous expression systems, triggered much greater FFA1-dependent GLP-1 release from primary murine intestinal cultures than agonists lacking $G_{\alpha s}$ activity (Hauge et al. 2015). Overall, the results suggest that in murine L-cells, isolated activation of $G_{\alpha q}$ by FFA1 is not a strong stimulus of GLP-1 release, but that the additional elevation of cAMP concentrations by $G_{\alpha s}$ recruitment enables a strong enhancement of secretion. As natural FFA ligands do not seem to activate FFA1-dependent $G_{\alpha s}$ signalling (Hauge et al. 2015), their activity in vivo might be enhanced by $G_{\alpha s}$-dependent signalling from GPR119 or the bile acid receptor GPBAR1.

In support of the effectiveness of the newer specific FFA1 agonists in primary intestinal cultures, these ligands triggered GLP-1 release from the perfused rat small intestine and in vivo in rodents (Christensen et al. 2015). Interestingly, in this perfused intestinal model, FFA1 agonists enhanced GLP-1 secretion when applied from the vascular but not the luminal direction, providing the best evidence yet that FFA1 receptors are located on the basolateral side of L-cells. These results support the idea that long chain FFA stimulates GLP-1 release after their absorption through the intestinal epithelium.

2.6 FFA4 Dependence of Gut Hormone Secretion in Primary Cultures and Rodent Models

Ffar4$^{-/-}$ mice were found to secrete significantly lower levels of GIP in response to small intestinal gavage of lard oil compared with control animals, whilst exhibiting normal glucose-induced GIP secretion (Iwasaki et al. 2015), supporting studies from cell lines that suggested a role for FFA4 in enteroendocrine cells of the upper GI tract. The role of FFA4 in GLP-1 secreting L-cells is less clear despite the identification of the receptor in this cell population by transcriptomic analysis (Reimann et al. 2008). Normal plasma GLP-1 excursions were observed after corn oil gavage in *ffar4*$^{-/-}$ mice (Xiong et al. 2013), and the FFA4-specific agonist cpdA did not elevate plasma GLP-1 levels in mice (Oh et al. 2014). α-Linolenic acid-triggered GLP-1 secretion was disrupted in primary cultures from *ffar1*$^{-/-}$ but not *ffar4*$^{-/-}$ mice (Hauge et al. 2015). However, the FFA4 agonist NCG21 did increase plasma GLP-1 concentrations when administered into the mouse colon (Sun et al. 2010).

In gastric somatostatin-secreting D-cells and gastric ghrelin-secreting cells, FFA4 appears to be linked to suppression of hormone secretion. The FFA4 agonist compound B inhibited somatostatin secretion from mixed primary cultured gastric epithelium from wild type mice, and this effect was abolished in cultures from *ffar4*$^{-/-}$ mice (Egerod et al. 2015). Compound B also inhibited ghrelin secretion from primary gastric cultures. This suppression of ghrelin release was prevented by pertussis toxin, indicating the involvement of $G_{\alpha i}$-dependent signalling pathways, and was abolished in tissues from *ffar4*$^{-/-}$ animals (Engelstoft et al. 2013). *Ffar4* deficient mice exhibited elevated fasting plasma ghrelin levels, suggesting that

FFA4 exhibits a tonic inhibitory effect on ghrelin release, but the lack of FFA4 did not prevent the suppression of ghrelin levels 2 h after an oral olive oil challenge (Engelstoft et al. 2013). A $G_{\alpha i}$ mediated inhibitory effect of FFA4 has also been reported for somatostatin secretion from pancreatic delta-cells (Stone et al. 2014).

2.7 Signalling Pathways Downstream of FFA Receptors in Primary Enteroendocrine Cells

There are only limited studies examining FFA-triggered intracellular signalling pathways in primary enteroendocrine cells. In I-cells purified by their cell-specific expression of a fluorescent protein, linolenic acid, linoleic acid and oleic acid triggered elevations in intracellular Ca^{2+} levels, and the response to linolenic acid was impaired in I-cells from *ffar1*$^{-/-}$ mice (Liou et al. 2011). GLP-1 secretion from primary small intestinal cultures was stimulated by GW9508 (Fig. 1), but a much greater enhancement of GLP-1 secretion was observed with mixed lipid micelles that contain oleic acid together with cholesterol, taurocholate, monooleoylglycerol and lysophosphatidylcholine. The latter three constituents are ligands of predominantly $G_{\alpha s}$ coupled receptors (GPBAR1 and GPR119) that elevate cAMP concentrations (Brighton et al. 2015; Moss et al. 2016) and likely enhance responses to FFA receptor agonism. GLP-1 secretion triggered by mixed micelles was impaired by the non-selective TRP channel inhibitors 2-APB and La^{3+} (Fig. 1), suggesting the involvement of a TRP channel dependent pathway, mirroring the TRPM5-dependent pathway identified in STC-1 cells (Shah et al. 2012). However, microarray analysis of primary L-cells revealed only very low levels of *trpm5* but high expression of members of the TRPC family (Emery et al. 2015), and in primary intestinal cultures GLP-1 secretory responses to GW9508 and mixed micelles were impaired by an inhibitor of TRPC3 channels, Pyr3 (Fig. 1). Plasma membrane TRPC3 channels are permeable to Ca^{2+} and Na^{+} ions, and activated by diacylglycerols produced downstream of $G_{\alpha q}$-coupled receptors (Abramowitz and Birnbaumer 2009), so could generate Ca^{2+} signals without the recruitment of L-type voltage-gated Ca^{2+} channels. Further work will be required to determine how the nature of the Ca^{2+} signal in different primary enteroendocrine cells and model cell lines is determined by their relative expression of TRP and voltage-gated Ca^{2+} channels. This type of model could, however, explain why cytoplasmic Ca^{2+} signals have variously been reported to be dependent or independent of voltage-gated Ca^{2+} channel entry (Fig. 1).

3 How Do Ingested Lipids Access FFA Receptors on Enteroendocrine Cells?

In human volunteers, oral ingestion of intralipid triggered elevations in plasma GLP-1 and GIP that were not reproduced when intralipid was infused intravenously at a rate that resulted in similar circulating triglyceride levels (Lindgren et al. 2011).

While this appears to suggest that fats stimulate L- and K-cells from the luminal rather than the vascular direction, triglycerides are not themselves believed to be detected in the gut. Supporting this idea, in patients with pancreatic insufficiency, duodenal perfusion of free fatty acids generated more pronounced and faster CCK release than triglycerides (Guimbaud et al. 1997). Human volunteers treated with orlistat, an inhibitor of gastric and pancreatic lipases (Sternby et al. 2002), also exhibited reduced post-prandial levels of CCK, GLP-1 and PYY (Ellrichmann et al. 2008). Whilst these studies demonstrate that lipid digestion is a prerequisite for triggering gut hormone secretion, they do not indicate how FFA and 2-monoglycerides access their receptors on enteroendocrine cells, and the role of epithelial transport.

Lipids are absorbed across the intestinal epithelium in the form of their component FFA and 2-monoglycerides (Yen et al. 2015; Abumrad and Davidson 2012). Protonated (uncharged) FFA can probably cross the membrane passively by a diffusional flip/flop mechanism, but transport is enhanced by membrane proteins such as CD36 and fatty acid transfer proteins (FATP). CD36 has been identified along the length of the GI tract from the stomach to the colon, exhibiting highest expression in the jejunum. Whether CD36 directly transports FFA across the membrane or concentrates FFA at the membrane and thereby enhances diffusional uptake remains uncertain.

Downstream of CD36, FATPs and fatty acid binding proteins (FABPs) contribute to FA uptake and trafficking. FATPs exhibit fatty acyl coA synthetase activity, which traps fatty acids within the cytoplasm. FABPs deliver fatty acids to the endoplasmic reticulum (ER) where they are re-esterified with 2-MG to form triglycerides. These are either stored in cytosolic lipid droplets or incorporated into chylomicrons in the ER-lumen. Re-esterification and chylomicron formation involve a series of enzymes, including monoacylglycerol acyl transferase (MGAT2), diacylglycerol acyl transferase (thought to be preferentially DGAT1 for chylomicrons and DGAT2 for lipid droplets in mice, whereas human intestine expresses little DGAT2) and microsomal triglyceride transfer protein (MTTP) (Abumrad and Davidson 2012; Yen et al. 2015).

A number of studies have demonstrated that gut hormone secretion is affected by interventions that disrupt lipid absorption. A CD36 inhibitor sulfo-N-succinimidyl oleate (SSO) impaired GIP release triggered by duodenal infusion of glycerol trioleate in rats (Shimotoyodome et al. 2012). Pluronic L-81 is a surfactant believed to interfere with MTTP activity, which disrupts lipid absorption and reduces post-prandial lymphatic concentrations of chylomicrons and FFA. Pluronic L-81 largely abolished fat-triggered CCK and GIP release in rodent models (Shimotoyodome et al. 2009; Lu et al. 2012a). Specific inhibitors of intestinal MTTP are used as a weight loss treatment in dogs, and were found in rodent models to increase plasma GLP-1 and PYY concentrations (Wren et al. 2007). The MTTP inhibitor JTT-130 similarly increased GLP-1 and PYY levels in rats through a mechanism that was reversed when lipid digestion was blocked using orlistat (Hata et al. 2011).

In analogous experiments targeting different stages of lipid absorption, mice lacking DGAT1 or MGAT2 exhibited impaired secretion of GIP. This matches the

findings with SSO and pluronic L-81, supporting an important role for a fully functional lipid absorption pathway as a prerequisite for hormone secretion from the upper GI tract. DGAT1 and MGAT2 knockout mice exhibited elevated GLP-1 secretion (Okawa et al. 2009), resembling the effect of MTTP inhibitors. Measurements of the absorption of labelled lipids revealed that the elevation of GLP-1 release by DGAT1 inhibition is an indirect effect, attributable to the impairment of lipid absorption in the upper gut which results in increased delivery of dietary fats to the distal GI tract where the density of GLP-1 secreting L-cells is higher (Liu et al. 2015). Lipid-triggered secretion of GLP-1 is highly dependent on GPR119 signalling (Moss et al. 2016), as demonstrated by the severe impairment of fat-triggered GLP-1 release in mice with L-cell specific *gpr119* knockout, and by the effectiveness of 1,3-dioctanoyl-2-oleoyl glycerol as a GLP-1 stimulant in humans (Mandøe et al. 2015). Even though lipid absorption would also be impaired in the distal gut when DGAT1 activity is reduced, any effect of reduced FFA uptake seems to be offset by increased 2-MG signalling via GPR119. Nevertheless, FFA uptake does seem to play some role even in the distal small intestine, as oleate was reported to stimulate GLP-1 release when infused directly into the ileal lumen and this was impaired in mice lacking FATP4 (Poreba et al. 2012).

Overall these results, taken together with the effectiveness of FFA1 ligands when applied from the vascular but not the luminal direction in perfused intestine (Christensen et al. 2015), are compatible with a model in which FFA receptors are located on the basolateral membrane of enteroendocrine cells, and FFA enhances gut hormone secretion only after their absorption through the intestinal epithelium. The GPR119 ligand oleoylethanolamide, by contrast, exhibited similar effectiveness on L-cell secretion after luminal or basolateral/vascular application (Lauffer et al. 2009; Patel et al. 2014), suggesting that access of 2-monoglycerides to GPR119 receptors might not be dependent on epithelial absorption pathways, although studies employing non-absorbable GPR119 ligands will be required to demonstrate this conclusively.

4 FFA1 and FFA4 as Candidate Targets for Increasing Endogenous Gut Hormone Release in Humans

In the light of the clinical success of GLP-1 mimetics for the treatment of type 2 diabetes and obesity, drugs are under development to increase the secretion of endogenous GLP-1 and PYY from the gut. Such treatments would aim to reproduce the post-prandial state of increased insulin secretion and reduced appetite. A widely adopted strategy is to target enteroendocrine cell receptors that are normally activated after lipid ingestion, including FFA1, FFA4, GPR119 and GBAR1.

Important considerations are which of these receptors are most important for normal post-prandial GLP-1 and PYY responses in humans, and which receptors could be activated to promote the largest secretory response. By analogy with the physiological state after gastric bypass surgery when post-prandial GLP-1 and PYY levels are elevated approximately tenfold (Jørgensen et al. 2013), it is reasonable to

suppose that higher levels of these gut hormones would be desirable. This also fits with the doses of GLP-1 mimetics used to treat obesity and type 2 diabetes, which aim to produce circulating equivalent concentrations several fold higher than normal post-prandial GLP-1 levels (Drucker and Nauck 2006).

The finding in humans that olive oil was no better as a GLP-1 stimulus than 1,3 dioctanoyl 2-oleoylglycerol (Mandøe et al. 2015) suggests that long chain 2-monoglycerides are sufficient for normal post-prandial GLP-1 secretion. This is consistent with findings in mice which showed that restricted knockout of *gpr119* in L-cells was sufficient to abolish the GLP-1 secretory response to a gavage oil challenge (Moss et al. 2016). However, GPR119 agonists have been developed and tested in type 2 diabetic human volunteers, and the first trials did not reveal a metabolic benefit (Katz et al. 2012). One possible explanation is that the drugs did not elevate GLP-1 levels sufficiently above the normal post-prandial range in this patient group.

FFA1 is expressed in enteroendocrine cells as well as pancreatic beta cells, making it good candidate target for mimicking the post-prandial metabolic state. The first FFA1 activator to reach phase 2 clinical trials was TAK-875 (Burant et al. 2012). This is an orally available and selective FFA1 agonist which increased GLP-1 levels ~2-fold in mice (Hauge et al. 2015) and also enhanced glucose-dependent insulin secretion from the pancreatic beta cells (Tsujihata et al. 2011). Despite having a beneficial metabolic effect in people with type 2 diabetes (Burant et al. 2012), trials on TAK-875 were discontinued because of concerns about liver toxicity (Mancini and Poitout 2015). As FFA1 is not known to be expressed in hepatocytes, it is hoped that this was a drug-specific side effect rather than a class effect. Other FFA1 agonists are therefore under development. Indeed, the identification of newer FFA1 agonists that target a second site on the FFA1 receptor and have in some cases been linked to elevated intracellular cAMP concentrations in heterologous expressions systems leads to the hope that the further development of FFA1 ligands will lead to successful clinical trials.

FFA4 agonism has been associated with protection from the development of diabetes and obesity, but it is not yet clear whether this is related to the enteroendocrine system. FFA4 knockout mice, for example, became obese, displayed impaired glucose tolerance and developed fatty liver disease when they were fed a high fat diet (Ichimura et al. 2012). In Europeans, a non-synonymous mutation in *ffar4* that impaired receptor signalling was associated with increased risk of morbid obesity (Ichimura et al. 2012), and a Japanese study has suggested that the combined effects of common genetic variations in the *ffar4* gene and fat intake might influence body mass index (Waguri et al. 2013).

5 Concluding Remarks

Changes in plasma gut hormones after lipid ingestion reflect where triglycerides are digested and absorbed, and the corresponding location of enteroendocrine cells and their receptors (Fig. 2). FFA1 and FFA4 link the release of gut hormones to the

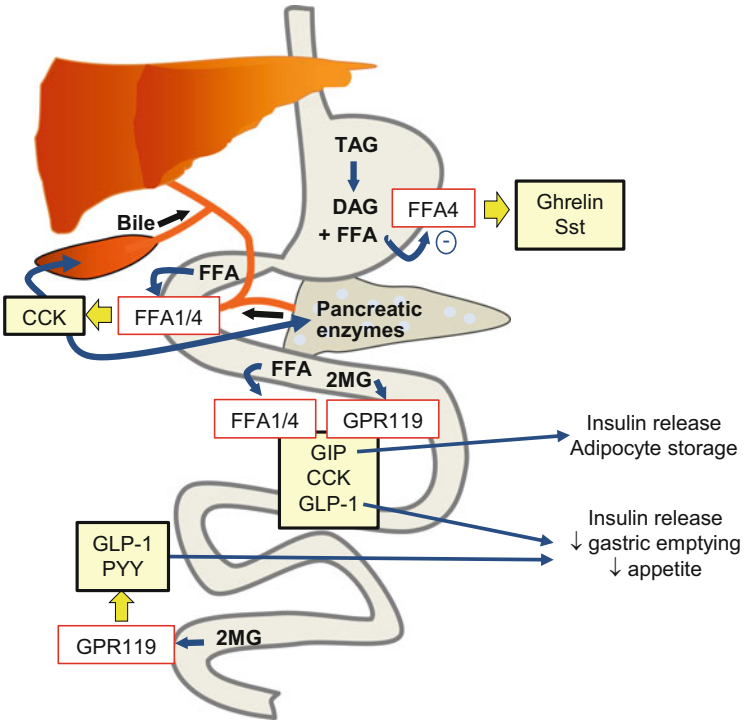

Fig. 2 Detection of triglyceride digestion products by enteroendocrine cells along the GI tract. Triglyceride (TAG) digestion in the stomach generates diacylglycerols (DAG) and FFA, which inhibit ghrelin and somatostatin (Sst) secretion, likely via FFA4. FFA formed in the stomach and duodenum also target I-cells to release CCK, and thereby stimulate gall bladder contraction and pancreatic enzyme secretion. Bile and pancreatic lipase promote small intestinal triglyceride digestion, generating 2-monoglycerides (2MG) and further FFA. FFA1 and FFA4 in enteroendocrine cells detect FFA after their absorption across the epithelial layer, triggering the release of hormones such as GIP, GLP-1 and PYY that coordinate the body's response to nutrients arriving in the bloodstream. 2MG target GPR119, and are particularly important for distal GLP-1 and PYY release

absorption of long chain FFA. Although enteroendocrine cells have a specialised morphology, with apical processes that extend to and make contact with the gut lumen, FFA1-dependent detection of fatty acids seems only to occur after their absorption across the enterocyte layer (Christensen et al. 2015). Bile acids are similarly detected by L-cells from the basolateral direction (Brighton et al. 2015) and require absorption before they can enhance GLP-1 secretion. Linking hormone secretion to fatty acid and bile acid absorption ensures that the circulating gut endocrine signal mirrors the rate of appearance of lipids in the bloodstream rather than their mere presence within the gut lumen.

FFA1 and FFA4 are exciting candidate targets for drug design to combat type 2 diabetes and obesity, as they have a variety of beneficial actions across the body.

In the stomach they may play a role in suppressing the hunger hormone ghrelin, and the negative regulator of hormone secretion somatostatin. In the small intestine, agonists of these receptors increase the release of CCK, GIP, GLP-1 and PYY, which variously promote lipid digestion, adipose fat storage, satiety and slow gastric emptying. GLP-1 and GIP also act in concert with FFA1 agonists on pancreatic beta cells to increase insulin release, and FFA4 agonists appear to increase insulin release indirectly by suppressing islet somatostatin secretion. Dissecting the relative physiological importance and therapeutic potential of FFA1 and FFA4 has been hindered by the lack of pharmacological tools specific for either receptor, but these studies will be possible in the future following the development of potent and specific FFA1 and FFA4 agonists. The challenge in this field will be to develop specific FFA1 or FFA4 agonists that trigger large increments in GLP-1 and PYY secretion in the fasting and/or the post-prandial states. Although development of TAK-875 was terminated because of off-target side effects, clinical data from human trials were promising and lend support to the therapeutic potential of new FFA receptor agonists.

