CARDIOVASCULAR PHARMACOLOGY

HEART AND CIRCULATION

EDITED BY

PAUL M. VANHOUTTE

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ADVANCES IN PHARMACOLOGY

CARDIOVASCULAR PHARMACOLOGY:
HEART AND CIRCULATION

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Foreword

The twentieth century has witnessed immense progress in preventing and treating cardiovascular disease. This has been fostered by improvements in lifestyle (exercise, diet) and the introduction of new therapeutics (antihypertensive and lipid-lowering drugs). Nonetheless, cardiovascular disease remains a major cause of death and disability in developed countries and, increasingly so, in the developing world. This is driven in part by demographics and the increase in longevity, and the obesity-metabolic syndrome-diabetes-atherosclerosis continuum that is reaching pandemic proportions. The hopes to address these issues using gene therapy have faded over the past decade. Stem cell therapy, while scientifically exciting, is still in its infancy, so will be, in the near-term, a treatment for the privileged. Given its cost, this approach is likely to be inaccessible to most patients with cardiovascular disease, in particular those in the emerging countries. Accordingly, the discovery of novel targets involved in cardiovascular disease, and the design of small molecules or biologics that interact with these sites, still holds the greatest promise for treating large numbers of individuals afflicted with these conditions.

Presented in this first volume of *Cardiovascular Pharmacology* are some of the most promising possibilities in that regard. Included are chapters on the treatment of heart failure. While current medications prolong life, they do little to improve its quality. As described in this volume, attempts to address this issue are centered on improving cardiac contractility. Other contributions focus on potassium channels and the manner in which their pharmacological manipulation can control heart rhythm and function. This is an area of compelling need, particularly in the wake of the CAST study. Other chapters describe new findings on the catecholamines, the major drivers of cardiovascular function and crucial factors in regulating the actions of calcium in vascular smooth muscle.

The emerging importance of angiotensin-converting enzyme 2 in counterbalancing the contributions of the renin-angiotensin system in the development and maintenance of hypertension and vascular disease is described in this work.

Contributors also consider new concepts relating to the impact of diabetes on platelet function. Presented in the volume is a summary of the evidence that tissue factor is crucial, not only for initiating the coagulation cascade but also for laying the groundwork for the atherosclerotic process.

I thank the contributors, all of whom are internationally recognized experts in the field, for their efforts in making this volume possible. Together with them, I sincerely hope these reports will be a source of inspiration, instruction, and ideas for graduate students, cardiovascular scientists, and physicians interested in the function and dysfunction of the heart and the blood vessel wall. Attainment of these goals will not only be personally satisfying for us but will hopefully provide a stimulus for further advances in this important area.

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The Ryanodine Receptor in Cardiac Physiology and Disease

Abstract

According to the American Heart Association it is estimated that the United States will spend close to $39 billion in 2010 to treat over five million Americans suffering from heart failure. Patients with heart failure suffer from dyspnea and decreased exercised tolerance and are at increased risk for fatal ventricular arrhythmias. Food and Drug Administration -approved pharmacologic therapies for heart failure include diuretics, inhibitors of the renin–angiotensin system, and β-adrenergic receptor antagonists. Over the past 20 years advances in the field of ryanodine receptor (RyR2)/calcium release channel research have greatly advanced our understanding of cardiac physiology and the pathogenesis of heart failure and arrhythmias. Here we

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review the key observations, controversies, and discoveries that have led to the development of novel compounds targeting the RyR2/calcium release channel for treating heart failure and for preventing lethal arrhythmias.

I. Introduction

In 1883 Sydney Ringer discovered that calcium (Ca^{2+}) is required for cardiac contraction (Ringer, 1883). Twenty four years later Locke and Rosenheim (1907) observed that Ca^{2+} is responsible for linking myocardial excitation with contraction. Following these seminal discoveries important advances have been made toward understanding the molecular determinants of cardiac Ca^{2+} regulation and its role in determining cardiac function.