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References

Abramowitz J, Birnbaumer L (2009) Physiology and pathophysiology of canonical transient receptor potential channels. FASEB J 23:297–328

Abumrad NA, Davidson NO (2012) Role of the gut in lipid homeostasis. Physiol Rev 92:1061–1085

Adriaenssens A, Lam BY, Billing L, Skeffington K, Sewing S, Reimann F, Gribble F (2015) A transcriptome-led exploration of molecular mechanisms regulating somatostatin-producing D-cells in the gastric epithelium. Endocrinology 156:3924–3936

Ahlman H, Nilsson (2001) The gut as the largest endocrine organ in the body. Ann Oncol 12(Suppl 2):S63–S68

Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. Gastroenterology 132:2131–2157

Bayliss WM, Starling EH (1902) The mechanism of pancreatic secretion. J Physiol 28:325–353

Brighton CA, Rievaj J, Kuhre RE, Glass LL, Schoonjans K, Holst JJ, Gribble FM, Reimann F (2015) Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G protein-coupled bile acid receptors. Endocrinology 156:3961–3970

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR, Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S, Muir AI (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311

Brubaker PL, Schloos J, Drucker DJ (1998) Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. Endocrinology 139:4108–4114

Burant CF, Viswanathan P, Marcinak J, Cao C, Vakilynejad M, Xie B, Leifke E (2012) TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial. Lancet 379:1403–1411

Carrière F, Rogalska E, Cudrey C, Ferrato F, Laugier R, Verger R (1997) In vivo and in vitro studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipases. Bioorg Med Chem 5:429–435

Chang CH, Chey WY, Chang TM (2000) Cellular mechanism of sodium oleate-stimulated secretion of cholecystokinin and secretin. Am J Physiol Gastrointest Liver Physiol 279: G295–G303

Christensen LW, Kuhre RE, Janus C, Svendsen B, Holst JJ (2015) Vascular, but not luminal, activation of FFAR1 (GPR40) stimulates GLP-1 secretion from isolated perfused rat small intestine. Physiol Rep 3

Christiansen E, Watterson KR, Stocker CJ, Sokol E, Jenkins L, Simon K, Grundmann M, Petersen RK, Wargent ET, Hudson BD, Kostenis E, Ejsing CS, Cawthorne MA, Milligan G, Ulven T (2015) Activity of dietary fatty acids on FFA1 and FFA4 and characterisation of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. Br J Nutr 113:1677–1688

Creutzfeldt W (1979) The incretin concept today. Diabetologia 16:75–85

Dockray GJ (2009) Cholecystokinin and gut-brain signalling. Regul Pept 155:6–10

Drucker DJ, Nauck MA (2006) The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368:1696–1705

Drucker DJ, Jin TR, Asa SL, Young TA, Brubaker PL (1994) Activation of proglucagon gene-transcription by protein kinase-A in a novel mouse enteroendocrine cell-line. Mol Endocrinol 8:1646–1655

Edfalk S, Steneberg P, Edlund H (2008) Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. Diabetes 57:2280–2287

Egerod KL, Engelstoft MS, Grunddal KV, Nohr MK, Secher A, Sakata I, Pedersen J, Windelov JA, Fuchtbauer EM, Olsen J, Sundler F, Christensen JP, Wierup N, Olsen JV, Holst JJ, Zigman JM, Poulsen SS, Schwartz TW (2012) A major lineage of enteroendocrine cells coexpress CCK, secretin, GIP, GLP-1, PYY, and neurotensin but not somatostatin. Endocrinology 153:5782–5795

Egerod KL, Engelstoft MS, Lund ML, Grunddal KV, Zhao M, Barir-Jensen D, Nygaard EB, Petersen N, Holst JJ, Schwartz TW (2015) Transcriptional and functional characterization of the G protein-coupled receptor repertoire of gastric somatostatin cells. Endocrinology 156:3909–3923

Ellrichmann M, Kapelle M, Ritter PR, Holst JJ, Herzig KH, Schmidt WE, Schmitz F, Meier JJ (2008) Orlistat inhibition of intestinal lipase acutely increases appetite and attenuates post-prandial glucagon-like peptide-1-(7-36)-amide-1, cholecystokinin, and peptide YY concentrations. J Clin Endocrinol Metab 93:3995–3998

Emery EC, Diakogiannaki E, Gentry C, Psichas A, Habib AM, Bevan S, Fischer MJ, Reimann F, Gribble FM (2015) Stimulation of GLP-1 secretion downstream of the ligand-gated ion channel TRPA1. Diabetes 64:1202–1210

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nøhr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW (2013) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2:376–392

Erdmann J, Lippl F, Schusdziarra V (2003) Differential effect of protein and fat on plasma ghrelin levels in man. Regul Pept 116:101–107

Geraedts MC, Troost FJ, Saris WH (2009) Peptide-YY is released by the intestinal cell line STC-1. J Food Sci 74:H79–H82

Gong Z, Yoshimura M, Aizawa S, Kurotani R, Zigman JM, Sakai T, Sakata I (2014) G protein-coupled receptor 120 signaling regulates ghrelin secretion in vivo and in vitro. Am J Physiol Endocrinol Metab 306:E28–E35

Gribble FM, Reimann F (2016) Enteroendocrine cells: chemosensors in the intestinal epithelium. Annu Rev Physiol 78:277–299

Guimbaud R, Moreau JA, Bouisson M, Durand S, Escourrou J, Vaysse N, Frexinos J (1997) Intraduodenal free fatty acids rather than triglycerides are responsible for the release of CCK in humans. Pancreas 14:76–82

Habib AM, Richards P, Cairns LS, Rogers GJ, Bannon CA, Parker HE, Morley TC, Yeo GS, Reimann F, Gribble FM (2012) Overlap of endocrine hormone expression in the mouse intestine revealed by transcriptional profiling and flow cytometry. Endocrinology 153:3054–3065

Habib AM, Richards P, Rogers GJ, Reimann F, Gribble FM (2013) Co-localisation and secretion of glucagon-like peptide 1 and peptide YY from primary cultured human L cells. Diabetologia 56:1413–1416

Hansen L, Deacon CF, Orskov C, Holst JJ (1999) Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. Endocrinology 140:5356–5363

Hara T, Hirasawa A, Sun Q, Sadakane K, Itsubo C, Iga T, Adachi T, Koshimizu TA, Hashimoto T, Asakawa Y, Tsujimoto G (2009) Novel selective ligands for free fatty acid receptors GPR120 and GPR40. Naunyn Schmiedebergs Arch Pharmacol 380:247–255

Hata T, Mera Y, Ishii Y, Tadaki H, Tomimoto D, Kuroki Y, Kawai T, Ohta T, Kakutani M (2011) JTT-130, a novel intestine-specific inhibitor of microsomal triglyceride transfer protein, suppresses food intake and gastric emptying with the elevation of plasma peptide YY and glucagon-like peptide-1 in a dietary fat-dependent manner. J Pharmacol Exp Ther 336:850–856

Hauge M, Vestmar MA, Husted AS, Ekberg JP, Wright MJ, Di Salvo J, Weinglass AB, Engelstoft MS, Madsen AN, Lückmann M, Miller MW, Trujillo ME, Frimurer TM, Holst B, Howard AD, Schwartz TW (2015) GPR40 (FFAR1) – combined Gs and Gq signaling in vitro is associated with robust incretin secretagogue action ex vivo and in vivo. Mol Metab 4:3–14

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 11:90–94

Holst JJ (2007) The physiology of glucagon-like peptide 1. Physiol Rev 87:1409–1439

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, Heathcote H, Tobin AB, Ulven T, Milligan G (2013) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential opportunity and possible challenges to therapeutic agonism. Mol Pharmacol 84:710–725

Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, Choquet H, Besnard P, Lecoeur C, Vivequin S, Ayukawa K, Takeuchi M, Ozawa K, Tauber M, Maffeis C, Morandi A, Buzzetti R, Elliott P, Pouta A, Jarvelin MR, Körner A, Kiess W, Pigeyre M, Caiazzo R, van Hul W, van Gaal L, Horber F, Balkau B, Lévy-Marchal C, Rouskas K, Kouvatsi A, Hebebrand J, Hinney A, Scherag A, Pattou F, Meyre D, Koshimizu TA, Wolowczuk I, Tsujimoto G, Froguel P (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 483:350–354

Iwasaki K, Harada N, Sasaki K, Yamane S, Iida K, Suzuki K, Hamasaki A, Nasteska D, Shibue K, Joo E, Harada T, Hashimoto T, Asakawa Y, Hirasawa A, Inagaki N (2015) Free fatty acid receptor GPR120 is highly expressed in enteroendocrine K cells of the upper small intestine and has a critical role in GIP secretion after fat ingestion. Endocrinology 156:837–846

Jørgensen NB, Dirksen C, Bojsen-Møller KN, Jacobsen SH, Worm D, Hansen DL, Kristiansen VB, Naver L, Madsbad S, Holst JJ (2013) Exaggerated glucagon-like peptide 1 response is important for improved β-cell function and glucose tolerance after Roux-en-Y gastric bypass in patients with type 2 diabetes. Diabetes 62:3044–3052

Karra E, Batterham RL (2010) The role of gut hormones in the regulation of body weight and energy homeostasis. Mol Cell Endocrinol 316:120–128

Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G (2005) Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. J Biol Chem 280:19507–19515

Katz LB, Gambale JJ, Rothenberg PL, Vanapalli SR, Vaccaro N, Xi L, Sarich TC, Stein PP (2012) Effects of JNJ-38431055, a novel GPR119 receptor agonist, in randomized, double-blind, placebo-controlled studies in subjects with type 2 diabetes. Diabetes Obes Metab 14:709–716

Koyama H, Iwakura H, Dote K, Bando M, Hosoda H, Ariyasu H, Kusakabe T, Son C, Hosoda K, Akamizu T, Kangawa K, Nakao K (2016) Comprehensive profiling of GPCR expression in ghrelin-producing cells. Endocrinology 157:692–704

Ku SK, Lee HS, Lee JH (2003) An immunohistochemical study of the gastrointestinal endocrine cells in the C57BL/6 mice. Anat Histol Embryol 32:21–28

Kuhre RE, Wewer Albrechtsen NJ, Deacon C, Balk-Møller E, Rehfeld JF, Reimann F, Gribble F, Holst JJ (2016) Peptide production and secretion in GLUTag, NCI-H716 and STC-1 cells: a comparison to native L-cells. J Mol Endocrinol 56:201–211

Lauffer LM, Iakoubov R, Brubaker PL (2009) GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell. Diabetes 58:1058–1066

Liao TH, Hamosh P, Hamosh M (1984) Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine. Pediatr Res 18:402–409

Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, Tran T, Dransfield PJ, Brown SP, Houze J, Vimolratana M, Jiao XY, Wang Y, Birdsall NJ, Swaminath G (2012) Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. Mol Pharmacol 82:843–859

Lindgren O, Carr RD, Deacon CF, Holst JJ, Pacini G, Mari A, Ahrén B (2011) Incretin hormone and insulin responses to oral versus intravenous lipid administration in humans. J Clin Endocrinol Metab 96:2519–2524

Lindström MB, Sternby B, Borgström B (1988) Concerted action of human carboxyl ester lipase and pancreatic lipase during lipid digestion in vitro: importance of the physicochemical state of the substrate. Biochim Biophys Acta 959:178–184

Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ, Raybould HE, Wank S (2011) The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. Gastroenterology 140:903–912

Little TJ, Isaacs NJ, Young RL, Ott R, Nguyen NQ, Rayner CK, Horowitz M, Feinle-Bisset C (2014) Characterization of duodenal expression and localization of fatty acid-sensing receptors in humans: relationships with body mass index. Am J Physiol Gastrointest Liver Physiol 307: G958–G967

Liu J, Mclaren DG, Chen D, Kan Y, Stout SJ, Shen X, Murphy BA, Forrest G, Karanam B, Sonatore L, He S, Roddy TP, Pinto S (2015) Potential mechanism of enhanced postprandial glucagon-like peptide-1 release following treatment with a diacylglycerol acyltransferase 1 inhibitor. Pharmacol Res Perspect 3:e00193

Lu WJ, Yang Q, Yang L, Lee D, D'Alessio D, Tso P (2012a) Chylomicron formation and secretion is required for lipid-stimulated release of incretins GLP-1 and GIP. Lipids 47:571–580

Lu X, Zhao X, Feng J, Liou AP, Anthony S, Pechhold S, Sun Y, Lu H, Wank S (2012b) Postprandial inhibition of gastric ghrelin secretion by long-chain fatty acid through GPR120 in isolated gastric ghrelin cells and mice. Am J Physiol Gastrointest Liver Physiol 303:G367–G376

Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, Vimolratana M, Houze JB, Wong S, Toteva M, Shan B, Li F, Zhuang R, Lin DC (2012) A potent class of GPR40 full agonists engages the enteroinsular axis to promote glucose control in rodents. PLoS One 7:e46300

Mancini AD, Poitout V (2015) GPR40 agonists for the treatment of type 2 diabetes: life after 'TAKing' a hit. Diabetes Obes Metab 17:622–629

Mandøe MJ, Hansen KB, Hartmann B, Rehfeld JF, Holst JJ, Hansen HS (2015) The 2-monoacylglycerol moiety of dietary fat appears to be responsible for the fat-induced release of GLP-1 in humans. Am J Clin Nutr 102:548–555

Mclaughlin JT, Lomax RB, Hall L, Dockray GJ, Thompson DG, Warhurst G (1998) Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca2+-dependent mechanism in the enteroendocrine cell line STC-1. J Physiol 513(Pt 1):11–18

Mentlein R (1999) Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 85:9–24

Moss CE, Glass LL, Diakogiannaki E, Pais R, Lenaghan C, Smith DM, Wedin M, Bohlooly-Y M, Gribble FM, Reimann F (2016) Lipid derivatives activate GPR119 and trigger GLP-1 secretion in primary murine L-cells. Peptides 77:16–20

Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W (1993) Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. J Clin Invest 91:301–307

Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, Yanase T, Nawata H, Kangawa K, Kojima M (2005) Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. Endocrinology 146:2255–2264

Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR, Sasik R, Hah N, Chi TJ, Cox JM, Powels MA, Di Salvo J, Sinz C, Watkins SM, Armando AM, Chung H, Evans RM, Quehenberger O, Mcnelis J, Bogner-Strauss JG, Olefsky JM (2014) A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med 20:942–947

Okawa M, Fujii K, Ohbuchi K, Okumoto M, Aragane K, Sato H, Tamai Y, Seo T, Itoh Y, Yoshimoto R (2009) Role of MGAT2 and DGAT1 in the release of gut peptides after triglyceride ingestion. Biochem Biophys Res Commun 390:377–381

Parker HE, Habib AM, Rogers GJ, Gribble FM, Reimann F (2009) Nutrient-dependent secretion of glucose-dependent insulinotropic polypeptide from primary murine K cells. Diabetologia 52:289–298

Patel S, Mace OJ, Tough IR, White J, Cock TA, Warpman Berglund U, Schindler M, Cox HM (2014) Gastrointestinal hormonal responses on GPR119 activation in lean and diseased rodent models of type 2 diabetes. Int J Obes (Lond) 38:1365–1373

Poreba MA, Dong CX, Li SK, Stahl A, Miner JH, Brubaker PL (2012) Role of fatty acid transport protein 4 in oleic acid-induced glucagon-like peptide-1 secretion from murine intestinal L cells. Am J Physiol Endocrinol Metab 303:E899–E907

Reimann F, Ward PS, Gribble FM (2006) Signalling mechanisms underlying the release of glucagon-like peptide-1. Diabetes 55(Suppl 2):S78–S85

Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM (2008) Glucose sensing in L cells: a primary cell study. Cell Metab 8:532–539

Reimann F, Tolhurst G, Gribble FM (2012) G-protein-coupled receptors in intestinal chemosensation. Cell Metab 15:421–431

Reimer RA, Darimont C, Gremlich S, Nicolas-Métral V, Rüegg UT, Macé K (2001) A human cellular model for studying the regulation of glucagon-like peptide-1 secretion. Endocrinology 142:4522–4528

Rindi G, Grant SG, Yiangou Y, Ghatei MA, Bloom SR, Bautch VL, Solcia E, Polak JM (1990) Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. Am J Pathol 136:1349–1363

Rocca AS, Brubaker PL (1995) Stereospecific effects of fatty acids on proglucagon-derived peptide secretion in fetal rat intestinal cultures. Endocrinology 136:5593–5599

Shah BP, Liu P, Yu T, Hansen DR, Gilbertson TA (2012) TRPM5 is critical for linoleic acid-induced CCK secretion from the enteroendocrine cell line, STC-1. Am J Physiol Cell Physiol 302:C210–C219

Shimotoyodome A, Fukuoka D, Suzuki J, Fujii Y, Mizuno T, Meguro S, Tokimitsu I, Hase T (2009) Coingestion of acylglycerols differentially affects glucose-induced insulin secretion via glucose-dependent insulinotropic polypeptide in C57BL/6J mice. Endocrinology 150:2118–2126

Shimotoyodome A, Osaki N, Onizawa K, Mizuno T, Suzukamo C, Okahara F, Fukuoka D, Hase T (2012) Dietary 1-monoolein decreases postprandial GIP release by reducing jejunal transport of glucose and fatty acid in rodents. Am J Physiol Gastrointest Liver Physiol 303:G298–G310

Sidhu SS, Thompson DG, Warhurst G, Case RM, Benson RS (2000) Fatty acid-induced cholecystokinin secretion and changes in intracellular Ca2+ in two enteroendocrine cell lines, STC-1 and GLUTag. J Physiol 528(Pt 1):165–176

Sjölund K, Sandén G, Håkanson R, Sundler F (1983) Endocrine cells in human intestine: an immunocytochemical study. Gastroenterology 85:1120–1130

Skibicka KP, Dickson SL (2013) Enteroendocrine hormones – central effects on behavior. Curr Opin Pharmacol 13:977–982

Sparks SM, Chen G, Collins JL, Danger D, Dock ST, Jayawickreme C, Jenkinson S, Laudeman C, Leesnitzer MA, Liang X, Maloney P, Mccoy DC, Moncol D, Rash V, Rimele T, Vulimiri P, Way JM, Ross S (2014) Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). Bioorg Med Chem Lett 24:3100–3103

Stengel A, Taché Y (2009) Regulation of food intake: the gastric X/A-like endocrine cell in the spotlight. Curr Gastroenterol Rep 11:448–454

Sternby B, Hartmann D, Borgström B, Nilsson A (2002) Degree of in vivo inhibition of human gastric and pancreatic lipases by Orlistat (Tetrahydrolipstatin, THL) in the stomach and small intestine. Clin Nutr 21:395–402

Stone VM, Dhayal S, Brocklehurst KJ, Lenaghan C, Sörhede Winzell M, Hammar M, Xu X, Smith DM, Morgan NG (2014) GPR120 (FFA4) is preferentially expressed in pancreatic delta-cells and regulates somatostatin secretion from murine islets of Langerhans. Diabetologia 57:1182–1191

Sun Q, Hirasawa A, Hara T, Kimura I, Adachi T, Awaji T, Ishiguro M, Suzuki T, Miyata N, Tsujimoto G (2010) Structure-activity relationships of GPR120 agonists based on a docking simulation. Mol Pharmacol 78:804–810

Sykaras AG, Demenis C, Case RM, Mclaughlin JT, Smith CP (2012) Duodenal enteroendocrine I-cells contain mRNA transcripts encoding key endocannabinoid and fatty acid receptors. PLoS One 7, e42373

Tanaka T, Katsuma S, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G (2008) Free fatty acids induce cholecystokinin secretion through GPR120. Naunyn Schmiedebergs Arch Pharmacol 377:523–527

Thomsen C, Rasmussen O, Lousen T, Holst JJ, Fenselau S, Schrezenmeir J, Hermansen K (1999) Differential effects of saturated and monounsaturated fatty acids on postprandial lipemia and incretin responses in healthy subjects. Am J Clin Nutr 69:1135–1143

Tsujihata Y, Ito R, Suzuki M, Harada A, Negoro N, Yasuma T, Momose Y, Takeuchi K (2011) TAK-875, an orally available G protein-coupled receptor 40/free fatty acid receptor 1 agonist, enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia in type 2 diabetic rats. J Pharmacol Exp Ther 339:228–237

Tsukahara T, Watanabe K, Watanabe T, Yamagami H, Sogawa M, Tanigawa T, Shiba M, Tominaga K, Fujiwara Y, Maeda K, Hirakawa K, Arakawa T (2015) Tumor necrosis factor α decreases glucagon-like peptide-2 expression by up-regulating G-protein-coupled receptor 120 in Crohn disease. Am J Pathol 185:185–196

Waguri T, Goda T, Kasezawa N, Yamakawa-Kobayashi K (2013) The combined effects of genetic variations in the GPR120 gene and dietary fat intake on obesity risk. Biomed Res 34:69–74

Weaver RE, Donnelly D, Wabitsch M, Grant PJ, Balmforth AJ (2008) Functional expression of glucose-dependent insulinotropic polypeptide receptors is coupled to differentiation in a human adipocyte model. Int J Obes (Lond) 32:1705–1711

Wen J, Phillips SF, Sarr MG, Kost LJ, Holst JJ (1995) PYY and GLP-1 contribute to feedback inhibition from the canine ileum and colon. Am J Physiol 269:G945–G952

Wren JA, Gossellin J, Sunderland SJ (2007) Dirlotapide: a review of its properties and role in the management of obesity in dogs. J Vet Pharmacol Ther 30(Suppl 1):11–16

Xiong Y, Swaminath G, Cao Q, Yang L, Guo Q, Salomonis H, Lu J, Houze JB, Dransfield PJ, Wang Y, Liu JJ, Wong S, Schwandner R, Steger F, Baribault H, Liu L, Coberly S, Miao L, Zhang J, Lin DC, Schwarz M (2013) Activation of FFA1 mediates GLP-1 secretion in mice. Evidence for allosterism at FFA1. Mol Cell Endocrinol 369:119–129

Yen CL, Nelson DW, Yen MI (2015) Intestinal triacylglycerol synthesis in fat absorption and systemic energy metabolism. J Lipid Res 56:489–501

Yip RG, Boylan MO, Kieffer TJ, Wolfe MM (1998) Functional GIP receptors are present on adipocytes. Endocrinology 139:4004–4007

FFA2 and FFA3 in Metabolic Regulation

Cong Tang and Stefan Offermanns

Contents

Abstract

The short-chain fatty acid receptors FFA2 (GPR43) and FFA3 (GPR41) are activated by acetate, propionate, and butyrate. These ligands are produced by bacteria in the gut. In addition, the body itself can in particular produce acetate, and acetate plasma levels have been shown to be increased, e.g., in diabetic patients or during periods of starvation. FFA2 and FFA3 are both expressed by enteroendocrine cells and pancreatic β-cells. In addition, FFA2 is found on

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immune cells and adipocytes, whereas FFA3 is expressed by some peripheral neurons. It has therefore been speculated that short-chain fatty acid receptors are involved in the regulation of various body functions under different nutritional and metabolic conditions. Here we summarize recent data on the role of FFA2 and FFA3 in the regulation of metabolic, immunological, and neuronal functions and discuss the potential pharmacological relevance of this receptor system.

Keywords
Acetate • Butyrate • Diabetes • Propionate • Short-chain fatty acids

1 Introduction

Plasma concentrations of metabolic intermediates such as free fatty acids largely depend on their availability from nutritional sources and on the metabolic state of the organisms. Their levels can vary under different physiological and pathological conditions. The body does not only use these metabolites as an energy source but takes also advantage of their changing levels to control body functions. Many of these metabolites can bind to particular G-protein-coupled receptors (GPCRs) to regulate a range of cellular processes (Blad et al. 2012; Peti-Peterdi et al. 2015; Stoddart et al. 2008). In the last decade, it has become clear that many of these metabolite GPCRs play important roles in the regulation of metabolic and other systems such as the immune system.

Short-chain fatty acids (SCFAs) have a carbon chain length of two to six, such as acetate (C2), propionate (C3), and butyrate (C4). They are produced in large quantities in the intestine by anaerobic bacterial fermentation of dietary fibers (Flint et al. 2008). In addition, body cells can generate in particular acetate via conversion from acetyl-coA or acetaldehyde (Shimazu et al. 2010). In addition to their roles as important substrates for cellular energy metabolism, work of the last decade has shown that they are also signaling molecules which can activate two structurally related receptors, free fatty acid receptor 2 (FFA2) and free fatty acid receptor 3 (FFA3) (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003). FFA2 and FFA3 have been implicated in the regulation of various processes, including immune cell function and control of lipid and energy metabolism. Based on some of these functions, FFA2 and FFA3 have been considered as potential drug targets for treatment of metabolic and inflammatory disorders. This chapter aims to review the latest insights into the role of FFA2 and FFA3 in metabolic regulation, with a particular focus on physiological and pathophysiological functions, pharmacological tools, and a therapeutic perspective.

2 Basic Properties of FFA2 and FFA3

Several GPCRs that are activated by free fatty acids with different carbon chain lengths have been identified. Long-chain fatty acids (LCFAs) with carbon chain lengths of C8–C22 activate free fatty acid receptor 1 (FFA1, also known as GPR40) and free fatty acid receptor 4 (FFA4, also known as GPR120), while short-chain fatty acids (SCFAs), including acetate (C2), propionate (C3), and butyrate (C4), activate two structurally related receptors, free fatty acid receptor 2 (FFA2, also known as GPR43) and free fatty acid receptor 3 (FFA3, also known as GPR41) (Briscoe et al. 2003; Brown et al. 2003; Oh et al. 2010). FFA2 and FFA3 were identified in 2003 by three independent groups (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003) and are evolutionally conserved across several mammalian species, having approximately 40% amino acid sequence identity (Kuwahara 2014). The genes encoding FFA2 and FFA3 are located in tandem on chromosome 7 in the mouse and on chromosome 19 in humans. In primates but not in other mammals, a tandem segmental duplication of the genomic region containing the open reading frame of FFA3 is present. The duplicated FFA3 gene was termed GPR42 and originally believed to be a pseudogene (Brown et al. 2003). Subsequent analyses revealed that both, FFA3 and GPR42, are highly polymorphic and that in the majority of cases, GPR42 is not only transcribed but also very likely to produce a functional protein with properties very similar to FFA3 (Liaw and Connolly 2009; Puhl et al. 2015).

FFA2 and FFA3 have different affinities for SCFAs, human FFA2 (hFFA2) binds acetate, propionate, and butyrate, while hFFA3 prefers propionate and butyrate (Brown et al. 2003; Hudson et al. 2012; Le Poul et al. 2003; Nilsson et al. 2003). In mice, acetate activates both FFA2 (mFFA2) and FFA3 (mFFA3) with similar potency, whereas propionate is more potent on mFFA3 than mFFA2 (Hudson et al. 2012). In addition, the endogenously produced ketone body β-hydroxybutyrate was initially reported to be an FFA3 antagonist (Kimura et al. 2011), whereas a later report showed that β-hydroxybutyrate is rather an FFA3 agonist (Won et al. 2013). FFA2 and FFA3 couple to G_i-type G-proteins (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003). Agonists of FFA2 can also induce G_q/G_{11}-mediated signaling, albeit with lower potency than G_i-dependent signaling (Le Poul et al. 2003; Lee et al. 2008). The pharmacological properties of FFA2 and FFA3 are summarized in Table 1.

3 Expression of FFA2 and FFA3

FFA2 and FFA3 have not only a similar spectrum of endogenous ligands; they also share some but not all of their expression sites. Both FFA2 and FFA3 are expressed by intestinal enteroendocrine cells, in particular L cells that secrete glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) in response to glucose (Engelstoft et al. 2013b; Karaki et al. 2006; Nohr et al. 2013; Tolhurst et al. 2012), and other

Table 1 Pharmacological properties of human and mouse FFA2 and FFA3

Receptor	Aliases	Endogenous ligands	pEC_{50} or pIC_{50}	References
hFFA2	hGPR43, hFFAR2	Acetate	4.5[a], 4.0[b], 3.4[c]	Brown et al. (2003), Hudson et al. (2012), Le Poul et al. (2003), and Nilsson et al. (2003)
		Propionate	3.5[c]	
		Butyrate	3.4[c]	
hFFA3	hGPR41, hFFAR3	Acetate	3.0[a], 3.0[b], 3.0[c]	
		Propionate	3.9[c]	
		Butyrate	3.8[c]	
mFFA2	mGPR43, mFFAR2	Acetate	5.0[b], 3.6[c]	Hudson et al. (2012) and Priyadarshini et al. (2015)
		Propionate	3.7[c]	
		Butyrate	3.5[c]	
mFFA3	mGPR41, mFFAR3	Acetate	3.7[c]	
		Propionate	4.9[c]	
		Butyrate	3.7[c]	

$pEC_{50} = -\log_{10}[EC_{50}]$; $pIC_{50} = -\log_{10}[IC_{50}]$, for cAMP assay
[a]cAMP assay
[b]Ca^{2+} assay
[c]$[^{35}S]GTP\gamma S$ binding assay

types of enteroendocrine cells, for instance, cholecystokinin (CCK) producing I cells and glucose-dependent insulinotropic peptide (GIP) producing K cells, seem to express FFA3 but not FFA2 (Nohr et al. 2013). Both receptors are also expressed in α- and β-cells of pancreatic islets (Bahar Halpern et al. 2012; Regard et al. 2008; Tang et al. 2015). A range of immune cells such as neutrophils, monocytes, eosinophils, and regulatory T cells (Treg cells) express FFA2 but not FFA3 (Brown et al. 2003; Le Poul et al. 2003; Maslowski et al. 2009; Smith et al. 2013; Trompette et al. 2014), whereas dendritic cells appear to express both FFA2 and FFA3 (Trompette et al. 2014). While FFA2 has been shown to be expressed by adipocytes of human and mouse white adipose tissue (WAT) (Brown et al. 2003; Le Poul et al. 2003; Maslowski et al. 2009), FFA3 expression in adipocytes is controversial. Initial studies showed FFA3 mRNA expression in human and rodent WAT; however, this was not confirmed by various other reports (Brown et al. 2003; Hong et al. 2005; Le Poul et al. 2003; Xiong et al. 2004). Nevertheless, FFA3 expression in adipocytes can be induced in FFA2-deficient mice fed normal chow as well as high-fat diet (Bjursell et al. 2011), suggesting that FFA3 may, at least to some degree, compensate the loss of FFA2 function in adipose tissue under various nutritional conditions. In contrast to the predominant expression of FFA2 in adipocytes and immune cells, FFA3, but not FFA2, is found on postganglionic sympathetic, enteric, and sensory neurons of the autonomic, enteric, and somatic peripheral nervous system (De Vadder et al. 2014; Kimura et al. 2011; Nohr et al. 2013, 2015). More recently, both FFA2 and FFA3 were also reported to be expressed by gastric brush cells in rodents (Eberle et al. 2014). The expression profiles of FFA2 and FFA3 are summarized in Table 2.

Table 2 Expression profiles of FFA2 and FFA3 in mammalian cells

Receptor	Enteroendocrine cells	Pancreatic islets	Immune system	Adipose tissue	Nervous system	Others	References
FFA2	L cells	α-cells, β-cells	Neutrophils, eosinophils, monocytes, dendritic cells, Treg cells	White adipocytes	–	Gastric brush cells	Bahar Halpern et al. (2012), Eberle et al. (2014), Engelstoft et al. (2013b), Karaki et al. (2006), Nohr et al. (2013), Regard et al. (2008), Smith et al. (2013), Tang et al. (2015), Tolhurst et al. (2012), and Trompette et al. (2014)
FFA3	L cells, K cells, I cells,	α-cells, β-cells	Dendritic cells	Not expressed, but expressed in the context of FFA2 absence	Sympathetic ganglia, enteric neurons	Gastric brush cells	Bahar Halpern et al. (2012), De Vadder et al. (2014), Eberle et al. (2014), Engelstoft et al. (2013b), Karaki et al. (2006), Kimura et al. (2011), Nohr et al. (2013, 2015), Regard et al. (2008), Tang et al. (2015), Tolhurst et al. (2012), and Trompette et al. (2014)

4 Physiological and Pathophysiological Functions of FFA2 and FFA3

4.1 Immune Cells

Multiple evidence indicates that fiber-rich diet has beneficial effects by reducing the risk of inflammatory bowel disease and metabolic disorders (Anderson et al. 2009; Campbell 2001; Mudgil and Barak 2013). Since high-fiber diet results in elevated plasma SCFA levels (Nilsson et al. 2010; Tarini and Wolever 2010), soon after their discovery, SCFA receptors FFA2 and FFA3 have been proposed to be involved in the immunological and metabolic benefits of fiber-rich diet (Cox et al. 2009). The first evidence for an involvement of FFA2 in the regulation of inflammation was shown in 2009, when Maslowski et al. reported that FFA2-deficient mice showed exacerbated inflammation in models of inflammatory colitis, arthritis, and allergic airway asthma and that this abnormality could be attributed to increased activation and recruitment of immune cells particularly neutrophils (Maslowski et al. 2009). FFA2 may thereby provide a link between diet, gut microbiota, and host immune homeostasis (Maslowski et al. 2009). Later, the same group found that the protective effects of SCFAs in dextran sodium sulfate (DSS)-induced colitis involve not only FFA2 but also HCA2 (GPR109A), a receptor activated by high concentrations of butyrate (Macia et al. 2015). The effect was reported to require both receptors on non-hematopoietic cells, probably epithelial cells, as well as the activation of NLRP3 inflammasome, a process which is important for the maintenance of intestinal epithelial integrity (Macia et al. 2015). The anti-inflammatory role of FFA2 in the mouse colitis model was confirmed by other studies (Masui et al. 2013; Smith et al. 2013). Another new link between SCFAs and anti-inflammatory mechanisms in the gut was provided by experimental evidence indicating that FFA2 is also expressed by intestinal Treg cells which suppress the response of inflammation-promoting immune cells. FFA2 in fact mediates the expansion and enhanced activity of Tregs induced by SCFAs, which leads to ameliorated colitis (Smith et al. 2013). Interestingly, another study demonstrated that mice fed a high-fiber diet were also protected against allergic inflammation in the lung, but a novel mechanism was suggested. In that case, SCFAs seemed to enhance the generation of dendritic cells (DC) with impaired immune response and the subsequent homing of this kind of DC in the lung via activation of FFA3 but not FFA2 (Trompette et al. 2014). While these studies suggest that both FFA2 and FFA3 function as anti-inflammatory receptors via different mechanisms, other studies have suggested a rather pro-inflammatory role. In models of acute and chronic colitis, DSS-treated mice lacking FFA2 showed reduced accumulation of intestinal polymorphonuclear leukocytes (PMN), decreased colonic inflammation, and attenuated colonic tissue damage (Sina et al. 2009). This is consistent with studies showing that FFA2 can mediate SCFA-induced mouse neutrophil chemotaxis (Vinolo et al. 2011) and that an FFA2 antagonist, GLPG0974, inhibited SCFA-induced migration and activation of human neutrophils in vitro (Vermeire et al. 2015). The latter report, however, also showed that a 4-week treatment of patients with inflammatory bowel disease

had no effect when compared to placebo (Vermeire et al. 2015). Another example for a pro-inflammatory pathway mediated by FFA2 came from studies in an acute mouse model of gout in which intra-articular injection of urate crystals causes arthritis. Interestingly, the response was greatly attenuated in germfree mice and in mice lacking FFA2 (Vieira et al. 2015). Recolonization or treatment with acetate restored the responsiveness to urate crystals in germfree mice (Vieira et al. 2015). In vitro, urate crystals induced the production of reactive oxygen species in macrophages and the assembly of the inflammasome, an effect which was greatly reduced in FFA2-deficient macrophages. It is currently unclear why different phenotypes of FFA2-deficient mice were observed in different models of inflammation. Mouse strains with different background may have contributed to this as mice on standard C57Bl/6 (Maslowski et al. 2009) and mixed C57Bl6/129 backgrounds were studied (Sina et al. 2009). Also, different inflammatory models were employed in these studies (Macia et al. 2015; Maslowski et al. 2009; Sina et al. 2009; Trompette et al. 2014; Vieira et al. 2015). Some of the data may also simply be due to the fact that FFA2 activation in different contexts can promote or prevent inflammation. In the future, more studies using specific FFA2/FFA3 agonists and antagonists are required to better understand the function of SCFA receptors in inflammation and to explore the potential of FFA2/FFA3 synthetic ligands in different inflammation conditions.

4.2 Enteroendocrine Cells

Enteroendocrine cells are specialized endocrine cells present in the epithelium of the gastrointestinal tract. They are critical for energy homeostasis as they are able to produce and release various hormones in response to different nutrients (Engelstoft et al. 2013a; Gribble and Reimann 2016). For example, upon stimulation by elevated glucose levels in the gut lumen after a meal, L cells that are distributed in the colon and small intestine can secrete GLP-1 to help control blood glucose level and reduce appetite; in contrast, ghrelin, produced by A cells of the stomach and released when the stomach is empty, induces feelings of hunger and prepares the body for food intake (Furness et al. 2013). Tolhurst et al. reported that enteroendocrine cells expressing FFA2, but not FFA3, mediates SCFA-induced secretion of gut hormone GLP-1 and that loss of FFA2 or FFA3 leads to reduced plasma levels of active GLP-1. Since GLP-1 is an incretin that promotes insulin secretion from pancreatic β-cells, this in turn results in diminished insulin secretion and impaired glucose tolerance in the FFA2- or FFA3-deficient mice (Tolhurst et al. 2012). Another report showed that butyrate induces GLP-1 secretion, an effect believed to be dependent on FFA3, but not FFA2. Interestingly, the authors could not confirm the metabolic phenotype described by Tolhurst et al., and no difference in glucose tolerance and insulin resistance was observed between wild-type and FFA3-deficient mice (Lin et al. 2012). Another study reported no effect of SCFAs on glucose-stimulated GLP-1 secretion in vivo (Tang et al. 2015), but observed that FFA2 and FFA3 mediated SCFA-inhibited secretion of glucose-dependent

insulinotropic peptide (GIP) from K cells (Tang et al. 2015), indicating that FFA2 and/or FFA3 predominantly couple to G_i-type G-proteins to exert an inhibitory effect on GIP secretion in K cells. The physiological relevance of this effect remains unclear since loss of FFA2 and FFA3 expression in enteroendocrine cells had no effect on glucose-stimulated insulin secretion in vivo (Tang et al. 2015). Recently, Park et al. reported that two selective inverse agonists of human FFA2 increase intracellular cAMP level in an endocrine cell line NCI-H716, thus resulting in an enhanced GLP-1 secretion, this provides evidence that inhibition of FFA2 can stimulate GLP-1 release (Park et al. 2015). Thus, whether FFA2 and FFA3 are associated with SCFA-stimulated incretin secretion and subsequent systemic effects including insulin secretion and how relevant this is in vivo is still unclear. Given that SCFA concentrations in the gut particularly in the colon are in the range of 100 mM (Nohr et al. 2013), which is several orders of magnitude higher than the EC_{50} of SCFAs at FFA2 and FFA3, it is also unclear how FFA2 and FFA3 expressed in enteroendocrine cells can sense SCFAs in the intestinal lumen as the receptors are expected to be constitutively activated. It is currently not known whether FFA2/FFA3 are localized on the apical, basolateral, or both membranes of enteroendocrine cells (Table 3).

4.3 Adipose Tissue and Body Weight Control

There is some evidence that FFA2 and FFA3 play a role in the control of adipocyte function and body weight. Under in vitro conditions, FFA2 and FFA3 mediate SCFA-induced adipocyte secretion of leptin (Zaibi et al. 2010), a hormone that

Table 3 Cellular effects mediated by FFA2 and FFA3

	FFA2	FFA3
Immune cells	Neutrophil chemotaxis and recruitment (Maslowski et al. 2009; Sina et al. 2009; Vinolo et al. 2011); regulation of colonic Treg cell homeostasis (Smith et al. 2013)	Dendritic cell production in the lung ↑ (Trompette et al. 2014)
Enteroendocrine cells	GLP-1 and PYY secretion ↑ (Samuel et al. 2008; Tolhurst et al. 2012)	Secretion of intestinal hormones ↑ (Lin et al. 2012)
Adipose tissue	Leptin secretion ↑ (Zaibi et al. 2010); lipolysis ↓ (Ge et al. 2008); inhibition of fat accumulation by suppressing insulin signaling (Kimura et al. 2013)	Leptin secretion ↑ (Xiong et al. 2004)
Pancreatic islets	Can mediate stimulation or inhibition of insulin secretion (McNelis et al. 2015; Priyadarshini et al. 2015; Tang et al. 2015)	Insulin secretion ↓ (Priyadarshini and Layden 2015; Tang et al. 2015)
Nervous system		Sympathetic tone and noradrenaline release ↑ (Inoue et al. 2012; Kimura et al. 2011)

inhibits food intake and helps control body weight. The in vivo relevance of this observation is, however, not clear. It was originally reported that FFA3-deficient mice developed a leaner phenotype, which was attributed to the decreased secretion of PYY, a gut hormone that inhibits gastric motility and increases extraction of energy substrates (Manning and Batterham 2014). No difference in body weight was observed when wild-type mice and FFA3-deficient mice were kept under germfree conditions, suggesting that SCFAs derived from fiber metabolism by gut microbiota may activate FFA3 to modulate PYY secretion and thus control body weight development (Samuel et al. 2008). In contrast to this study, it was later reported that FFA3-deficient male mice have increased adiposity accompanied by increased plasma levels of leptin (Bellahcene et al. 2012). Finally, Lin et al. described an FFA3-independent protection against diet-induced obesity and insulin resistance by all types of SCFA (Lin et al. 2012). Similar contradictory results were found in studies carried out on mice lacking FFA2. Bjursell et al. described that FFA2-deficient mice fed high-fat diet (HFD), but not normal chow diet (NC), were somewhat protected against development of obesity compared to wild-type mice and that mice lacking FFA2 have improved glucose tolerance and increased energy expenditure (Bjursell et al. 2011). In contrast, Kimura et al. reported that FFA2-deficient mice have significantly increased body weight accompanied by glucose intolerance and increased inflammatory macrophage infiltration of adipose tissue when kept on normal chow or high-fat diet (Kimura et al. 2013). They also provided evidence that mice overexpressing FFA2 were leaner and were more glucose tolerant compared to WT mice (Kimura et al. 2013). It is worth noting that, again, knockout mice based on different targeting strategies and kept on different genetic backgrounds were used in these studies (Bjursell et al. 2011; Kimura et al. 2013).