In cardiomyocytes Ca^{2+} is stored in an intracellular vesicular network called the sarcoplasmic reticulum (SR) (Hasselbach & Makinose, 1961, 1963; Martonosi & Feretos, 1964) and is available for immediate release into the cytosol, where it binds to Troponin C and enables actin–myosin binding and sliding of the myofilaments that results in sarcomere shortening and myocardial contraction (Ebashi & Lipmann, 1962; Otsuka et al., 1964; Weber, 1959). The key roles that cyclical SR Ca^{2+} release and reuptake play in cardiac contraction underscore the importance of exquisite regulation of the proteins involved in these processes.

Cardiac contraction can be divided into electrical (excitation) and contractile phases. The electrical phase begins with depolarization of the sinoatrial node (SAN), situated near the junction of the superior vena cava and the right atrium, which causes a wave of depolarization to spread via the conduction system through the atria and ventricles. On the cellular level current flows between a depolarized cardiomyocyte and its resting neighbor through specialized low-resistance channels called gap junctions (Weidmann, 1952) causing depolarization of the membrane potential of the resting cell (Rohr, 2004). As the membrane potential of the resting cell increases from –90 mV (Draper & Weidmann, 1951) to –70 mV voltage-gated Na^+ channels (SCN5A) begin to open allowing an influx of sodium ions into the myocyte, further depolarizing the cell to ~+10 mV (Gibbons & Zygmunt, 1992). As the membrane potential rises above –40 mV L-type calcium channels (Cav1.2) on the sarcolemma begin opening leading to an influx of Ca^{2+} into the myocyte (Bean, 1985; Gibbons & Zygmunt, 1992). At ~0 mV voltage-gated K^+ channels (e.g., KCNH2 and KCNQ1) open allowing K^+ to efflux from the cell (Oudit et al., 2004). The influx of Na^+ and Ca^{2+} balanced by the efflux of K^+ causes the membrane potential to plateau at ~0 mV. Na^+ channels and L-type calcium channels inactivate as a function of time, membrane potential, and [Ca^{2+}] (Campbell et al., 1988) which reduces inward current leaving the unopposed efflux of potassium to repolarize the membrane to resting potential.

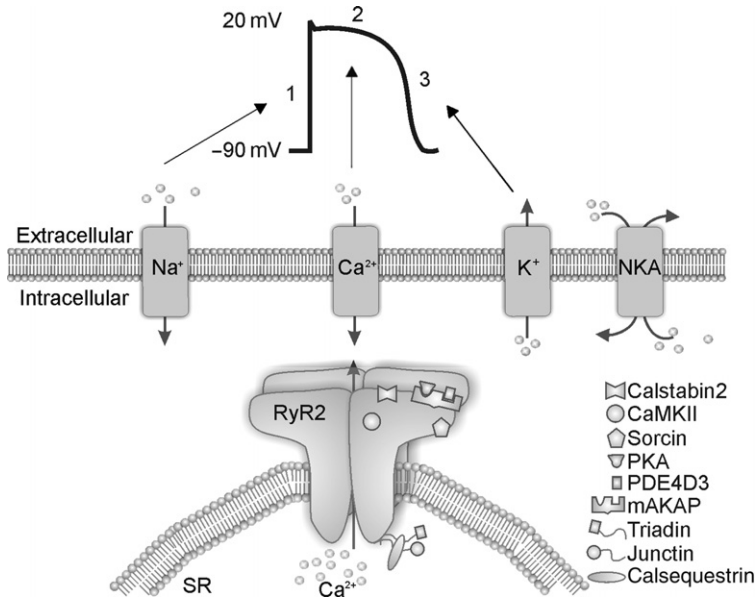

FIGURE 1 Cardiac action potential: Depolarization is initiated by opening of voltage-gated Na^+ channels followed by the opening of voltage-gated Ca^{2+} and K^+ channels. Influx of Ca^{2+} into the cytosol during phase 2 of the cardiac action potential triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR2). Baseline $[Na^+]$ and $[K^+]$ are restored by the Na^+/K^+ ATPase (NKA). RyR2 is a homotetrameric macromolecular complex.