4.4 Pancreatic β-Cells

Since both FFA2 and FFA3 are expressed in pancreatic β-cells (Bahar Halpern et al. 2012; Regard et al. 2008; Tang et al. 2015), the role of FFA2 and FFA3 in the regulation of β-cell function in the pancreas has been analyzed. It has been known that propionate, an endogenous ligand for FFA2 and FFA3 with a slight selectivity toward FFA3, inhibits glucose-stimulated insulin secretion (GSIS) in isolated rat pancreatic islets (Ximenes et al. 2007). Recently, Priyadarshini et al. provided evidence that FFA3 mediates propionate inhibited GSIS in isolated mouse islets by using a FFA3 knockout mouse (Priyadarshini and Layden 2015). This is consistent with the classic concept that activation of G_i-coupled GPCRs leads to an inhibition of insulin secretion (Regard et al. 2007). Due to the fact that the plasma level of propionate is below 10 μM (Pouteau et al. 2001), which is at least one order of magnitude lower than its EC_{50} at FFA3, it is unclear whether FFA3 is able to sense blood propionate under in vivo conditions to regulate insulin secretion. In fact, two independent studies could not observe any difference in insulin secretion and glucose homeostasis between WT and FFA3-deficient mice (Lin

et al. 2012; Tang et al. 2015), suggesting that FFA3 alone does not significantly contribute to the regulation of insulin secretion. It is known that the plasma concentration of acetate, another ligand of FFA2 and FFA3, can reach levels as high as 300 μM (Pouteau et al. 2001). This makes it a reasonable ligand for FFA2 and FFA3 expressed by pancreatic β-cells in vivo. It has been described that acetate inhibits both first phase and second phase insulin secretion induced by glucose or arginine in the perfused rat pancreas (Tiengo et al. 1981). This was confirmed in a study showing that acetate inhibits GSIS from mouse pancreatic islets via activation of both FFA2 and FFA3 (Tang et al. 2015). This indicates that both FFA2 and FFA3 preferably couple to G_i protein to exert an inhibition on insulin secretion, though acetate binding to FFA2 also activates $G_{q/11}$-mediated signaling; however, the potency is considerably lower than for FFA2-mediated G_i signaling (Le Poul et al. 2003). Interestingly, McNelis et al. reported that activation of FFA2 by a synthetic agonist but not by acetate leads to increased insulin secretion in murine insulinoma cells, but not in rat insulinoma cells (McNelis et al. 2015), suggesting that activation of FFA2 can induce different G-protein-mediated signaling pathways in different cell lines depending on the agonist. Similar observations were described in another study, which showed that FFA2 is able to signal by different pathways depending on the applied ligand to induce stimulation or inhibition of insulin secretion (Priyadarshini et al. 2015). Notably, McNelis et al. described major metabolic defects in FFA2-deficient mice fed HFD, including impaired glucose tolerance and diminished insulin secretion; this, however, was not observed in other studies (Bjursell et al. 2011; McNelis et al. 2015; Priyadarshini et al. 2015; Tang et al. 2015). Again, genetically modified mouse lines generated via different strategies and bred onto different genetic backgrounds were used in these studies, which may contribute to the differences in the observed phenotypes. Given that FFA2 and FFA3 share the same endogenous ligands and are co-expressed by pancreatic β-cells, one receptor may be able to, at least partially, substitute the loss of the other receptor's function in FFA2 or FFA3 single receptor-deficient mice. Results from analysis of mice with single FFA2 or FFA3 deficiency may therefore be difficult to interpret. A study on mice with global and β-cell-specific loss of both FFA2 and FFA3 indicated that both receptors mediate an inhibition of insulin secretion in obese mice, but not in lean mice (Tang et al. 2015). In this study, evidence was provided that some of the glucose taken up by β-cells is converted to acetate which is then released and functions in an autocrine or paracrine fashion to reduce GSIS. As acetate formation by β-cells increases with increasing glucose concentrations, this mechanism may function as a negative feedback control of insulin release to avoid overshooting insulin secretion. It also explains why FFA2- and FFA3-mediated inhibition of GSIS in vivo is more prominent under diabetic conditions when glucose levels are increased (Tang et al. 2015). This study indicates that antagonists of FFA2 and FFA3 are useful to improve insulin secretion in type 2 diabetes. However, more studies are needed to better understand the pharmacological potential of these receptors, as in particular FFA2 when activated by appropriate biased ligands can also augment GSIS.

4.5 Nervous System

Neither FFA2 nor FFA3 appear to be expressed in the central nervous system (Nohr et al. 2015). However, FFA3 expression has been found in various parts of the peripheral nervous system, including sympathetic ganglia, the enteric nervous system, and sensory neurons. This suggests that SCFAs can modulate activity in various parts of the peripheral nervous system. Loss of FFA3 in mice resulted in a decreased heart rate, which was attributed to reduced norepinephrine release by sympathetic neurons (Kimura et al. 2011). Later the same group reported that this effect is due to the ability of FFA3 to stimulate norepinephrine release from sympathetic nerve terminals via a $G_{\beta\gamma}$-phospholipase C (PLC)-β3-ERK1/2-synapsin 2 signaling pathway (Inoue et al. 2012). However, another study reported that in rat sympathetic neurons, activation of FFA3 inhibits N-type Ca^{2+} channels through $G_{\beta\gamma}$, an effect which would rather decrease neuronal catecholamine release (Won et al. 2013). More recently, evidence was provided that FFA3 is present in portal nerves and mediates propionate-induced gluconeogenesis through a gut-brain neural circuit (De Vadder et al. 2014). In this study, it was shown that propionate induced in the jejunum of rats an increase in glucose-6-phosphatase activity as an indicator of gluconeogenesis which was blocked by the putative FFA3 antagonist β-hydroxybutyrate. However, no direct in vivo evidence was provided to prove the concept that FFA3 is critical for SCFA-induced gluconeogenesis. Nevertheless, the study still suggests a possible role of FFA3 in linking the nervous system and metabolic system.

5 Pharmacological Potential of FFA2 and FFA3

Several synthetic ligands of FFA2 and FFA3 with agonistic and antagonistic properties have been developed (see Chapter 2 by Milligan et al.). In vivo studies so far mostly performed with mice genetically engineered to lack FFA2 and/or FFA3 have not been fully conclusive with regard to the therapeutic potential of both receptors. There are, for instance, several reports based on often different genetic mouse lines, which suggest in part contradictory roles of short-chain fatty acid receptors in inflammatory processes, body weight regulation, and regulation of insulin secretion from β-cells (see above). There are several reasons explaining these discrepancies. They may be due to different genetic backgrounds used in mouse genetics studies. There is also evidence that different targeting strategies to delete one of the two genes encoding FFA2 and FFA3, which are located next to each other on chromosome 7 in mice, can affect the expression of the other gene (Bjursell et al. 2011). Since both receptors have overlapping expression patterns and endogenous ligands, a constitutive single knockout of one receptor may, to different degrees, be compensated by the other receptor. Finally, since most studies are done with non-induced constitutive knockouts, there may also be different more complex and indirect compensatory mechanisms in play. To avoid these pitfalls, it

would be advisable that genetic studies in the future rely more on inducible systems as well as on single and FFA2/FFA3 double-deficient models. Despite the problems in the interpretation of data resulting from genetic mouse models, there is some evidence for a pharmacological potential of FFA2 and FFA3. For instance, an increasing number of data suggest that, depending on the context, FFA2 can have pro- and anti-inflammatory properties. However, no beneficial effect has so far been shown in humans. Similarly, there is clear data that FFA2 and FFA3 can regulate insulin secretion from β-cells with FFA3 being a secretion-inhibiting receptor, whereas FFA2 may have, depending on the ligand, both secretion-inhibiting and secretion-stimulating effects. The FFA2 allosteric agonist AMG-7703, for example, increases insulin secretion from mouse and human islets (McNelis et al. 2015), whereas other studies described an inhibitory effect mediated by the endogenous ligand acetate on insulin secretion (Tang et al. 2015). Future studies with agonists, antagonists, as well as allosteric modulators with high selectivity or selectivity for both, FFA2 and FFA3, and with increased potency will be required to better understand the function of FFA2 and FFA3 in regulation of various pathological conditions such as obesity, type 2 diabetes, dyslipidemia, and inflammatory diseases in vivo.

6 Conclusions

The largely overlapping expression profiles of FFA2 and FFA3 and their somewhat shared ligand spectrum suggest that the functions of both receptors to some degree overlap. Studies in mice lacking either FFA2 or FFA3 and both, FFA2 and FFA3, have in some cases yielded controversial results. In addition, the relatively low potency of endogenous ligands also limits elucidating the function of FFA2 and FFA3, particularly under in vivo conditions. Thus, it is still not clear, whether FFA2 and FFA3 would be promising drug targets for the treatment of inflammatory and metabolic diseases. Nevertheless, there is now some evidence that immune cell functions and the release of insulin from pancreatic β-cells can be regulated through short-chain fatty acid receptors. However, the exact involvement of each of the receptors and the involved downstream signaling pathways are not yet fully elucidated. Another question which is still open pertains to GPR42, a highly polymorphic third short-chain fatty acid receptor found only in primates. To better understand the physiological and pathophysiological role of these receptors, and to further test their relevance as therapeutic targets, more and better synthetic ligands with mono- and bispecific properties, which allow for in vivo analysis, are required.

References

Anderson JW, Baird P, Davis RH Jr, Ferreri S, Knudtson M, Koraym A, Waters V, Williams CL (2009) Health benefits of dietary fiber. Nutr Rev 67:188–205

Bahar Halpern K, Veprik A, Rubins N, Naaman O, Walker MD (2012) GPR41 gene expression is mediated by internal ribosome entry site (IRES)-dependent translation of bicistronic mRNA encoding GPR40 and GPR41 proteins. J Biol Chem 287:20154–20163

Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, Smith DM, Cawthorne MA, Stocker CJ, Arch JRS (2012) Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. Br J Nutr 109:1755–1764

Bjursell M, Admyre T, Goransson M, Marley AE, Smith DM, Oscarsson J, Bohlooly YM (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. Am J Physiol Endocrinol Metab 300:E211–E220

Blad CC, Tang C, Offermanns S (2012) G protein-coupled receptors for energy metabolites as new therapeutic targets. Nat Rev Drug Discov 11:603–619

Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT, Murdock PR, Sauls HR Jr, Shabon U, Spinage LD, Strum JC, Szekeres PG, Tan KB, Way JM, Ignar DM, Wilson S et al (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278:11303–11311

Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, Muir AI, Wigglesworth MJ, Kinghorn I, Fraser NJ, Pike NB, Strum JC, Steplewski KM, Murdock PR, Holder JC, Marshall FH, Szekeres PG, Wilson S, Ignar DM, Foord SM et al (2003) The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278:11312–11319

Campbell AP (2001) Health benefits of dietary fiber for people with diabetes. Diabetes Educ 27:511–514

Cox MA, Jackson J, Stanton M, Rojas-Triana A, Bober L, Laverty M, Yang X, Zhu F, Liu J, Wang S, Monsma F, Vassileva G, Maguire M, Gustafson E, Bayne M, Chou CC, Lundell D, Jenh CH (2009) Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. World J Gastroenterol 15:5549–5557

De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Backhed F, Mithieux G (2014) Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell 156:84–96

Eberle JA, Widmayer P, Breer H (2014) Receptors for short-chain fatty acids in brush cells at the "gastric groove". Front Physiol 5:152

Engelstoft MS, Egerod KL, Lund ML, Schwartz TW (2013a) Enteroendocrine cell types revisited. Curr Opin Pharmacol 13:912–921

Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM et al (2013b) Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2:376–392

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 6:121–131

Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B (2013) The gut as a sensory organ. Nat Rev Gastroenterol Hepatol 10:729–740

Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, Tian H, Li Y (2008) Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 149:4519–4526

Gribble FM, Reimann F (2016) Enteroendocrine cells: chemosensors in the intestinal epithelium. Annu Rev Physiol 78:277–299

Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, Choi KC, Feng DD, Chen C, Lee HG, Katoh K, Roh SG, Sasaki S (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinology 146:5092–5099

Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G (2012) Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. J Biol Chem 287:41195–41209

Inoue D, Kimura I, Wakabayashi M, Tsumoto H, Ozawa K, Hara T, Takei Y, Hirasawa A, Ishihama Y, Tsujimoto G (2012) Short-chain fatty acid receptor GPR41-mediated activation of sympathetic neurons involves synapsin 2b phosphorylation. FEBS Lett 586:1547–1554

Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, Kuwahara A (2006) Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 324:353–360

Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyauchi S, Kobayashi M, Hirasawa A, Tsujimoto G (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc Natl Acad Sci U S A 108:8030–8035

Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, Terasawa K, Kashihara D, Hirano K, Tani T, Takahashi T, Miyauchi S, Shioi G, Inoue H, Tsujimoto G (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829

Kuwahara A (2014) Contributions of colonic short-chain Fatty Acid receptors in energy homeostasis. Front Endocrinol 5:144

Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278:25481–25489

Lee T, Schwandner R, Swaminath G, Weiszmann J, Cardozo M, Greenberg J, Jaeckel P, Ge HF, Wang YC, Jiao XY, Liu J, Kayser F, Tian H, Li Y (2008) Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2. Mol Pharmacol 74:1599–1609

Liaw CW, Connolly DT (2009) Sequence polymorphisms provide a common consensus sequence for GPR41 and GPR42. DNA Cell Biol 28:555–560

Lin HV, Frassetto A, Kowalik EJ Jr, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G, Marsh DJ (2012) Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One 7, e35240

Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA et al (2015) Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun 6:6734

Manning S, Batterham RL (2014) The role of gut hormone peptide YY in energy and glucose homeostasis: twelve years on. Annu Rev Physiol 76:585–608

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461:1282–1286

Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, Izawa S, Kondo Y, Ito Y, Tamura Y, Yanamoto K, Noda H, Tanabe A, Okaniwa N, Yamaguchi Y, Iwamoto T, Kasugai K (2013) G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflamm Bowel Dis 19:2848–2856

McNelis JC, Lee YS, Mayoral R, van der Kant R, Johnson AM, Wollam J, Olefsky JM (2015) GPR43 potentiates beta-cell function in obesity. Diabetes 64:3203–3217

Mudgil D, Barak S (2013) Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: a review. Int J Biol Macromol 61:1–6

Nilsson NE, Kotarsky K, Owman C, Olde B (2003) Identification of a free fatty acid receptor, FFA (2)R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 303:1047–1052

Nilsson AC, Ostman EM, Knudsen KE, Holst JJ, Bjorck IM (2010) A cereal-based evening meal rich in indigestible carbohydrates increases plasma butyrate the next morning. J Nutr 140:1932–1936

Nohr MK, Pedersen MH, Gille A, Egerod KL, Engelstoft MS, Husted AS, Sichlau RM, Grunddal KV, Poulsen SS, Han S, Jones RM, Offermanns S, Schwartz TW (2013) GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinology 154:3552–3564

Nohr MK, Egerod KL, Christiansen SH, Gille A, Offermanns S, Schwartz TW, Moller M (2015) Expression of the short chain fatty acid receptor GPR41/FFAR3 in autonomic and somatic sensory ganglia. Neuroscience 290:126–137

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142:687–698

Park BO, Kim SH, Kong GY, Kim DH, Kwon MS, Lee SU, Kim MO, Cho S, Lee S, Lee HJ, Han SB, Kwak YS, Lee SB, Kim S (2015) Selective novel inverse agonists for human GPR43 augment GLP-1 secretion. Eur J Pharmacol 771:1–9

Peti-Peterdi J, Kishore BK, Pluznick JL (2015) Regulation of vascular and renal function by metabolite receptors. Annu Rev Physiol 78:391–414

Pouteau E, Meirim I, Metairon S, Fay LB (2001) Acetate, propionate and butyrate in plasma: determination of the concentration and isotopic enrichment by gas chromatography/mass spectrometry with positive chemical ionization. J Mass Spectrom 36:798–805

Priyadarshini M, Layden BT (2015) FFAR3 modulates insulin secretion and global gene expression in mouse islets. Islets: e1045182

Priyadarshini M, Villa SR, Fuller M, Wicksteed B, Mackay CR, Alquier T, Poitout V, Mancebo H, Mirmira RG, Gilchrist A, Layden BT (2015) An acetate-specific GPCR, FFAR2, regulates insulin secretion. Mol Endocrinol 29:1055–1066

Puhl HL 3rd, Won YJ, Lu VB, Ikeda SR (2015) Human GPR42 is a transcribed multisite variant that exhibits copy number polymorphism and is functional when heterologously expressed. Sci Rep 5:12880

Regard JB, Kataoka H, Cano DA, Camerer E, Yin L, Zheng YW, Scanlan TS, Hebrok M, Coughlin SR (2007) Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. J Clin Invest 117:4034–4043

Regard JB, Sato IT, Coughlin SR (2008) Anatomical profiling of G protein-coupled receptor expression. Cell 135:561–571

Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI (2008) Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A 105:16767–16772

Shimazu T, Hirschey MD, Huang JY, Ho LT, Verdin E (2010) Acetate metabolism and aging: an emerging connection. Mech Ageing Dev 131:511–516

Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, Raabe B, Chalaris A, Scheller J, Rehmann A, Franke A, Ott S, Hasler R, Nikolaus S, Folsch UR, Rose-John S, Jiang HP, Li J, Schreiber S, Rosenstiel P (2009) G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 183:7514–7522

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341:569–573

Stoddart LA, Smith NJ, Milligan G (2008) International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1,-2, and-3: pharmacology and pathophysiological functions. Pharmacol Rev 60:405–417

Tang C, Ahmed K, Gille A, Lu S, Grone HJ, Tunaru S, Offermanns S (2015) Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nat Med 21:173–177

Tarini J, Wolever TM (2010) The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. Appl Physiol Nutr Metab 35:9–16

Tiengo A, Valerio A, Molinari M, Meneghel A, Lapolla A (1981) Effect of ethanol, acetaldehyde, and acetate on insulin and glucagon secretion in the perfused rat pancreas. Diabetes 30:705–709

Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM (2012) Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 61:364–371

Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, Marsland BJ (2014) Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20:159–166

Vermeire S, Kojecký V, Knoflícek V, Reinisch W, Van Kaem T, Namour F, Beetens J, Vanhoutte F (2015) DOP030. GLPG0974, an FFA2 antagonist, in ulcerative colitis: efficacy and safety in a multicenter proof-of-concept study. J Crohns Colitis 9:S39

Vieira AT, Macia L, Galvao I, Martins FS, Canesso MC, Amaral FA, Garcia CC, Maslowski KM, De Leon E, Shim D, Nicoli JR, Harper JL, Teixeira MM, Mackay CR (2015) A role for gut microbiota and the metabolite-sensing receptor GPR43 in a murine model of gout. Arthritis Rheumatol 67:1646–1656

Vinolo MA, Ferguson GJ, Kulkarni S, Damoulakis G, Anderson K, Bohlooly YM, Stephens L, Hawkins PT, Curi R (2011) SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. PLoS One 6, e21205

Won YJ, Lu VB, Puhl HL 3rd, Ikeda SR (2013) beta-Hydroxybutyrate modulates N-type calcium channels in rat sympathetic neurons by acting as an agonist for the G-protein-coupled receptor FFA3. J Neurosci 33:19314–19325

Ximenes HM, Hirata AE, Rocha MS, Curi R, Carpinelli AR (2007) Propionate inhibits glucose-induced insulin secretion in isolated rat pancreatic islets. Cell Biochem Funct 25:173–178

Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101:1045–1050

Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, Brown AJ, Smith DM, Arch JR (2010) Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett 584:2381–2386

Anti-Inflammatory and Insulin-Sensitizing Effects of Free Fatty Acid Receptors

Junki Miyamoto, Mayu Kasubuchi, Akira Nakajima, and Ikuo Kimura

Contents

Abstract

Chronic low-grade inflammation in macrophages and adipose tissues can promote the development of obesity and type 2 diabetes. Free fatty acids (FFAs) have important roles in various tissues, acting as both essential energy sources and signaling molecules. FFA receptors (FFARs) can modulate inflammation in various types of cells and tissues; however the underlying mechanisms mediating these effects are unclear. FFARs are activated by specific FFAs; for example, GPR40 and GPR120 are activated by medium and long chain FFAs, GPR41 and GPR43 are activated by short chain FFAs, and GPR84 is activated by medium-chain FFAs. To date, a number of studies associated with the physiological functions of G protein-coupled receptors (GPCRs) have reported

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that these GPCRs are expressed in various tissues and involved in inflammatory and metabolic responses. Thus, the development of selective agonists or antagonists for various GPCRs may facilitate the establishment of novel therapies for the treatment of various diseases. In this review, we summarize current literature describing the potential of GPCRs as therapeutic targets for inflammatory and metabolic disorders.

Keywords
Anti-inflammation • GPCRs • Insulin sensitivity

1 Introduction

G protein-coupled receptors (GPCRs) are widely expressed in various tissues and contribute to multiple physiological functions. Free fatty acids (FFAs) can act as ligands for several GPCRs, including GPR40, GPR41, GPR43, GPR84, and GPR120; GPR41 and GPR43 are activated by short chain fatty acids (SCFAs), whereas GPR40 and GPR120 are activated by medium and long chain fatty acids (MCFAs and LCFAs, respectively) (Brown et al. 2003; Briscoe et al. 2003; Itoh et al. 2003; Kotarsky et al. 2003; Le Poul et al. 2003; Nilsson 2003). In addition, GPR84 is activated by MCFAs (Wang et al. 2006) (Table 1).

Obesity is the most common cause of insulin resistance (Facchini et al. 2001; Kahn et al. 2006; Seidell 2000), and chronic low-grade metabolic inflammation is a key component in obesity-induced insulin resistance owing to the infiltration of pro-inflammatory immune cells, such as macrophages, into the adipose tissue and liver (Osborn and Olefsky 2012). The contribution of inflammation to insulin resistance has been widely studied, and immunological changes occurring in various tissues are thought to be etiological factors affecting the development of insulin resistance (Gregor and Hotamisligil 2011; Donath and Shoelson 2011). Donath et al. have reported that among the immune cells present in adipose tissue, macrophages are the most abundant leukocytes and generally contribute to inflammation-mediated insulin resistance (Donath and Shoelson 2011). Various GPCRs are expressed in macrophages and modulate physiological functions such as immunity and metabolism. Importantly, the expression patterns of GPCRs differ in pro-inflammatory and anti-inflammatory macrophages (Lattin et al. 2008); thus, the identification of ligands for each GPCRs is critical for the treatment of diseases.

Recent studies have investigated the beneficial effects of ω3 and ω9 fatty acids (FAs) on obesity-induced insulin resistance. Oliveira et al. reported that α-linolenic (ω3) or oleic (ω9) acids reduce the expression of inflammatory markers and improve insulin resistance in animal models of diet-induced obesity and type 2 diabetes (Oliveira et al. 2015). In addition, ω3 and ω9 FAs exert beneficial effects in the steatotic liver by inducing interleukin (IL)-10, an anti-inflammatory cytokine (Morari et al. 2010). Thus, ω3 and ω9 FAs play a key role in metabolic activities by decreasing pro-inflammatory mediators and increasing IL-10. In this review, we

Table 1 Affinities of free fatty acids for GPR40, GPR41, GPR43, GPR84, and GPR120

	EC_{50} of ligand affinity (μM)				
Ligand	GPR41	GPR43	GPR84	GPR40	GPR120
Fatty acids					
Acetic acid (C2:0)	>1,000	35–431			
Propionic acid (C3:0)	6–127	14–290			
Butyric acid (C4:0)	42–158	28–371			
Valeric acid (C5)	42–142	>1,000			
Caproic acid (C6:0)	102–134			46	
Caprylate (C8)				38	
Nonanoic acid (C9:0)			52.3		
Capric acid (C10:0)			4.5	14–43	
Undecanoic acid (C11:0)			7.7		
Lauric acid (C12:0)			8.8	6–12	
Tridecanoic acid (C13:0)			24.8		
Myristic acid (C14:0)			93.2	8–14	30
Palmitic acid (C16:0)				5–7	52
Stearic acid (C18:0)				17	18
Unsaturated fatty acids					
Palmitoleic acid (C16:1, n-7)				14	0.7–3
Oleic acid (C18:1, n-9)				2–40	31
ω3 fatty acids					
α-Linolenic acid (C18:3)				2–13	0.5
cis-11,14,17-eicosatrienoic acid (C20:3)				11	1
cis-5,8,11,14,17-eicosapentaenoic acid (C20:5, EPA)				2–7	2–3
Docosahexaenoic acid (22:6, DHA)				1–4	4
ω6 fatty acids					
Linoleic acid (C18:2)				2–10	1
γ-Linolenic acid (C18:3)				5–9	1
Dihomo-γ-linolenic acid (C20:4)				7	14
Arachidonic acid (C20:4)				2–12	
Docosatetraenoic acid (C22:4)				13	16

summarize recent progress that GPCRs have potential therapeutic targets for inflammatory and metabolic responses.

2 GPR120-Mediated Anti-Inflammatory Mechanisms

GPR120 is widely expressed in many tissues and cell types, such as the intestine, adipocytes, and immune cells. Various ω3 or ω6 polyunsaturated fatty acids, including docosahexaenoic acid C22:6 (DHA, ω3) and eicosapentaenoic acid C20:5 (EPA, ω3), activate GPR120 at micromolar concentrations. GPR120 is a

Gq-coupled receptor and induces cellular responses via second messenger pathways, such as Ca^{2+} flux and mitogen-activated protein kinase (MAPK) (Gether 2000; Schulte and Fredholm 2003). Intestinal K and L cells express GPR120 and produce incretin hormones, such as glucagon-like peptide (GLP)-1 and gastric inhibitory polypeptide (GIP), respectively (Oh et al. 2010; Parker et al. 2009). Recently, Ichimura et al. reported that GPR120 dysfunction leads to obesity and glucose intolerance in humans and mice (Ichimura et al. 2012). These results indicate that GPR120 has an important role in the regulation of metabolic disorders.

Oh et al. reported that GPR120 acts as an anti-inflammatory receptor in pro-inflammatory macrophages and mature adipocytes. GPR120 signaling activated by DHA and EPA inhibits Toll-like receptor (TLR) signaling and tumor necrosis factor (TNF)-α-induced inflammatory responses. Interestingly, the anti-inflammatory effects of GPR120 are entirely dependent on β-arrestin 2, but not Gq, despite the fact that GPR120 is a Gq-coupled receptor. Although *Gpr120*-deficient and wild-type mice become equally obese and insulin resistant when consuming a high-fat diet, *Gpr120*-deficient mice developed glucose intolerance and hyperinsulinemia compared with wild-type mice on a normal diet. Importantly, supplementation with ω3 FAs significantly increases insulin sensitivity in wild-type mice but not in *Gpr120*-deficient mice. Consistent with these results, treatment with ω3 FAs decreases the infiltration of macrophages into adipose tissue in wild-type mice, but not in *Gpr120*-deficient mice. Thus, GPR120 strongly induces anti-inflammatory effects in macrophages. The activation of GPR120 by ω3 FAs inhibits transforming growth factor β-activated kinase 1 (TAK1) binding protein 1 (TAB1)-mediated activation of TAK1 and provides a mechanism for inhibition of both TLR signaling and TNF-α induced pro-inflammatory pathways. These results strongly support that GPR120-mediated anti-inflammatory effects reduce the infiltration of pro-inflammatory macrophages into the adipose tissue and improve insulin resistance in the context of obesity. Taken together, these studies have shown that GPR120 has an important role in the anti-inflammatory and insulin-sensitizing responses, which may be useful in the development of new therapeutic targets for the treatment of insulin resistance (Oh et al. 2010).

The pharmacology and activities of cpdA, a selective small-molecule agonist for GPR120, have been investigated in various studies (da Oh et al. 2014). Oral administration of cpdA regulates systemic insulin sensitivity by enhancing muscle and liver insulin activity. In addition, cpdA treatment exerts beneficial effects on hepatic lipid metabolism by decreasing hepatic steatosis and triglyceride levels. Furthermore, these effects of cpdA treatment are abolished in *Gpr120*-deficient mice. In the context of high-fat diet-induced obesity, adipose tissue macrophage (ATM) activation is promoted, and inflammatory gene expression is increased; however, treatment with cpdA or ω3 FAs inhibits the activation of ATM and reduces monocyte migration. In addition, in murine primary macrophages, the expression of pro-inflammatory genes, such as *Tnf*, *Il6*, *Ccl2* (also known as *Mcp1*), and *Il1b*, are decreased, whereas the expression of anti-inflammatory genes, such as *Il10*, *Clec7a*, *Clec10a* (also known as *Mgl1*), and *Chil3* (also known as *Ym1*), are increased in response to cpdA treatment. Therefore, although

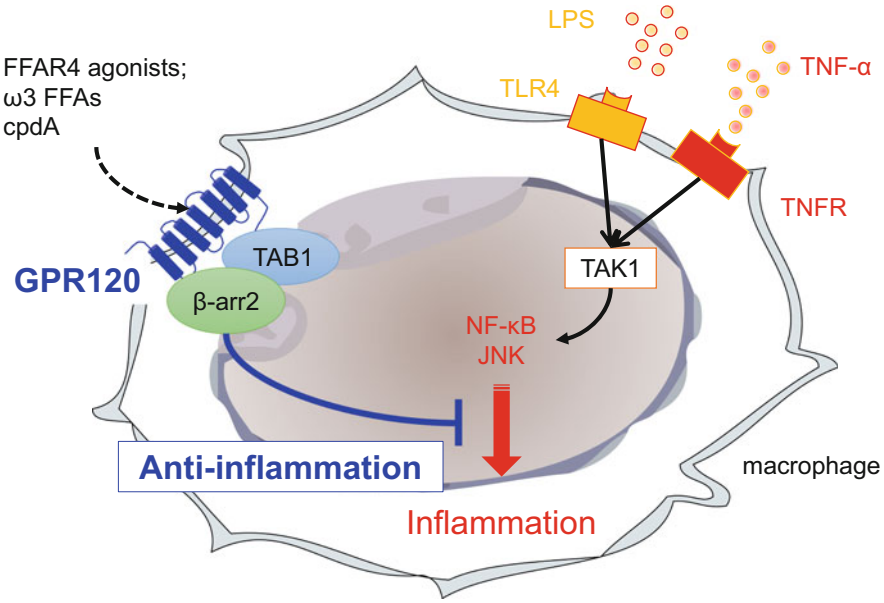

Fig. 1 GPR120 activation inhibits pro-inflammatory pathways

high circulating cytokine levels are elevated in the context of obesity, cpdA-treated mice were significantly reduced inflammatory features (Oh et al. 2010). Importantly, cpdA treatment stimulates strong anti-inflammatory and insulin-sensitizing effects in vitro and in vivo compared with the effects of treatment with ω3 FAs treatment. Collectively, these data support that GPR120 agonists, particularly the selective small-molecule cpdA, could have applications as insulin-sensitizing agents in the treatment of metabolic disorders (da Oh et al. 2014) (Fig. 1).

3 GPR40-Mediated Anti-Inflammatory Mechanisms

GPR40 is highly expressed in pancreatic β cells and enteroendocrine cells and is coupled with a Gq protein. GPR40 is activated by MCFAs and LCFAs and induces phospholipase C activation and intracellular Ca^{2+} efflux. The activation of GPR40 signaling enhances glucose-stimulated insulin secretion (GSIS) directly via stimulation of insulin secretion from pancreatic β cells and indirectly via production of incretin hormones, such as GLP-1 and GIP, from enteroendocrine cells (Hara et al. 2014; Tomita et al. 2014).

Oliveira et al. reported that α-linolenic (ω3) or oleic (ω9) acids improved insulin resistance in the context of high-fat diet-induced obesity and type 2 diabetes (Oliveira et al. 2015). Specifically, ω3 and ω9 FAs exert their anti-inflammatory effects through GPR40 and GPR120, respectively. GPR120 affects the adipose tissues and liver, whereas GPR40 affects the muscles. Additionally, activation of

GPR40 signaling increases insulin sensitivity and reduces the expression of inflammatory markers. Although additional studies are needed, these results provide novel insights into the beneficial effects of diets containing ω3 and ω9 FAs through GPR40 and GPR120 (Oliveira et al. 2015).

The anti-inflammatory effects of GPR40 signaling have been also reported in various other types of tissues and cells. The human intestinal epithelial cell line, Caco-2, secretes excessive amounts of IL-8, a leukocyte chemotactic factor, upon stimulation with TNF-α. Endogenous ligands, such as linoleic acid and gut microbial metabolites, suppress IL-8 production through GPR40 activation (Miyamoto et al. 2015). In addition, topical administration of the GPR40 agonist, GW9508, inhibits allergic inflammation and downregulates pro-inflammatory cytokine expression induced in the hapten application model (Fujita et al. 2011). Although GPR40 is weakly expressed in macrophages, the anti-inflammatory effects of GPR40 in macrophages remain unclear (Oh et al. 2010). Thus, additional studies are needed to determine the function of GPR40 in macrophages.

4 GPR84-Mediated Anti-Inflammatory Mechanisms

MCFAs seem to improve lipotoxicity and insulin resistance in high-fat diet-induced obese rats (Wein et al. 2009). In addition, Turner et al. reported that MCFAs reduce fat accumulation and maintain insulin sensitivity in muscle and adipose tissues in high-fat diet-induced obese rats (Turner et al. 2009.).

GPR84 is coupled with a Gi/o protein and activated by MCFAs (C9–C14) (Wang et al. 2006). In addition, GPR84 is widely expressed in the heart, lung, liver, and immune cells (Yousefi et al. 2001; Venkataraman and Kuo 2005). Interestingly, macrophage activation by lipopolysaccharide (LPS) induces GPR84 expression (Wang et al. 2006), and GPR84 agonists, such as capric acid, undecanoic acid, and lauric acid, strongly stimulate secretion of the IL-12p40 subunit and enhance IL-8 and TNF-α production in LPS-stimulated RAW264.7 cells (a macrophage-like cell line) and human polymorphonuclear leukocytes (Bouchard et al. 2007). Conversely, these effects are not observed following treatment with SCFAs and LCFAs in LPS-stimulated RAW264.7 cells. Thus, only MCFAs can modulate IL-12p40 expression through GPR84 in LPS-stimulated macrophages (Wang et al. 2006). Based on these findings, GPR84 expression may have a role in the immune system. Furthermore, GPR84 expression is also enhanced in adipocytes stimulated by macrophages that have infiltrated into the adipose tissue, and the activation of GPR84 by MCFAs inhibits TNF-α-induced adiponectin mRNA expression (Nagasaki et al. 2012). In contrast, a previous report has shown that consumption of a medium-chain triglyceride-rich diet, which elevates plasma MCFA levels, significantly enhances adiponectin expression in rats and can help burn excess calories, resulting in weight loss (Takeuchi et al. 2006). Further studies are needed to determine the function of GPR84 in adipocytes.

5 SCFAs Regulate Inflammatory and Metabolic Responses

SCFAs contain less than six carbons and are produced by gut microbiota fermentation of dietary fibers. In a previous study, dietary fibers played a key role in the development of various disorders, such as inflammatory and metabolic responses (Galisteo et al. 2008; den Besten et al. 2013). For example, butyrate derived from dietary fibers alleviates colitis (Park et al. 2015) and adipose inflammation (Wang et al. 2015) and improves insulin sensitivity (Gao et al. 2009). Thus, SCFAs derived from dietary fibers may play an important role in the development of inflammatory and metabolic disorders (Donohoe et al. 2011; Harig et al. 1989; Fukuda et al. 2013).

6 GPR43-Mediated Anti-Inflammatory Mechanisms

GPR43 is a receptor for SCFAs, primarily acetate (C2), and propionate (C3), and is coupled with Gi/o and Gq proteins. GPR43 is predominantly expressed in enteroendocrine cells, adipose tissues, and pancreatic β cells. Activation of GPR43 in these tissues regulates metabolic disorders; for example, secretion of peptide YY (PYY) and GLP-1 is increased in enteroendocrine cells (Hudson et al. 2013), and insulin secretion is stimulated by Ca^{2+} induction through a Gq-coupled signaling (Tolhurst et al. 2012). In addition, although *Gpr43*-deficient mice exhibit obesity after consumption of a high-fat diet or normal chow diet, adipose-specific *Gpr43* transgenic mice are lean, regardless of diet. Moreover, activation of GPR43 increases energy expenditure and preferentially enables fat consumption by inhibition of insulin signaling in adipose tissues (Kimura et al. 2013).

Because GPR43 has been reported to regulate intestinal mucosal inflammation (Maslowski et al. 2009; Sina et al. 2009), McNelis et al. investigated whether GPR43 could be involved in inflammation in metabolic tissues (McNelis et al. 2015). In the context of high-fat diet-induced obesity, GPR43 expression is strongly induced in the pancreas and colocalizes with insulin-positive β cells. In short-term (8-week-long) high-fat diet treatment, glucose tolerance and insulin sensitivity do not differ between *Gpr43*-deficient and wild-type mice; however, in long-term (14-week-long) treatment, *Gpr43*-deficient mice develop fasting hyperglycemia and glucose intolerance compared with wild-type mice. In contrast, the expression of pro-inflammatory markers, such as *Tnf*, *Il1b*, *inos*, and *Mcp-1*, do not differ in the liver and epididymal adipose tissue, and no differences are observed in hepatic histological assessments. Moreover, McNelis et al. showed that GPR43 expressed in immune cells is not important for this phenotype because glucose tolerance and insulin secretion are not improved in *Gpr43*-deficient mice transplanted with bone marrow cells from wild-type mice. These results suggest that GPR43 expression in immune cells does not contribute to glucose tolerance and insulin sensitivity. However, several reports have shown that the expression of GPR43 in neutrophils (Maslowski et al. 2009) and mononuclear cells (Masui et al. 2013) regulates intestinal homeostasis and inflammation. Therefore, GPR43 expression in

pancreatic β cells may regulate metabolic activity in the context of high-fat diet-induced obesity, and GPR43 may be a potential target for therapeutic intervention (McNelis et al. 2015).

7 GPR41-Mediated Anti-Inflammatory Mechanisms

GPR41 is also a receptor for SCFAs, primarily propionate (C3) and butyrate (C4), and is coupled with a Gi/o protein. GPR41 is widely expressed in enteroendocrine cells, adipose tissues, and the peripheral nervous system. GPR41 activation induces PYY and GLP-1 secretion from enteroendocrine cells (Tazoe et al. 2009) and increases energy expenditure and heart rate to maintain the energy balance in the sympathetic nervous system (Kimura et al. 2011). In addition, the activation of GPR41 in adipose tissues promotes the secretion of leptin, a polypeptide that regulates appetite and energy balance (Xiong et al. 2004). These results indicate that GPR41 activation is a potential therapeutic target for the treatment of obesity.

GPR41 is also expressed in peripheral blood mononuclear cells, monocytes, and macrophages. Although GPR41 expression has been reported to be correlated with inflammatory and metabolic markers (Pivovarova et al. 2015), the effects of GPR41 on inflammation have not been widely recognized. Trompette et al. reported that expression of GPR41, but not GPR43, in lung tissues and dendritic cells suppresses allergic inflammation (Trompette et al. 2014). Conversely, expression of GPR41 in intestinal epithelial cells promotes the expression of pro-inflammatory markers through extracellular signal-regulated kinase 1/2 and p38 MAPK. Since these pathways contribute to protection against bacterial infection, GPR41 may promote acute inflammatory responses in the intestine that have beneficial effects on host homeostasis (Kim et al. 2013.). Thus, although GPR41 would regulate both inflammatory and anti-inflammatory responses, further studies are needed to clarify the role of GPR41 in inflammation.

8 Conclusion

FFAs derived from the diet regulate the development of inflammatory and metabolic disorders through activation of GPCRs. These receptors are expressed in various cell types or tissues and act as anti-inflammatory mediators by regulating inflammatory responses. Indeed, expression of GPR120 in macrophages suppresses inflammation through inhibition of TLR and TNF-α pro-inflammatory signaling (Fig. 1). Thus, GPCR signaling may be involved in mediating insulin sensitivity through regulation of inflammatory responses. The number of patients exhibiting obesity and type 2 diabetes has increased worldwide, representing a major threat to public health. If the activation of GPCRs can suppress tissue inflammation, including inflammation of adipose tissues, such an approach may have potential applications in the management of inflammatory and metabolic disorders. Further

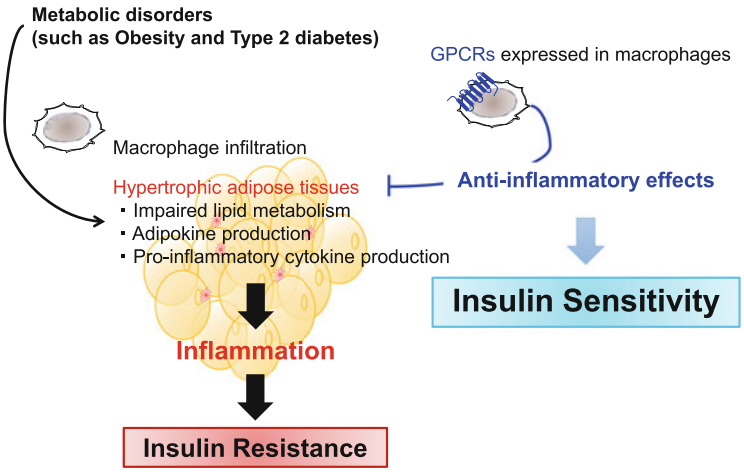

Fig. 2 Schematic model of the regulation of insulin sensitivity through the anti-inflammatory effects of GPCRs

studies are needed to fully elucidate the medical and pharmaceutical roles of GPCRs (Fig. 2).