Ca^{2+} that enters the cell through $Ca_v1.2$ during the excitation phase initiates contraction by binding to and activating the ryanodine receptor (RyR2), the major cardiac SR Ca^{2+} release channel, to release Ca^{2+} from the SR into the cytosol. As cytosolic Ca^{2+} levels increase from less than 100 nM to ~1 μM Ca^{2+} binds to troponin C, a component of the myofilaments, causing a conformational change in the troponin/tropomyosin complex, which enables myosin to interact with actin leading to myofilament shortening. Ca^{2+} is subsequently pumped back into the SR through the sarco/endoplasmic reticulum ATPase (SERCA2a) and is removed from the cell through the sarcolemmal sodium–calcium exchanger (NCX) and plasmalemmal Ca^{2+} ATPase (Fig. 1).

II. Ryanodine Receptors

Ryanodine receptors were originally identified using the plant alkaloid ryanodine, isolated from *Ryania speciosa* found in Central and South America. At the time ryanodine was being tested as a potential insecticide (Rogers

et al., 1948) owing to its paralyzing effect on insects (Edwards et al., 1948). Ryanodine was subsequently found to induce profound paralysis of cardiac and skeletal muscle and that it bound to a component of the SR (Jenden & Fairhurst, 1969). Ryanodine was used as a high-affinity ligand to track the purification of its receptor from SR preparations. The purified ryanodine receptors were shown to be Ca^{2+} release channels in skeletal (Fleischer et al., 1985; Meissner, 1986) and cardiac (Meissner & Henderson, 1987) muscles. Ryanodine was shown to lock the channel open in a characteristic subconductance state resulting in an SR Ca^{2+} leak that provided a mechanism for the paralytic action of the drug (Fill & Copello, 2002; Fleischer et al., 1985).

The cardiac ryanodine receptor isoform, RyR2, is a homotetramer comprising four ~565 kDa monomers. Each monomer contains a transmembrane segment located at the carboxy terminus that is formed by ~10% of the linear sequence, whereas the remaining 90% of the protein sequence encodes an enormous cytoplasmic domain that serves as a scaffold for regulatory subunits and enzymes that modulate the function of the channel (Zalk et al., 2007). It is estimated that each monomer contributes 6–8 transmembrane segments that form the pore region of the channel (Du et al., 2002). Cryo-electron microscopic (cryo-EM) analysis of the RyR2 pore combined with predictions based on sequence and structural similarities with the bacterial K^+ channels suggests that the luminal loops of the RyR2 monomers contribute to the pore structure (Balshaw et al., 1999; Samso et al., 2009) and that amino acids Glu4832, Ile4829, Gly4826, and Gln4881 (Rabbit RyR2) directly mediate Ca^{2+} passage through the pore (Welch et al., 2004)

RyR2, first cloned from rabbit heart in 1990 (Nakai et al., 1990; Otsu et al., 1990), shares close to 70% homology with two other mammalian RyR isoforms: RyR1 and RyR3. RyR1 is found predominantly in skeletal muscle where it is activated directly by the L-type calcium channel (Cav1.1) to release SR Ca^{2+} stores during skeletal muscle contraction. All three isoforms have been identified in smooth muscle and in the brain, though the physiologic role of RyR in these tissues has not been fully elucidated. All three channels share similar permeation properties with large conductance for both monovalent and divalent cations and relatively low Ca^{2+} selectivity (Fill & Copello, 2002). Under physiologic conditions RyR2 predominantly conducts Ca^{2+} because Ca^{2+} is present at millimolar concentrations in the SR.

RyR2 is normally closed at low cytosolic diastolic [Ca^{2+}] (~100–200 nM) (Copello et al., 1997). At submicromolar cytosolic [Ca^{2+}] Ca^{2+} binds to high-affinity binding sites on RyR2 which increases the open probability (Po) of the channel. Channel activity is maximal at $[Ca^{2+}]_{cyto}$ ~10 μM and elevating $[Ca^{2+}]_{cyto}$ beyond this point leads to a reduction in Po (Copello et al., 1997; Laver et al., 1995) possibly due to Ca^{2+} binding to

low-affinity inhibitory binding sites (Fill & Copello, 2002). Regulation of RyR2 Po by luminal [Ca^{2+}] between 10 μM and 1 mM has also been demonstrated (Ching et al., 2000). However, whether this regulation is due to Ca^{2+} flow through the channel and binding to cytosolic Ca^{2+}-binding sites remains uncertain (Fill & Copello, 2002; Gyorke & Gyorke, 1998; Liu et al., 2010; Tripathy & Meissner, 1996).