References

Bouchard C et al (2007) G protein-coupled receptor 84, a microglia-associated protein expressed in neuroinflammatory conditions. Glia 55(8):790–800

Briscoe CP et al (2003) The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278(13):11303–11311

Brown AJ et al (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 278(13):11312–11319

da Oh Y et al (2014) A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med 20(8):942–947

den Besten G et al (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54(9):2325–2340

Donath MY, Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11(2):98–107

Donohoe DR et al (2011) The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 13(5):517–526

Facchini FS et al (2001) Insulin resistance as a predictor of age-related diseases. J Clin Endocrinol Metab 86(8):3574–3578

Fujita T et al (2011) A GPR40 agonist GW9508 suppresses CCL5, CCL17, and CXCL10 induction in keratinocytes and attenuates cutaneous immune inflammation. J Invest Dermatol 131(8):1660–1667

Fukuda S et al (2013) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469(7331):543–547

Galisteo M, Duarte J, Zarzuelo A (2008) Effects of dietary fibers on disturbances clustered in the metabolic syndrome. J Nutr Biochem 19(2):71–84

Gao Z et al (2009) Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes 58(7):1509–1517
Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21(1):90–113
Gregor MF, Hotamisligil GS (2011) Inflammatory mechanisms in obesity. Annu Rev Immunol 29:415–445
Hara T, Ichimura A, Hirasawa A (2014) Therapeutic role and ligands of medium- to long-chain fatty acid receptors. Front Endocrinol (Lausanne) 5:83
Harig JM et al (1989) Treatment of diversion colitis with short-chain-fatty acid irrigation. N Engl J Med 320(1):23–28
Hudson BD et al (2013) Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. J Biol Chem 288(24):17296–17312
Ichimura A et al (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature 483(7389):350–354
Itoh Y et al (2003) Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422(6928):173–176
Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444(7121):840–846
Kim MH et al (2013) Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 145(2):396–406
Kimura I et al (2011) Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc Natl Acad Sci U S A 108 (19):8030–8035
Kimura I et al (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 4:1829
Kotarsky K et al (2003) A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem Biophys Res Commun 301(2):406–410
Lattin JE et al (2008) Expression analysis of G protein-coupled receptors in mouse macrophages. Immunome Res 4:5
Le Poul E et al (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278(28):25481–25489
Maslowski KM et al (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461(7268):1282–1286
Masui R et al (2013) G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflamm Bowel Dis 19(13):2848–2856
McNelis JC et al (2015) GPR43 potentiates β-cell function in obesity. Diabetes 64(9):3203–3217
Miyamoto J et al (2015) A gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, ameliorates intestinal epithelial barrier impairment partially via GPR40-MEK-ERK pathway. J Biol Chem 290(5):2902–2918
Morari J et al (2010) The role of proliferator-activated receptor gamma coactivator-1alpha in the fatty-acid-dependent transcriptional control of interleukin-10 in hepatic cells of rodents. Metabolism 59(2):215–223
Nagasaki H et al (2012) Inflammatory changes in adipose tissue enhance expression of GPR84, a medium-chain fatty acid receptor: TNFα enhances GPR84 expression in adipocytes. FEBS Lett 586(4):368–372
Nilsson NE (2003) Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 303(4):1047–1052
Oh DY et al (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142(5):687–698
Oliveira V et al (2015) Diets containing α-linolenic (ω3) or oleic (ω9) fatty acids rescues obese mice from insulin resistance. Endocrinology 156(11):4033–4046
Osborn O, Olefsky JM (2012) The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med 18(3):363–374

Park J et al (2015) Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol 8(1):80–93
Parker HE et al (2009) Nutrient-dependent secretion of glucose-dependent insulinotropic polypeptide from primary murine K cells. Diabetologia 52(2):289–298
Pivovarova O et al (2015) Regulation of nutrition-associated receptors in blood monocytes of normal weight and obese humans. Peptides 65:12–19
Schulte G, Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 15(9):813–827
Seidell JC (2000) Obesity, insulin resistance and diabetes—a worldwide epidemic. Br J Nutr 83 (Suppl 1):S5–S8
Sina C et al (2009) G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 183(11):7514–7522
Takeuchi H et al (2006) Lower weight gain and higher expression and blood levels of adiponectin in rats fed medium-chain TAG compared with long-chain TAG. Lipids 41(2):207–212
Tazoe H et al (2009) Expression of short-chain fatty acid receptor GPR41 in the human colon. Biomed Res 30(3):149–156
Tolhurst G et al (2012) Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 61(2):364–371
Tomita T et al (2014) The G-protein-coupled long-chain fatty acid receptor GPR40 and glucose metabolism. Front Endocrinol (Lausanne) 5:152
Trompette A et al (2014) Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20(2):159–166
Turner N et al (2009) Enhancement of muscle mitochondrial oxidative capacity and alterations in insulin action are lipid species dependent: potent tissue-specific effects of medium-chain fatty acids. Diabetes 58(11):2547–2554
Venkataraman C, Kuo F (2005) The G-protein coupled receptor, GPR84 regulates IL-4 production by T lymphocytes in response to CD3 crosslinking. Immunol Lett 101(2):144–153
Wang J et al (2006) Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem 281(45):34457–34464
Wang X et al (2015) Sodium butyrate alleviates adipocyte inflammation by inhibiting NLRP3 pathway. Sci Rep 5:12676
Wein S et al (2009) Medium-chain fatty acids ameliorate insulin resistance caused by high-fat diets in rats. Diabetes Metab Res Rev 25(2):185–194
Xiong Y et al (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 101(4):1045–1050
Yousefi S et al (2001) Cloning and expression analysis of a novel G-protein-coupled receptor selectively expressed on granulocytes. J Leukoc Biol 69(6):1045–1052

Free Fatty Acid Receptors and Cancer: From Nutrition to Pharmacology

Mandi M. Hopkins and Kathryn E. Meier

Contents

Abstract

The effects of fatty acids on cancer cells have been studied for decades. The roles of dietary long-chain n-3 polyunsaturated fatty acids, and of microbiome-generated short-chain butyric acid, have been of particular interest over the years. However, the roles of free fatty acid receptors (FFARs) in mediating effects of fatty acids in tumor cells have only recently been examined. In

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reviewing the literature, the data obtained to date indicate that the long-chain FFARs (FFA1 and FFA4) play different roles than the short-chain FFARs (FFA2 and FFA3). Moreover, FFA1 and FFA4 can in some cases mediate opposing actions in the same cell type. Another conclusion is that different types of cancer cells respond differently to FFAR activation. Currently, the best-studied models are prostate, breast, and colon cancer. FFA1 and FFA4 agonists can inhibit proliferation and migration of prostate and breast cancer cells, but enhance growth of colon cancer cells. In contrast, FFA2 activation can in some cases inhibit proliferation of colon cancer cells. Although the available data are sometimes contradictory, there are several examples in which FFAR agonists inhibit proliferation of cancer cells. This is a unique response to GPCR activation that will benefit from a mechanistic explanation as the field progresses. The development of more selective FFAR agonists and antagonists, combined with gene knockout approaches, will be important for unraveling FFAR-mediated inhibitory effects. These inhibitory actions, mediated by druggable GPCRs, hold promise for cancer prevention and/or therapy.

Keywords
Cancer • Free fatty acids • G-protein-coupled receptors • Omega-3 fatty acids • Proliferation

1 Introduction

> Those who are given to the luxuries of the table are preparing for the pleasures of the operator's table—(Hubbard 1927)

As evidenced by the quotation above, the effects of diet on human health have been considered for many years. The impact of nutrition in cancer is currently a major focus, for scientists as well as for the public. Accordingly, the influences of dietary fatty acids (FAs) on cancer prevention, promotion, and treatment have been extensively studied. Nonetheless, the groundbreaking de-orphanization of FFA1 and FFA4 as receptors for omega-3 FAs (n-3 FAs) and other long-chain fatty acids occurred in the context of inflammation and metabolic disease (Oh et al. 2010). FFA2 and FFA3 are receptors for butyrate and other short-chain fatty acids, which are produced by intestinal microbiota, and have long been known to be important in the physiology and pathophysiology of the digestive tract as well as in immune function (Ang and Ding 2016). Differences in the expression of FFARs between different tissues also directed early investigations into the physiologic roles of these receptors in particular organs. Thus, it is logical that the first studies of FFARs, as well as the development of pharmacologic ligands for these receptors, were focused on conditions other than cancer. Fortunately, it took only a few years for investigations of the newly identified FFARs to progress into the pathophysiologic

realm of cancer. As the field moves forward, the roles of FFARs in neoplastic cells are beginning to provide intriguing new insights.

The situation with regard to cancer is particularly complex. While fatty acid metabolism in cancer cells has been studied for decades, epidemiologic studies examining fatty acid intake and cancer risk have yielded mixed results that are variable between different cancers. For example, omega-3 FAs are generally viewed as preventative or therapeutic for breast cancer (Chung et al. 2014; Hopkins et al. 2016; Yang et al. 2014), but may be detrimental for colon cancer (Wu et al. 2013). In the case of prostate cancer, most studies suggest that n-3 FAs lower risk; one analysis to the contrary generated a firestorm of response from scientists and the public (reviewed by Hopkins and Meier 2016). Clinical data indicate that n-3 FAs can increase response to chemotherapy (reviewed by Laviano et al. 2013). Dietary intake is one aspect of people's lives over which they have a great deal of control. Thus, potential dietary interventions are of great interest to the public with respect to health maintenance and cancer prevention. The effects of dietary fatty acids have been frequently reviewed and are not the major focus of the current analysis. Not all research concerning fatty acids in cancer involves dietary intake, however, since variations in lipid metabolism within malignant cells can also affect cell signaling and tumor growth (reviewed by Currie et al. 2013).

Potential mechanisms for anticancer effects of n-3 FAs have been reviewed in detail (Gu et al. 2013). Until recently, mechanistic explanations for the effects of fatty acids on cancer cells have focused primarily on the metabolism of the FAs to other products that either enhance or inhibit cellular signaling. Foremost among these theories is the idea that n-3 FAs compete for metabolic pathways that convert n-6 FAs to pro-inflammatory products and that the n-3 products are either less inflammatory or anti-inflammatory. Since inflammation generally promotes cancer, this mechanism is logical, and much evidence has accumulated in its support. However, in the field of lipid signaling, it has historically been the case that non-receptor mechanisms have initially predominated, only to be overturned or reconsidered once a receptor for a lipid mediator is identified. The potential roles for G-protein-coupled receptors (GPCRs) (Audigier et al. 2013), and lipid GPCRS in particular (Van Jaarsveld et al. 2015), as druggable targets for cancer therapy have recently been reviewed.

The discovery of FFARs, and our evolving view of their cellular roles, is only beginning to provide perspective on the roles of these GPCRs in cancer cells. We have reached the point where it is important to distinguish receptor-mediated events from events mediated by fatty acid metabolism, in order to obtain a more complete picture of the actions of fatty acids on cancer cells. In this review, we will organize the discussion by receptor type, and then by cancer type.

2 FFAR Subtypes

In the last decade, four orphaned receptors were classified into a new GPCR family, the free fatty acid receptor (FFAR) family. The FFAR family is comprised of free fatty acid (FFA) receptors 1–4, formerly known as GPR40, 43, 41, and

120, respectively. Activation of these receptors appears to have many health benefits, ranging from stimulation of insulin secretion, improvement of adipose function and pancreatic cell viability, and modulation of inflammatory responses and energy intake (Butcher et al. 2014; Dranse et al. 2013; Gotoh et al. 2007). In the last few years, there has been an intensification of interest in this family of receptors, mostly due to their promise as targets for metabolic diseases such as diabetes and obesity. While most of the research has been focused on metabolic diseases, several groups have begun to examine the role of FFARs in other areas and particularly with respect to inhibition of inflammation. Inflammation has roles in multiple diseases, including cancer, cardiovascular disease, multiple sclerosis, and rheumatoid arthritis (Butcher et al. 2014; Talukdar et al. 2011; Wu et al. 2013). The following discussion focuses on the roles of FFARs in cancer, which in some cases overlap with their roles in inflammation.

FFAs 1, 2, and 3 are closely related GPCRs, sharing 30–40% of their amino acid sequence, which are encoded in tandem on chromosome 19q13.1. FFA2 and 3 are activated by short-chain fatty acids, such as formate, acetate, butyrate, propionate, and pentanoate. The EC_{50} values of the endogenous ligands range from 0.1 to 1.0 mM, with physiological serum levels ranging from 50 to 200 μM (Dranse et al. 2013; Feng et al. 2012). However, FFA2 and 3 exhibit obvious differences in the rank-order potency for these ligands. For instance, acetate and propionate have similar potencies at FFA2, while propionate has greater potency than acetate for FFA3. Another difference between the two receptors concerns G-protein coupling: FFA2 couples to both the $G_{i/o}$ and $G_{q/11}$ families, whereas FFA3 couples solely to the $G_{i/o}$ family (Burns et al. 2014; Dranse et al. 2013; Hara et al. 2011; Hirasawa et al. 2008; Holliday et al. 2012; Hudson et al. 2013a). FFA2 can also signal via arrestins (reviewed by Bolognini et al. 2016).

FFA1, while sharing genomic similarities to FFA2 and 3, is more similar in function to FFA4. FFA1 and 4 are both receptors for long-chain fatty acids, with physiological levels of the endogenous ligands ranging from 200 to 500 μM in serum and EC_{50} values ranging from 1 to 30 μM (Dranse et al. 2013). Long-chain fatty acids include saturated FAs such as palmitic acid, monounsaturated FAs such as oleic acid, and polyunsaturated omega-3 fatty acids (n-3 FAs). FFA1 and 4 also share G-protein coupling mechanisms; they both couple through $G_{q/11}$, although FFA1 also signals through $G_{i/o}$ (Burns et al. 2014; Dranse et al. 2013; Hara et al. 2011; Hirasawa et al. 2008; Holliday et al. 2012).

The discussion that follows will be organized by individual FFARs, beginning with the long-chain FFARs because there is more information available about their roles in cancer cells than for the short-chain FFARs.

3 FFA1/GPR40

3.1 Overview of FFA1

FFA1 was the first FFAR to be identified as a long-chain fatty acid receptor and, as such, has been the most studied of the four receptors in the FFAR family. Aside from being the first de-orphanized, FFA1 has also been studied extensively due to its potential as a target for the treatment of metabolic diseases such as diabetes. FFA1 activation enhances glucose-stimulated insulin secretion, which makes it a therapeutic target in type 2 diabetes (Butcher et al. 2014; Feng et al. 2012; Hudson et al. 2013a; Qian et al. 2014; Watterson et al. 2014).

While FFA1 is widely expressed throughout the body, it is most highly expressed in the pancreas and gut, contributing to the interest in FFA1 as a target for diabetes and other metabolic diseases (Butcher et al. 2014; Dranse et al. 2013; Hirasawa et al. 2008; Hudson et al. 2013a, b; Talukdar et al. 2011; Watterson et al. 2014). Currently, of the FFARs, FFA1 has the widest range of synthetic ligands, including both agonists and antagonists (Milligan et al. 2015). Synthetic agonists of FFA1 include GW9508; AMG 837; MEDICA16; TUG424; Cpd B, C and 37; AM-1638; and TAK-875, while synthetic antagonists include GW1100, DC260126, and Pfizer compound 15i (Briscoe et al. 2006; Du et al. 2014; Feng et al. 2012; Hara et al. 2014; Holliday et al. 2012; Hudson et al. 2013b, 2014; Kebede et al. 2012; Sun et al. 2010; Talukdar et al. 2011; Watterson et al. 2014).

To date, the roles of FFA1 in cancer have been investigated in several different types of cancer cells. Breast cancer has been of particular interest, in part, because of the known association between obesity and breast cancer. Obesity leads to hyperlipidemia and increases in free fatty acid levels in plasma (Felber and Golay 2002). In addition, there is considerable epidemiological evidence that dietary n-3 FAs are of benefit for breast cancer prevention and treatment (Fabian et al. 2015), although the mechanism has not been established.

3.2 FFA1 in Breast Cancer

The expression of FFA1/GPR40 mRNA in human breast cancer cell lines was first demonstrated in 2004 by Yonezawa et al. (2004). They showed that oleate and linoleate increased intracellular calcium levels in these cells in a manner that was partially pertussis toxin sensitive, consistent with a role for a GPCR. However, knockdown experiments were not performed to specifically investigate the role of FFA1 in this early study.

Another report, published in 2005, reported that the n-3 FA eicosapentaenoic acid (EPA) inhibits proliferation of MCF-7 human breast cancer cells, using mouse tumor xenografts perfused in situ (Sauer et al. 2005). This inhibitory effect, which was measured by thymidine incorporation, was pertussis toxin sensitive. The authors reference possible roles for FFA1 and/or FFA4, but did not directly test them within their study.

In one study, Hardy et al. (2005) showed that 100 μM oleate stimulates proliferation of serum-starved MDA-MB-231 cells. The response was blocked by pertussis toxin, suggesting GPCR involvement. The authors further demonstrated that MDA-MD-231, T47D, and MCF-7 cells express mRNA for FFA1/GPR40. When FFA1 expression was knocked down by siRNA in MDA-MB-231 cells, the dose-response curve for oleate-induced proliferation was shifted to the right, with the most profound decreases in response seen with 1 and 5 μM oleate. The maximal response (50–100 μM oleate) was also significantly reduced, although only partially. This study was conducted before pharmacologic agonists for FFA1 were commercially available. In summary, this early study established that FFA1 is expressed in breast cancer cells, and suggested that activation of this receptor enhances proliferation.

Two additional studies, published in 2008, showed that both FFA1 and FFA4 are expressed in breast cancer cells. In one of these reports, expression of FFA1 and FFA4 was demonstrated in both MCF-7 and MCF10A cells by flow cytometry using anti-receptor antibodies (Soto-Guzman et al. 2008). Interestingly, 400 μM oleate increased DNA synthesis in MCF-7 and MDA-MB-231 breast cancer cells, and not in MCF10A cells which are non-tumorigenic (Navarro-Tito et al. 2008). The potential roles for the FFARs were not investigated by knockdown studies. In the subsequent report, from the same group, the expression of FFA1 and FFA4 was again demonstrated by flow cytometry. The major point of this second manuscript was to characterize responses (FAK activation, migration) to arachidonic acid, which were shown to be pertussis toxin sensitive. However, these responses were presumed to be independent of FFA1 and FFA4 since these GPCRs are not activated by arachidonic acid.

Data concerning FFA1 function in breast cancer cells were presented as part of a 2014 study that focused on melanoma (Nehra et al. 2014). The authors showed that FFA1 mRNA was expressed in MCF-7 cells, although the major focus of the manuscript was that the FFA1 mRNA levels were much higher in melanoma cells than in breast cancer cells. The FFA1-selective agonist TAK-875 decreased cell numbers for melanoma cells, but not MCF-7 cells, as measured after 72 h in medium containing 1% serum. The cell loss was referred to as both an "inhibitory effect on growth" as well as a "toxic effect"; the protocol used did not distinguish between inhibition of proliferation and loss of viability.

More recently, our group showed that EPA (20 μM) and pharmacologic FFAR agonists inhibit proliferation and migration of MCF-7 and MDA-MB-231 cells in response to lysophosphatidic acid (LPA) or epidermal growth factor (EGF) (Hopkins et al. 2016). Dose-response studies comparing the inhibitory effects of the FFA1-selective agonist GW9508, and the FFA4-selective agonist TUG-891, suggested that FFA1 may play a major role in inhibiting growth in these cell lines. In other words, the IC_{50} values for the inhibitory effects of the agonists were most consistent with a role for FFA1. Expression of FFA1 was demonstrated at the mRNA level, and expression of FFA4 was demonstrated at the mRNA and protein levels in this study. However, knockdown studies were not performed to further test the relative roles of FFA1 and FFA4. The inhibitory effects of FFAR agonists on

proliferation in this study are consistent with previous literature concerning effects of n-3 fatty acids on breast cancer cells (Manna et al. 2008; Zou et al. 2013; Xue et al. 2014; Pogash et al. 2015). The study design also investigated whether the inhibitory action of FFAR agonists on EGF response might be mediated by LPA receptors, which are also GPCRs. The results were consistent with such a mechanism, but not definitive.

In summary, expression of both FFA1 and FFA4 has been demonstrated in breast cancer cell lines by several different groups. The effects of FFA1 agonists have been variable, with different dose ranges and FAs tested in different studies. It is not yet clear that growth-stimulating effects of oleate are mediated predominantly by FFA1. There is a consensus between two groups that EPA inhibits breast cancer cell proliferation, with our group implicating FFA1 in this response. Further studies are needed to delineate the relative roles, whether stimulatory or inhibitory, of FFA1 and FFA4 in breast cancer cells.

3.3 FFA1 in Other Cancers

Ishii et al. (2015a) recently published a study of the role of FFA1 in migration and invasion in HT1080 fibrosarcoma cells. This report is related to another study published by the same group earlier in the same year, concerning FFA1 and FFA4 in WB-F344 liver epithelial cells (Ishii et al. 2015b). The study of the liver epithelial cell line indicated that FFA4 activation promotes cell motility, while FFA1 inhibits motility (Ishii et al. 2015b). In this study, the investigators also indicated that the effects might involve modulation of LPA receptors. In their study of the fibrosarcoma cell line (Ishii et al. 2015a), the investigators detected expression of FFA1 but not FFA4. Treatment of these cells with the FFA1-selective agonist GW9508 decreased motility and invasion. This study is another example of an inhibitory effect mediated by FFA1 in cancer cells.

The same research group examined the roles of FFA1 and FFA4 in pancreatic cancer cells (Fukushima et al. 2015). In this study, the investigators used shRNA-mediated knockdown to modulate levels of both receptors, which were endogenously expressed. Cells lacking FFA4 showed decreased motility and reduced colony formation, as compared to control cells. In contrast, cells lacking FFA1 showed enhanced motility, increased MMP-2 activity, and enhanced colony formation. Taken together, the results published by this group of collaborators provide interesting insights into potentially opposing effects of FFA1 and FFA4 within a single cell line and confirm that FFAR agonists can exert inhibitory actions on cancer cells.

FFA1 has been recently shown to be expressed at elevated levels in A2058, A375, and SKMel3, all of which are human melanoma cell lines, when compared to a control fibroblast cell line and to several human neuroblastoma and breast cancer cell lines (Nehra et al. 2014). In their cell culture studies, this group demonstrated that GW9508, an agonist for FFA1 and FFA4, and TAK-875, a selective FFA1 agonist, both inhibited the proliferation of the three melanoma cell lines, while

showing no such inhibition in the control fibroblast cell line. In addition, the researchers tested the effects of treating mice with subcutaneous xenografts of human melanoma with 100 mg/kg of TAK-875. These mice developed tumors with significantly lower weights and volumes when compared to the tumors in the placebo control mice; there was no significant difference in total body weight between the two groups of mice. The studies with the FFA1 agonists were conducted in both cell lines (treatments with DHA) and animal models (diets with high n-3 fatty acid contents); the results of these studies mirrored one another, leading the authors to conclude that DHA is acting through FFA1 to inhibit the growth of malignant melanoma. This is another key example of the ability of long-chain FFAR agonists to inhibit cancer growth.

Results presented in a meeting abstract indicate that FFA1 is expressed in ~80% of high-grade serous ovarian carcinomas (Munkarah et al. 2016). The researchers report that exposure of ID8, A2780, C200, OVCAR3, and SKOV3 human ovarian cancer cell lines to adipocytes, a source of FFAs, results in increased expression of FFA1. The investigators further state that the FFA1 antagonist, GW1000, inhibits proliferation of ovarian cancer cell lines, while the agonist CAY10587 has no effect on proliferation. The proliferation study results appear to contradict results from previous reports, showing that n-3 FAs inhibit proliferation of ovarian cancer cells (Sharma et al. 2005, 2009). Further work is needed to resolve these contradictory findings.

Our group has shown that both FFA1 and FFA4 are expressed in human prostate cancer cell lines, PC-3 and DU145 (Liu et al. 2015a). As will be discussed further below, the inhibitory role of FFA4 is more prominent than that of FFA1 in these particular cells.

4 FFA4/GPR120

4.1 Overview of FFA4

Like FFA1, FFA4 is a receptor for long-chain fatty acids, although it does not share homology with the other three receptors in its family, having only 10% identity with FFA1. It was included in the FFAR family after being identified by high-throughput screening. FFA4 has been described as a selective n-3 FA receptor (Dranse et al. 2013; Hirasawa et al. 2008). Similar to FFA1, FFA4 is expressed throughout the body, with high levels of expression in the GI tract, adipocytes, taste buds, immune cells, and lungs. The highest expression in humans is in the lungs (Zhang and Leung 2014). FFA4 has been reported to play a role in regulating inflammation, insulin sensitization, and obesity reduction, as well as regulating lipid and glucose metabolism by stimulating the release of hormones (Liu et al. 2015a). It is believed to regulate inflammation and metabolism through suppression of macrophage-induced tissue inflammation and β–arrestin-2-mediated augmentation of insulin sensitivity, respectively (Burns et al. 2014; Dranse et al. 2013; Gu et al. 2013;

Hara et al. 2014; Hirasawa et al. 2008; Hudson et al. 2014; Oh et al. 2014; Watterson et al. 2014; Wu et al. 2013; Zhang and Leung 2014).

There are two FFA4 isoforms: FFA4 long (FFA4L) and FFA4 short (FFA4S). FFA4S is found in humans, as well as in other species, whereas FFA4L is believed to be present only in humans. FFA4S contains 361 residues, while FFA4L contains an additional 16 residues between positions 231 and 247 in intracellular loop 3. As stated above, FFA4 signals through $G_{q/11}$, which it does only through its short isoform. However, both isoforms signal through the arrestin pathway (Dranse et al. 2013; Holliday et al. 2012; Hudson et al. 2013b; Milligan et al. 2015; Oh et al. 2010; Oh and Walenta 2014; Watson et al. 2012; Zhang and Leung 2014). FFA4 is thought to signal through β-arrestin-2, as it is a Class A or rhodopsin-like GPCR. Class A GPCRs preferentially bind β-arrestin-2 (arrestin-3) over β-arrestin-1 (arrestin-2) (Luttrell and Gesty-Palmer 2010; Oh and Walenta 2014). In addition, the Milligan group has shown that FFA4 does recruit arrestin-3 in Chinese hamster ovary (CHO) cells when stimulated with ALA or a synthetic FFA4 agonist, TUG-891 (Butcher et al. 2014).

The growing interest in FFA4 has prompted the discovery of several synthetic agonists to FFA4. There are two selective synthetic agonists with high affinity thus far: TUG-891 (chemical name 4-[(4-Fluoro-4′-methyl[1,1′-biphenyl]-2-yl) methoxy]-benzenepropanoic acid), which was developed by the Ulven group (Butcher et al. 2014; Milligan et al. 2015; Shimpukade et al. 2012), and GPR120 cpdA (chemical name 3-[2-chloro-5-(trifluoromethoxy)phenyl]-3-azaspiro[5.5] undecane-9-acetic acid), developed by Olefsky and co-workers (Oh et al. 2014). Other synthetic agonists are available; however, they are far less selective and/or potent. GW9508, mentioned above as an agonist for FFA1, is also an agonist for FFA4 but has much higher potency for FFA1 than for FFA4. Additional early FFA4 synthetic agonists include NCG21, NCG46, and AH7614, all of which, like GW9508, have significant dual agonism for FFA1 (Hara et al. 2014; Hudson et al. 2014; Oh et al. 2014; Shimpukade et al. 2012; Sparks et al. 2014; Watterson et al. 2014; Milligan et al. 2014). Although TUG-891 is currently one of the best available synthetic FFA4 ligands, it is limited to human FFA4, as it has limited selectivity for the mouse ortholog of FFA4 over the mouse ortholog of FFA1 (Hudson et al. 2014; Zhang and Leung 2014). CpdA has been shown to recruit β-arrestin-2 with equal intensity in both human and mouse cells (Oh et al. 2014).

4.2 FFA4 in Prostate Cancer

Our group used two human prostate cancer cell lines, PC-3 and DU145, to test whether previously reported inhibitory effects of n-3 FAs on prostate cancer cells are mediated by FFARs (Liu et al. 2015a). Our initial observations were that treatment of DU145, a human prostate cancer cell line, with n-3 FAs (EPA or DHA) inhibited responses to growth factors (EGF and LPA). These responses included signal transduction events such as Erk and Akt activation, as well as

proliferation and migration. The inhibitory effect occurred too rapidly to be easily attributed to alterations in lipid metabolism. Further experiments showed that these inhibitory responses on proliferation and migration were mimicked by the synthetic FFAR agonists TUG-891 and GW9508; TUG-891 exhibited much higher potency, suggesting a major role for FFA4. FFAR agonists had similar inhibitory effects in PC-3, another human prostate cancer cell line. The inhibitory effects on proliferation were sustained as long as the agonists were present, but were readily reversible. Since these data pointed toward an FFAR-mediated effect, we tested for FFA1 and FFA4 mRNA expression and found that both transcripts were present in both cell lines, albeit at different levels. When FFA4 knockdown was accomplished in the prostate cancer cell lines using siRNA, the inhibitory responses to EPA and TUG-891 were lost. In summary, this study showed for the first time that inhibitory effects of n-3 FAs on prostate cancer cells can be mediated by an FFAR.

4.3 FFA4 in Breast Cancer

The roles of FFA4 in breast cancer cells are only beginning to be addressed. FFA4 is expressed in MDA-MB-231 and MCF-7 breast cancer cells (Hopkins et al. 2016; Navarro-Tito et al. 2008; Soto-Guzman et al. 2008). Navarro-Tito et al. (2008) concluded that although FFA4 is present in MDA-MB-231 cells, it likely does not play a role in facilitating FAK activation in these cells.

Using an obese mouse model to study the effects of n-3 FAs on breast cancer, Chung et al. (2014) determined that, in a mouse model, expression of FFA4 in the host mouse (i.e., host immune system) does not mediate the inhibition of xenograft mammary tumor progression observed in response to n-3 FAs. These investigators showed direct inhibitory effects of n-3 FAs on breast tumor cells, but these effects were independent of FFA4 expression.

The authors' research group recently reported the results of cell culture studies (MCF-7 and MDA-MB-231) indicating that the inhibitory effects of FFAR agonists on breast cancer cell proliferation and migration are most consistent with a role for FFA1, even though FFA4 was also expressed in these cell lines (Hopkins et al. 2016). In summary, the findings for breast cancer cells recapitulate the general situation reported for prostate cancer cells, but are more complex and raise the possibility that FFA1 can also mediate inhibitory effects on growth factor action.

Based on the studies published to date, FFA4 can be expressed in breast cancer cells, but its roles in these cells remain to be defined.

4.4 FFA4 in Other Cancers

FFA4 is highly expressed in colorectal cancer cell lines (Liu et al. 2015a; Navarro-Tito et al. 2008). In a very comprehensive study, Wu et al. (2013) showed that FFA4 is overexpressed in colorectal cancer tissue as compared to adjacent normal tissue. Using GW9508 as agonist, these investigators used two established colorectal cell

lines, with and without FFA4 knockdown, to show that FFA4 mediates Akt activation and enhanced migration in these cells (Wu et al. 2013). Taken together, the results of this study indicate that (FFA4?) promotes tumor growth in colorectal cancer. Results from our group, using the Caco2 colorectal cell line, also indicate that FFAR agonists activate Akt in these cells and do not inhibit growth factor response (Liu et al. 2015a), in contrast to their inhibitory effects in prostate and breast cancer cells (Hopkins et al. 2016; Liu et al. 2015a).

The varying responses to FFA4 activation in different cancer cell types, tissues, and models demonstrate the need for further investigation into the role of FFA4 in cancer (Oh and Walenta 2014).

5 FFA2/GPR43

5.1 Overview of FFA2

FFA2 and FFA3 are activated by short-chain (less than six carbon) fatty acids (SCFAs) and are sometimes referred to as "butyrate receptors". These receptors were "de-orphanized" in 2003 (Le Poul et al. 2003). FFA2 is expressed rather selectively within the body, with highest expression in the GI tract, adipocytes, monocytes, and neutrophils. The transcriptional regulation of FFA2 expression has therefore been investigated (Ang et al. 2015). Since butyrate is an anti-inflammatory molecule produced by fermentation of dietary fiber by colonic bacteria, and since dietary fiber reduces colon cancer, the roles of FFA2 and FFA3 are of particular interest with respect to colon cancer (reviewed by Bultman 2013). It is not only the direct effects of SCFAs on cancer cells that are of relevance. Both FFA2 and FFA3 can mediate pro-inflammatory responses to SCFAs in colon epithelial cells, which may play a role in protective immunity (Kim et al. 2013). In addition, FFA2/FFA3-independent effects of SCFAs on T-cells may regulate immune response (Park et al. 2015). Other physiological roles of FFA2 and FFA3 have been reviewed (Offermanns 2014).

Selective agonists and antagonists for FFA2 have been under development, as discussed in a review that included the patent literature (Ulven 2012). Chloro-α-(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide (CMTB) is a selective agonist for FFA2 (Milligan et al. 2009). A more recent report detailed the synthesis and characterization of new FFAR agonists that are phenylthiazole-carboxamido acid derivatives (Ma et al. 2016).

5.2 FFA2 in Cancer

In an early study of the roles of FFARs in the response to short-chain fatty acids in breast cancer cells, one group showed that both FFA2 and FFA3 are expressed in MCF-7 cells (Yonezawa et al. 2006). These investigators found that the short-chain fatty acids (10 mM acetate, butyrate, or propionate) induce an acute increase in

intracellular calcium in MCF-7 cells, and also caused activation of p38 MAPK. Knockdown of FFA2 resulted in a loss of both the calcium and p38 responses. All three SCFAs (10 mM) decreased cell numbers in the absence of serum; only butyrate inhibited proliferation in response to serum. Effects on proliferation were not a major focus of this study.

As mentioned earlier, short-chain fatty acids, which are products of bacterial fermentation in the gut, are known to play a protective and preventative role against colon cancer (reviewed by Bindels et al. 2013). This represents one aspect of the burgeoning interest in the roles of gut microbiota in health and disease (reviewed by Louis et al. 2014). Tang et al. (2010) therefore examined expression of FFA2 in human colorectal cancer and found it to be markedly reduced in colon adenocarcinomas as compared to normal colon tissue. These investigators proceeded to restore FFA2 expression to HCT8, a colon cancer cell line, and then showed that propionate and butyrate-induced apoptosis in the FFA2-expressing cells. The authors concluded that FFA2 functions as a tumor suppressor in the colon.

In a particularly interesting study, Bindels et al. (2012) tested the hypothesis that production of SCFAs by gut microbiota, in response to consumption of nondigestible carbohydrates, might inhibit tumor growth by activating FFARs. Mice were injected with Ba/F3 transfected with Bcr-Abl, a leukemic cell line that expresses FFA2 and invades the liver. The reasoning was that the liver is directly exposed to SCFAs that are taken up by the gut, and thus an effect of SCFAs might be detected in this xenograft model. Accordingly, they found that treatment of the mice with nondigestible carbohydrates decreased the infiltration of the tumor cells into the liver. The authors also showed that acetate, propionate, and butyrate inhibit Ba/F3 cell proliferation in culture; this effect was mimicked by CMTB, a synthetic FFA2 agonist. In summary, this study provides another example of an antiproliferative effect on cancer cells, mediated by an FFAR.

Another important story concerning FFA2, with contrasting conclusions, is described in work published by Hatanaka et al. (2009). This study concerned gallbladder cancer. Retroviral expression screening was used to identify FFA2 as a transcript with oncogenic potential. Overexpression of FFA2 mRNA and protein enhanced gallbladder carcinogenesis; FFA2 mutations were not correlated with oncogenesis. Moreover, overexpression of FFA2 in 3T3 fibroblasts increased acetate and butyrate-induced proliferation of these cells. Evidence for upregulation of FFA2 in digestive tract tumors was also presented. In summary, this study showcases FFA2 as a transforming receptor with respect to digestive cancers, rather than an inhibitory receptor.

As pointed out in a review by Bindels et al. (2013), published studies have provided contradictory results, albeit with different model systems. Further work is needed to resolve the roles of FFA2 in cancer cells.

6 FFA3/GPR41

6.1 Overview of FFA3

Before GPR41 was de-orphanized as an FFAR, and assigned the alternative nomenclature FFA3, it was identified as a "hypoxia-induced apoptosis receptor" (Kimura et al. 2001). This work was done in H9c2, a rat cell line derived from embryonic heart. Although the study did not concern cancer, it is worthy of mention because overexpression of FFA3 resulted in increased p53 and apoptosis. A deletion mutant of FFA3 that was unable to couple to G proteins caused the opposite effect, inhibiting hypoxia-induced apoptosis. The authors also noted that transcript for FFA3 increased within 2 h after ischemia followed by reoxygenation, indicating that FFA3 is inducible.

6.2 FFA3 in Cancer

As noted earlier, Yonezawa et al. (2006) showed that FFA3 was expressed in MCF-7 human breast cancer cells. However, since mRNA for FFA3 was expressed at much lower levels than that for FFA2, these investigators subsequently focused on the role of FFA2.

Wu et al. (2012) stably transfected CHO cells with FFA3 and then examined responses to butyrate. They reported that butyrate was antiproliferative and pro-apoptotic in untransfected cells, but that these effects of butyrate were inhibited in cells transfected with FFA3. The roles of histone acetylation and cell cycle progression were also examined. However, it is not clear whether the potential role of endogenously expressed FFA2 was considered in this study.

As can be seen from the limited studies of FFA3-mediated effects on proliferation in both tumor and non-tumor cells, the roles of FFA3 in cancer cells are understudied relative to those of the other three FFARs.

7 Conclusions

At the time when this review was compiled, there were relatively few studies published examining the roles of FFARs in cancer cells. However, several conclusions emerge from the available data.

First, several studies demonstrate that FFAR agonists, either dietary or pharmacologic, exert inhibitory effects on cancer cell proliferation. The best-established examples for long-chain fatty acid receptors are the inhibitory role of FFA4 in prostate cancer cells (Liu et al. 2015a, b), of FFA1 and/or FFA4 in breast cancer cells (Hopkins et al. 2016), and FFA1-mediated inhibition in fibrosarcoma (Ishii et al. 2015a), melanoma (Nehra et al. 2014), and pancreatic cancer (Fukushima et al. 2015) cells. For short-chain fatty acid receptors, FFA2 activation is inhibitory

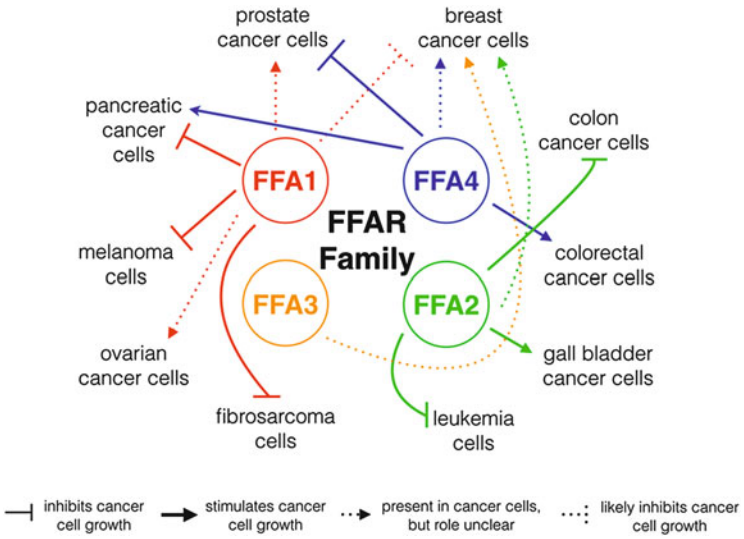

Fig. 1 Scheme summarizing the published reports discussed in this review. The terms "inhibits" and "stimulates" refer to proliferation, migration, and/or motility of the cancer cells. The term "likely" refers to studies in which agonist effects were consistent with the indicated action at a specific receptor, but not yet proven

in colon cancer cells (Tang et al. 2010) and in a leukemia cell line (Bindels et al. 2012).

Second, FFARs have been shown to enhance proliferation in some types of cancer cells. The most prominent examples are FFA4-mediated proliferation in colorectal tumor lines (Wu et al. 2013), and FFA2-mediated carcinogenesis in gallbladder (Hatanaka et al. 2009). Results from the published studies that have been discussed in this review are summarized in Fig. 1.

The third comment concerns potential mechanisms of action. Many GPCRs, when activated, can enhance the growth of tumor cells (Dorsam and Gutkind 2007; O'Hayre et al. 2013). Thus, it is atypical to see GPCR agonists under consideration for cancer therapy; typically GPCR antagonists are used to block pro-mitogenic signaling. The mechanisms for GPCR-mediated proliferation that are usually invoked involve activation of canonical signaling pathways such as Erk, Akt, and EGFR; these pathways are typically activated by $G_{q/11}$-coupled GPCRs. It is strikingly unusual that FFARs such as FFA1 and FFA4 can inhibit proliferation, despite their known coupling to $G_{q/11.}$ There are other published examples of FFA4-mediated inhibition of signaling; these examples concern inflammation (e.g., Li et al. 2013; Mobraten et al. 2013; Yan et al. 2013). The key to the inhibitory mechanism may lie in the very prominent role of arrestins in mediating responses to some FFARs, and particularly to FFA4 (Burns et al. 2014; Hudson et al. 2014; Prihandoko et al. 2016). Arrestins can mediate diverse responses to GPCR activation, and can interact with non-GPCR signaling proteins, but the full range of their

signaling activities has not been determined (Kaparianos et al. 2013; Luttrell 2013). It is therefore possible that some or all of the inhibitory effects noted for FFARs are arrestin dependent. From a GPCR perspective, this would be a very intriguing mechanism that could involve receptor-receptor interactions.

Further studies are needed to better establish the roles of FFARs in diverse types of cancer cells, and to pinpoint their mechanisms of action. With additional knowledge, we will eventually be able to progress from dietary interventions to pharmacologic ones to fully explore the potential of FFAR ligands as preventative or therapeutic agents in cancer.