Studies employing single-particle cryo-EM of RyR1 have revealed conformational changes which occur when the channel opens including expansion of the clamp domain (situated between domains 9 and 10 and depicted as the "corners" of the RyR1 tetramer), rotation of the transmembrane domains relative to the cytoplasmic region, and expansion of the pore to 18 Å (Orlova et al., 1996; Samso et al., 2005, 2009). Cryo-EM has also been useful for identifying binding sites for several RyR modulatory proteins which interact with the cytoplasmic domain of the channel (Samso et al., 2005).

A. Modulatory Proteins

Many proteins complex directly and indirectly with the N-terminal cytoplasmic domain of RyR2 including the 12.6 kDa FK506-binding protein (calstabin2 or FKBP12.6) (Timerman et al., 1994), protein kinase A (PKA) (Marx et al., 2000), calcium/calmodulin-dependent kinase II (CaMKII) (Wehrens et al., 2004b), phosphodiesterase 4D3 (PDE4D3) (Lehnart et al., 2005), calmodulin (CaM) (Meissner & Henderson, 1987), protein phosphatases 1 and 2A (PP1 and PP2A) (Marx et al., 2000), and Sorcin (Farrell et al., 2004). Calsequestrin, junctin, and triadin have been proposed to complex with the C-terminus of RyR2 (Gyorke & Terentyev, 2008) (Fig. 1).

1. Calstabin2 (FKBP12.6)

Calstabin2 (FKBP12.6) is a 12.6 kDa immunophilin originally identified by its ability to bind to FK506, a common immunosuppressant used in organ transplantation. FKBP12.6 has peptidyl-prolyl *cis/trans*-isomerase (rotamase) activity that is highly conserved among all FKBP isoforms (Marks, 1996). FKBP12 and FKBP12.6 have been renamed calstabin1 and calstabin2 reflecting their roles as RyR1 and RyR2 Ca^{2+} channel stabilizers, respectively (calcium channel-stabilizing binding proteins) (Wehrens & Marks, 2003).

In 1992 calstabin1 (FKBP12), which is 85% similar to calstabin2 (FKBP12.6), was found to be associated with RyR1 in skeletal muscle (Jayaraman et al., 1992). Co-expressing calstabin1 with RyR1 stabilized the closed state of the channel and eliminated subconductance states of single RyR1 channels reconstituted in planar lipid bilayer (Brillantes et al., 1994). Calstabin1 also increased the probability of coupled gating, a

phenomenon whereby RyR1 channels exhibit simultaneous openings and closings (Marx et al., 1998). Pharmacologic depletion of calstabin1 from RyR1 shifted the Ca^{2+} dependence of the channel leftward resulting in increased Po (Brillantes et al., 1994). Calstabin2 was subsequently shown to colocalize with RyR2 in the heart (Timerman et al., 1994) and dissociating calstabin2 from RyR2 enhanced channel Po (Kaftan et al., 1996; Xiao et al., 1997). Genetic deletion of calstabin2 enhanced SR Ca^{2+} release in isolated cardiomyocytes (Xin et al., 2002).

Cryo-EM studies on RyR1 have demonstrated that calstabin1 binds to the channel at least 130 Å away from the pore (Samso et al., 2009), and it has been proposed that clastabin1 does not directly interact with the pore. The calstabin2-binding site on RyR2 is controversial. Using yeast two-hybrid and site-directed mutagenesis Ile2427 and Pro2428 were identified as the peptidyl–prolyl bond to which FKBP12.6 binds (Marx et al., 2000). Ile2427 was proposed to be responsible for the selectivity of RyR2 for calstabin2, as replacing this residue with a valine, which is naturally present in RyR1, causes calstabin1 to bind to RyR2 instead of calstabin2 (Gaburjakova et al., 2001). This selectivity may explain why calstabin2 colocalizes with RyR2 in the heart despite the higher concentration of calstabin1 in heart muscle cytosol. However, others using serial carboxyl terminal deletions identified a region between amino acids 1815 and 1855 as the calstabin2-binding site on RyR2 (Masumiya et al., 2003). A crystal structure of the ryanodine receptor providing atomic resolution may be necessary before consensus can be achieved. Isomerase activity does not appear to be necessary for the RyR stabilizing properties of these immunophilins (Huang et al., 2006; Timerman et al., 1995).

Others have challenged the findings that calstabin2 stabilizes the closed state of RyR2 (Timerman et al., 1996; Xiao et al., 2007a). Chen and colleagues observed that ryanodine binding to cell lysates of HEK293 cells expressing RyR2 was similar whether or not RyR2 was co-expressed with calstabin2 (Xiao et al., 2007a). Additionally, the Po of recombinant RyR2 reconstituted in planar lipid bilayer was similar in the absence and presence of calstabin2 (Xiao et al., 2007a). Methodological differences between single channel studies including the use of different charge carriers (K^+ vs. Ba^{2+} vs. Ca^{2+}), absence or presence of potential difference across the bilayer membrane, [ATP] and [Mg^{2+}], [Ca^{2+}] in the *cis* and *trans* compartments may partially explain these conflicts. However, many other studies now suggest that calstabin2 modulates RyR2 activity (Chen et al., 2010; Gellen et al., 2008; Hu et al., 2010; Noguchi et al., 2008; Zhang et al., 2008, 2009).

2. *PKA and CaMKII*

The PKA holoenzyme comprises two catalytic and two regulatory subunits that is targeted to the RyR2 channel via binding to the muscle A kinase anchoring protein (mAKAP) (Marx et al., 2000). mAKAP binds to leucine/

isoleucine zipper domains on RyR2 at amino acids 3003–3039 (Marx et al., 2001). PKA becomes activated when cyclic adenosine monophosphate (cAMP) binds to the regulatory subunits causing them to dissociate from the catalytic subunits. CaMKII is a dodecameric holoenzyme activated by Ca^{2+}-bound CaM (Couchonnal & Anderson, 2008). Activated PKA and CaMKII phosphorylate Ser2809 and 2815, respectively, on RyR2 (Marx et al., 2000; Wehrens et al., 2004b).

Ser2809 on canine and human RyR2 (Ser2808 on murine RyR2) was originally identified as a CaMKII phosphorylation site (Witcher et al., 1991). However, in these experiments the RyR2 peptide that was identified as being phosphorylated by endogenous CaMKII included both Ser2809 and Ser2815 (Ser2814 in murine RyR2). Moreover, experiments using phosphospecific antibodies have suggested that both PKA and CaMKII phosphorylate Ser2809 (Rodriguez et al., 2003). However, studies using site-directed mutagenesis of recombinant RyR2 have demonstrated that PKA specifically phosphorylates Ser2809 (Marx et al., 2000; Wehrens et al., 2004b, 2006) and CaMKII specifically phosphorylates Ser2815 (Kushnir et al., 2010b; Wehrens et al., 2004b). The observations that PKA but not CaMKII phosphorylates RyR1 and that RyR1 contains only a serine residue corresponding to Ser2809 (Ser2845) but not to Ser2815 (Witcher et al., 1991) further support the proposed specificity of these phosphorylation sites. Other reports suggest that serine 2030 (murine RyR2) is the major PKA phosphorylation site on RyR2 and that phosphorylation at this site increases the sensitivity of the channel to luminal Ca^{2+} (Xiao et al., 2005, 2007b). Studies supporting (Benkusky et al., 2007) and challenging (Huke & Bers, 2008; Wehrens et al., 2006) Ser2030 as a physiological RyR2 PKA phosphorylation site have been reported.

PKA phosphorylation of RyR2 activates the channel (Valdivia et al., 1995), at least in part by increasing the sensitivity of RyR2 to cytosolic Ca^{2+} (Marx et al., 2000). It has been proposed that PKA phosphorylation of RyR2 causes dissociation of calstabin2 from the channel complex as a result of steric repulsion between the negatively charged phosphate group that is covalently linked to RyR2 and Asp37 on calstabin2 (Huang et al., 2006; Marx et al., 2000). Supporting this hypothesis, recombinant mutant RyR2 engineered with aspartic acid in place of Ser2808 (RyR2-S2808D, murine RyR2) has reduced binding of calstabin2 and increased Po at diastolic $[Ca^{2+}]$ (Wehrens et al., 2006).