References

Ang Z, Ding JL (2016) GRP41 and GPR43 in obesity and inflammation – protective or causative? Front Immunol 7. doi:10.3389/fimmu.2016.00028

Ang Z, Er JZ, Ding JL (2015) The short-chain fatty acid receptor GPR43 is transcriptionally regulated by XBP1 in human monocytes. Sci Rep. 5 doi:10.1038/srep08134

Audigier Y, Picault F-X, Chaves-Almagro C, Masri B (2013) G protein-coupled receptors in cancer: biochemical interactions and drug design. Prog Mol Biol Transl Sci 115:143–173. doi:10.1016/B978-0-12-394587-7.00004-X

Bindels LB, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, Scott KP, Calderon PB, Feron O, Muccioli GG, Sonveaux P, Cani PD, Delzenne NM (2012) Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. Br J Cancer 107:1337–1344. doi:10.1038/bjc.2012.409

Bindels LB, Dewulf EM, Delzenne NM (2013) GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci 34:226–232. doi:10.1016/j.tips.2013.02.002

Bolognini D, Tobin AB, Milligan G, Moss CE (2016) The pharmacology and function of receptors for short-chain fatty acids. Mol Pharm 89:388–398. doi:10.1124/mol.115.10230

Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, McCoy DC, Kenakin TP, Andrews JL, Ammala C, Fornwald JA, Ignar DM, Jenkinson S (2006) Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Br J Pharmacol 178:619–628. doi:10.1038/sj.bjp.0706770

Bultman SJ (2013) Molecular pathways: gene-environment interactions regulating dietary fiber induction of proliferation and apoptosis via butyrate for cancer prevention. Clin Cancer Res 20:1–5. doi:10.1158/1078-0432.CCR-13-1483

Burns RN, Singh M, Senatorov IS, Moniri NH (2014) Mechanisms of homologous and heterologous phosphorylation of FFA receptor 4 (GPR120): GRK6 and PKC mediate phosphorylation of Thr347, Ser350, and Ser357 in the C-terminal tail. Biochem Pharmacol 87:650–659. doi:10.1016/j.bcp.2013.12.016

Butcher AJ, Hudson BD, Shimpukade B, Alvarez-Curto E, Prihandoko R, Ulven T, Milligan G, Tobin AB (2014) Concomitant action of structural elements and receptor phosphorylation determine arrestin-3 interaction with the free fatty acid receptor FFA4. J Biol Chem 289:18451–18465. doi:10.1074/jbc.M114.568816

Chung H, Lee YS, Mayoral R, Oh DY, Siu JT, Webster NJ, Sears DD, Olefsky JM, Ellies LG (2014) Omega-3 fatty acids reduce obesity-induced tumor progression independent of GPR120 in a mouse model of postmenopausal breast cancer. Oncogene 34:1–10. doi:10.1038/onc.2014.283

Currie E, Schulze A, Zechner R, Walther TC, Farese RV (2013) Cellular fatty acid metabolism and cancer. Cell Metab 18:153–161. doi:10.1016/j.cmet.2013.05.017

Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. Nat Rev Cancer 7:79–94. doi:10.1038/nrc2069

Dranse HJ, Kelly MEM, Hudson BD (2013) Drugs or diet?--developing novel therapeutic strategies targeting the free fatty acids family of GPCRs. Br J Pharmacol 170:696–711. doi:10.1111/bph.12327

Du X, Dransfield PJ, Lin SCH, Wong S, Wang Y, Wand Z, Kohn T, Yu M, Brown SP, Vimolratana M, Zhu L, Li AR, Su Y, Jiao X, Liu J, Swaminath G, Tran T, Luo J, Zhuang R, Hang J, Guo Q, Li F, Connors R, Medina JC, Houze JB (2014) Improving the pharmacokinetics of GPR40/FFA1 agonists. ACS Med Chem Lett 5:384–389. doi:10.1021/ml4005123

Fabian CJ, Kimler BF, Hursting SD (2015) Omega-3 fatty acids for breast cancer prevention and survivorship. Breast Cancer Res 17. doi:10.1186/s13058-015-0571-6

Felber JP, Golay A (2002) Pathways from obesity to diabetes. Int J Obes Relat Metab Disord 26 (Suppl 2):S39–S45

Feng X-T, Leng J, Xie Z, Li S-L, Zhao W, Tang Q-L (2012) GPR40: a therapeutic target for mediating insulin secretion. Int J Mol Med 30:1261–1266. doi:10.3892/ijmm.2012.1142

Fukushima K, Yamasaki E, Ishii S, Tomimatsu A, Takahashi K, Hirane M, Fukushima N, Honoki K, Tsukiuchi T (2015) Different roles of GPR120 and GPR40 in the acquisition of malignant properties in pancreatic cancer cells. Biochem Biophys Res Commun 465:512–515. doi:10.1016/j.bbrc.2015.08.050

Gotoh C, Hong Y-H, Iga T, Hishikawa D, Suzuki Y, Song S-H, Choi K-C, Adachi T, Hirasawa A, Tsujimoto G, Sasaki S-I, Roh S-G (2007) The regulation of adipogenesis through GPR120. Biochem Biophys Res Commun 354:591–597. doi:10.1016/j.bbrc.2007.01.028

Gu Z, Suburu J, Chen H, Chen YQ (2013) Mechanisms of omega-3 polyunsaturated fatty acids in prostate cancer prevention. Biomed Res Int. doi:10.1155/2013/824563

Hara T, Hirasawa A, Ichimura A, Kimura I, Tsujimoto G (2011) Free fatty acid receptors FFAR1 and GPR120 as novel therapeutic targets for metabolic disorders. J Pharm Sci 100:3594–3601. doi:10.1002/jps.22639

Hara T, Ichimura A, Hirasawa A (2014) Therapeutic role and ligands of medium- to long-chain fatty acid receptors. Front Endocrinol (Lausanne) 5. doi:10.3389/fendo.2014.00083

Hardy S, St-Onge GG, Joly E, Langelier Y, Prestki MM (2005) Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. J Biol Chem 280:13285–13291. doi:10.1074/jbc.M410922200

Hatanaka H, Tsukui M, Takada S, Kurashina K, Choi YL, Soda M, Yamashita Y, Haruta H, Hamada T, Ueno T, Tamada K, Hosoya Y, Sata N, Yasuda Y, Nagai H, Sugano K, Mano H (2009) Identification of transforming activity of free fatty acid receptor 2 by retroviral expression screening. Cancer Sci 101:54–59. doi:10.1111/j.1349-7006.2009.01348.x

Hirasawa A, Hara T, Katsuma S, Adachi T, Tsujimoto G (2008) Free fatty acid receptors and drug discovery. Biol Pharm Bull 31:1847–1851. doi:10.1248/bpb.31.1847

Holliday ND, Watson S-J, Brown AJH (2012) Drug discovery opportunities and challenges at G protein coupled receptors for long chain free fatty acids. Front Endocrinol (Lausanne) 2:1–12. doi:10.3389/fendo.2011.00112

Hopkins MM, Meier KE (2016) Omega-3 fatty acids and their impact on prostate cancer risk. Curr Nutr Rep. doi:10.1007/s13668-016-1250-1

Hopkins MM, Zhang Z, Liu Z, Meier KE (2016) Eicosopentaneoic acid and other free fatty acid receptor agonists inhibit lysophosphatidic acid- and epidermal growth factor-induced proliferation of breast cancer cells. J Clin Med 5. doi:10.3390/jcm5020016

Hubbard EG (1927) The note book of Elbert Hubbard. Hubbard EG II (ed) Wm Wise & Co, New York, p 118

Hudson BD, Murdoch H, Milligan G (2013a) Minireview: the effects of species ortholog and SNP variation on receptors for free fatty acids. Mol Endocrinol 27:1177–1187. doi:10.1210/m3.2013-1085

Hudson BD, Shimpukade B, Mackenzie AE, Butcher AJ, Pediani JD, Christiansen E, Heathcote H, Tobin AB, Ulven T, Milligan G (2013b) The pharmacology of TUG-891, a potent and selective agonist of the free fatty acid receptor 4 (FFA4/GPR120), demonstrates both potential

opportunity and possible challenges to therapeutic agonism. Mol Pharm 84:710–725. doi:10.1124/mol.113.087783

Hudson BD, Shimpukade B, Milligan G, Ulven T (2014) The molecular basis of ligand interaction at free fatty acid receptor 4 (FFA4/GPR120). J Biol Chem 289:20345–20358. doi:10.1074/jbc.M114.561449

Ishii S, Kitamura Y, Hirane M, Tomimatsu A, Fukushima K, Takahashi K, Fukushima N, Honoki K, Tsujiuchi T (2015a) Negative effects of G-protein-coupled free fatty acid receptor GPR40 on cell migration and invasion in fibrosarcoma HT1080 cells. Mol Carcinog. doi:10.1002/mc.22408

Ishii S, Hirane M, Kato S, Fukushima N, Tsujiuchi T (2015b) Opposite effects of GPR 120 and GPR 40 on cell motile activity induced by ethionine in liver epithelial cells. Biochem Biophys Res Commun 456:135–138. doi:10.1016/j.bbrc.2014.11.047

Kaparianos A, Argyropoulou E, Spiropoulos K (2013) The role of β-arrestins in respiratory pathophysiology and tumorigenesis: going a step beyond the cell surface. Eur Rev Med Pharmacol Sci 16:1781–1794

Kebede M, Ferdaoussi M, Mancini A, Alquier T, Kulkarni RN, Walker MD, Poitout V (2012) Glucose activates free fatty acid receptor 1 gene transcription via phosphatidylinositol-3-kinase-dependent O-GlcNAcylation of pancreas-duodenum homeobox-1. Proc Natl Acad Sci U S A 109:2376–2381. doi:10.1073/pnas.1114350109

Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH (2013) Short-chain fatty acids activate GPR41 and GPR 43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 145:396–406. doi:10.1053/gastro.2013.04.056

Kimura M, Mizukami Y, Miura T, Fujimoto K, Kobayashi S, Matsuzaki M (2001) Orphan G protein-coupled receptor, GPR41, induces apoptosis via a p53/Bax pathway during ischemic hypoxia and reoxygenation. J Biol Chem 276:26453–26460. doi:10.1074/jbc.M101289200

Laviano A, Rianda S, Molfino A, Fanelli FR (2013) Omega-3 fatty acids in cancer. Curr Opin Clin Nutr Metab Care 16:156–161. doi:10.1097/MCO.0b013e32835d2d99

Le Poul E, Loison C, Struy S, Springael J-Y, Lannoy V, Decobecq M-E, Brezillon S, Dupriez V, Vassart G, Van Damme J, Parmentier M, Detheux M (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 278:25481–25489. doi:10.1074/jbc.M301403200

Li X, Yu Y, Funk CD (2013) FASEB J 27:4987–4997

Liu Z, Hopkins MM, Zhang Z, Quisenberry CR, Fix L, Galvan BM, Meier KE (2015a) Omega-3 fatty acids and other FFA4 agonists inhibit growth factor signaling in human prostate cancer cells. J Pharmacol Exp Ther 352:380–394. doi:10.1124/jpet.114.218974

Liu HD, Wang WB, Xu ZG, Liu CH, He DF, Du LP, Li MY, Yu X, Sun JP (2015b) FFAR receptor (GPR120): a hot target for the development of anti-diabetic therapies. Eur J Pharmacol 763:120–168. doi:10.1016/j.ejphar.2015.06.028

Louis P, Hold GL, Flint HJ (2014) The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 12:661–672. doi:10.1038/nrmicro3344

Luttrell LM (2013) Arrestin pathways as drug targets. Prog Mol Biol Transl Sci 118:469–497. doi:10.1016/B978-0-12-394440-5.00018-8

Luttrell LM, Gesty-Palmer D (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. Pharmacol Rev 62:305–327. doi:10.1124/pr.109.002436

Ma L, Wang T, Shi M, Fu P, Pei H, Ye H (2016) Synthesis, activity and docking study of novel phenylthiazole-carboxamido acid derivatives as FFA2 agonists. Chem Biol Drug Des. doi:10.1111/cbdd.12729

Manna S, Chakraborty T, Ghosh B, Chatterjee M, Panda A, Srivastava S, Rana A, Chatterjee M (2008) Dietary fish oil associated with increased apoptosis and modulated expression of Bax and Bcl-2 during 7,12-dimethylbenz(α)anthracene-induced mammary carcinogenesis in rats. Prostaglandins Leukot Essent Fatty Acids 79:5–14. doi:10.1016/j.plefa.2008.05.005

Milligan G, Stoddart LA, Smith NJ (2009) Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3. Br J Pharmacol 158:146–153. doi:10.1111/j.1476-5381,2009,00421.x

Milligan G, Alvarez-Curto E, Watterson KR, Ulven T, Hudson BD (2015) Characterising pharmacological ligands to study the long chain fatty acid receptors GPR40/FFA1 and GPR120/FFA4. Br J Pharmacol 172:3254–3265. doi:10.1111/bph.12879

Mobraten K, Haug TM, Klelveland CR, Lea T (2013) Lipids Health Dis 12:101–108

Munkarah A, Hamid S, Chhina J, Mert I, Jackson L, Hensley-Alford S, Chitale D, Giri S, Rattan R (2016) Abstract 81: targeting of free fatty acid signaling in ovarian cancer may serve as a potential therapeutic approach. Clin Cancer Res 22:A81

Navarro-Tito N, Robledo T, Salazar EP (2008) Arachidonic acid promotes FAK activation and migration in MDA-MB-231 breast cancer cells. Exp Cell Res 314:3340–3355. doi:10.1016/j.yexcr.2008.08.018

Nehra D, Pan AH, Le HD, Fallon EM, Carlson SJ, Kalish BT, Puder M (2014) DHA, G-protein coupled receptors and melanoma: is GPR40 a potential therapeutic target? J Surg Res 188:451–451–458. doi:10.1016/j.jss.2014.01.037

O'Hayre M, Vazquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S, Gutkind JS (2013) The emerging mutational landscape of G-proteins and G-protein coupled receptors in cancer. Nat Rev Cancer 13:412–424. doi:10.1038/nrc3521

Offermanns S (2014) Free fatty acid (FFA) and hydroxyl carboxylic acid (HCA) receptors. Annu Rev Pharmacol Toxicol 54:407–434. doi:10.1146/annurev-pharmtox-011613-135945

Oh DY, Walenta E (2014) Omega-3 fatty acids and FFAR4. Front Endocrinol 5:1–5. doi:10.3389/fendo.2014.00115

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan WQ, Li P, Lu WJ, Watkins SM, Olefsky JM (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin sensitizing effects. Cell 142:687–698. doi:10.1016/j.cell.2010.07.041

Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR, Sasik R, Hah N, Chi TJ, Cox JM, Powels MA, Salvo JD, Sinz C, Watkins SM, Armando AM, Chung H, Evans RM, Quehenberger O, McNelis J, Bogner-Strauss JG, Olefsky JM (2014) A GPR120 selective agonist improves insulin resistance and chronic inflammation. Nat Med 20:942–947. doi:10.1038/nm.3614

Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, Kim CH (2015) Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol 8:80–93. doi:10.1038/mi.2014.44

Pogash TJ, El-Bayoumy K, Amin S, Gowda K, de Cicco RL, Barton M, Su Y, Russo IH, Himmelberger JA, Slifker M, Manni A, Russo J (2015) Oxidized derivative of docosahexaenoic acid preferentially inhibit cell proliferation in triple negative over luminal breast cancer cells. In Vitro Cell Dev Biol Anim 51:121–127. doi:10.1007/s1626-014-9822-6

Prihandoko R, Alvarez-Curto E, Hudson BD, Butcher AJ, Ulven T, Miller AM, Tobin AB, Milligan G (2016) Distinct phosphorylation clusters determine the signaling outcome of the free fatty acid receptor FFA4/GPR120. Mol Pharmacol. doi:10.1124/mol.115.101949

Qian J, Wu C, Chen X, Li X, Ying G, Jin L, Ma Q, Li G, Shi Y, Zhang G, Zhou N (2014) Differential requirements of arrestin-3 and clathrin for ligand-dependent and –independent internalization of human G protein-coupled receptor 40. Cell Signal 26:2412–2423. doi:10.1016/j.cellsig.2014.07.019

Sauer LA, Dauchy RT, Blask DE, Krause JA, Davidson LK, Dauchy EM (2005) Eicosapentaenoic acid suppresses cell proliferation in MCF-7 human breast cancer xenografts in nude rats via a pertussis toxin-sensitive signal transduction pathway. J Nutr 135:2124–2129

Sharma A, Belna J, Logan J, Espat J, Hurteau JA (2005) The effects of omega-3 fatty acids on growth regulation of epithelial ovarian cancer cell lines. Gynecol Oncol 99:58–64

Sharma A, Belna J, Espat J, Rodriguez G, Cannon VT, Hurteau JA (2009) Effects of omega-e fatty acids on components of the transforming growth factor beta-1 pathway: implication for dietary modification and prevention in ovarian cancer. Am J Obstet Gynecol 200:516.e1–516.e6. doi:10.1016/j.ajog.2008.12.023

Shimpukade B, Hudson BD, Hovgaard CK, Milligan G, Ulven T (2012) Discovery of a potent and selective GPR120 agonist. J Med Chem 55:4511–4515. doi:10.1021/jm300215x

Soto-Guzman A, Tobledo T, Lopez-Perez M, Salazar EP (2008) Oleic acid induces ERK1/2 activation and AP-1 DNA binding activity through a mechanism involving Src kinase and EGFR transactivation in breast cancer cells. Mol Cell Endocrinol 294:81–91. doi:10.1016/j.mce.2008.08.003

Sparks SM, Chen G, Collins JL, Ganger D, Dock ST, Jayawickreme C, Jenkinson S, Laudeman C, Leesnitzer MA, Liang X, Maloney P, McCoy DC, Moncol D, Rash V, Rimele T, Vulimiri P, Way JM, Ross S (2014) Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). Bioorg Med Chem Lett 24:3100–3103. doi:10.1016/j.bmci.2014.05.012

Sun Q, Hirasawa A, Hara T, Kimura I, Adachi T, Awaji T, Ishiguro M, Suzuki T, Miyata N, Tsujimoto G (2010) Structure-activity relationships of GPR120 agonists based on a docking simulation. Mol Pharmacol 78:804–810. doi:10.1124/mol.110.066324

Talukdar S, Olefsky JM, Osborn O (2011) Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. Trends Pharmacol Sci 32:543–550. doi:10.1016/j.tips.2011.-4.004

Tang Y, Chen Y, Jiang H, Robbins GT, Nie D (2010) G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. Int J Cancer 128:847–856. doi:10.1002/ijc.25638

Ulven T (2012) Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. Front Endocrinol 3. doi:10.3389/fendo.2012.00111

Van Jaarsveld MTM, Houthuijzen JM, Voest EE (2015) Molecular mechanisms of target recognition by lipid GPCRs: relevance for cancer. Oncogene 2015:2–15. doi:10.1038/onc.2015.467

Watson S-J, Brown AJH, Holliday ND (2012) Differential signaling by splice variants of the human free fatty acid receptor GPR120. Mol Pharmacol 81:631–642. doi:10.1124/mol.111.077388

Watterson KR, Hudson BD, Ulven T, Milligan G (2014) Treatment of type 2 diabetes by free fatty acid receptor agonists. Front Endocrinol 5:1–9. doi:10.3389/fendo.2014.00137

Wu J, Zhou Z, Hu Y, Dong S (2012) Butyrate-induced GPR41 activation inhibits histone acetylation and cell growth. J Genet Genomics 39:375–384. doi:10.1016/j.jgg.2012.05.008

Wu Q, Wang H, Zhao X, Shi Y, Jin M, Wan R, Xu H, Cheng Y, Ge H, Zhang Y (2013) Identification of G protein-coupled receptor 120 as a tumor-promoting receptor that induces angiogenesis and migration in human colorectal carcinoma. Oncogene 32:5541–5550. doi:10.1038/0nc.2013.264

Xue M, Wang Q, Zhao J, Dong L, Ge Y, Hou L, Liu Y, Zheng Z (2014) J Nutr Biochem 25:104–110

Yan Y, Jian W, Spinetti T, Tardivel A, Castillo R, Barquin C, Guarda G, Tian Z, Tschopp J, Zhou R (2013) Immunity 38:1154–1163

Yang B, Ren X-L, Fu Y-Q, Gao J-L, Li D (2014) Ratio of n-3/n-6 PUFAs and risk of breast cancer: a meta-analysis of 274135 adult females from 11 independent prospective studies. BMC Cancer 14:105–119. doi:10.1186/1471-2407-14-105

Yonezawa T, Katoh K, Obara Y (2004) Existence of GPR40 functioning in a human breast cancer cell line, MCF-7. Biochem Biophys Res Commun 314:805–809. doi:10.1016/j.bbrc.2003.12.175

Yonezawa T, Kogayashi Y, Obara Y (2006) Short-chain fatty acids induce acute phosphorylation of the p38 mitogen-activated protein kinase/heat shock protein 27 pathway via GPR43 in the MCV-7 human breast cancer cell line. Cell Signal 19:185–193. doi:10.1016/j.cellsig.2006.06.004

Zhang D, Leung PS (2014) Potential roles of GPR120 and its agonists in the management of diabetes. Drug Des Devel Ther 8:1013–1027. doi:10.2147/DDDT.S53892

Zou Z, Bellenger S, Massey KA, Nicolaou A, Geissler A, Bidu C, Bonnotte B, Pierre A-S, Minville-Walz M, Rialland M, Seubert J, Kang JX, Lagrost L, Narce M, Bellenger J (2013) Inhibition of the HER2 pathway by n-3 polyunsaturated fatty acids prevents breast cancer in fat-1 transgenic mice. J Lipid Res 54:3453–3463. doi:10.1194/jlr.Mo42754

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