The observation that calstabin2 depleted channels have increased Po which can be further enhanced by PKA phosphorylation (Wehrens et al., 2003) supports the proposal that PKA phosphorylation of RyR2 also enhances RyR2 activity independent of calstabin2 depletion. CaMKII phosphorylation of RyR2 at Ser2815 increases the Po of RyR2 by sensitizing the channel to cytosolic Ca^{2+}, though unlike PKA this does not dissociate calstabin2 from the channel (Wehrens et al., 2004b). Cellular evidence that

CaMKII phosphorylation of RyR2 enhances the activity of the channel is based on the observation that Ca^{2+} transient amplitude is reduced by pharmacologic inhibition of CaMKII with KN-93, under conditions of matched $I_{CaV1.2}$ (L-type Ca^{2+} channel current) and SR Ca^{2+} load (Li et al., 1997). Furthermore, cardiomyocytes isolated from cardiac-specific CaMKII overexpressing mice have enhanced fractional release of SR Ca^{2+} (Maier et al., 2003).

Studies measuring small spontaneous RyR-mediated Ca^{2+} release events, Ca^{2+} sparks, in permeabilized cardiomyocytes have suggested that PKA phosphorylation of RyR2 does not play a role in isoproterenol (Iso) enhancement of Ca^{2+} release (Li et al., 2002). However, in this study the investigators artificially clamped cytosolic [Ca^{2+}] at either 10 or 50 nM in order to reduce spark frequency to facilitate their measurements (Li et al., 2002).

While some groups have demonstrated that PKA phosphorylation of RyR2 causes depletion of calstabin2 from the channel (Blayney et al., 2010; George et al., 2003) others have not (Stange et al., 2003; Xiao et al., 2004). Recently it has become apparent that nitrosylation (Aracena et al., 2005; Bellinger et al., 2009) and oxidation (Zissimopoulos et al., 2007) of RyR also affect the binding of calstabin to the channel. Additionally variations in the molar ratios of RyR2 and calstabin2 in heterologous systems can influence the amount of calstabin2 bound to PKA phosphorylated channels. Thus, differences in these parameters, which are sometimes not assessed, may explain the divergent findings that have been reported.

Cryo-EM studies have reported a 105–120 Å distance between the PKA phosphorylation site, Ser2808, and the putative calstabin2-binding site (Meng et al., 2007). This challenges the hypothesis that phosphorylation at one site directly interferes with binding at the other. Similar analysis has demonstrated that Ser2030 is too far from the calstabin2-binding site for phosphorylation at this site to affect calstabin2 binding (Jones et al., 2008). However, while these studies are certainly informative, in the absence of an atomic resolution structure, they fall short of providing definitive assignments for the locations of these regulatory sites on the RyR2 channel cytoplasmic domain. These results do not rule out the possibility that PKA phosphorylation at Ser2808 indirectly modulates calstabin2 binding to RyR2.

3. *Phosphodiesterase 4D3 and Protein Phosphatases 1 and 2a*

PDE4D3 degrades cAMP and plays an important role in regulating spatial and temporal cAMP levels (Zaccolo & Pozzan, 2002). Both PDE4D3 and PKA bind to mAKAP (Dodge et al., 2001) as part of the RyR2 macromolecular complex (Lehnart et al., 2005). PKA phosphorylation of PDE4D3 at Ser54 and Ser13 increases enzymatic activity twofold (Sette & Conti, 1996) and enhances the affinity of PDE4D3 to mAKAP (Carlisle Michel et al., 2004), respectively. This may provide negative

feedback for modulating the activating effects of PKA. The localization of PDE4D3 to RyR2 enables regulation of local cAMP levels near the channel (Lehnart et al., 2005).

PP1 and PP2a indirectly associate with RyR2 through spinophilin and PR130, respectively, and regulate channel activity by dephosphorylating phosphorylated channels (Marx et al., 2001). It has been proposed that PP1 is the predominant phosphatase which dephosphorylates Ser2808 and Ser2814 and that PP2a contributes to dephosphorylating Ser2814 (Huke & Bers, 2008).

4. *Calmodulin*

CaM is a ubiquitously expressed, highly conserved, 17 kDa protein that contains four Ca^{2+}-binding EF hands, two on each end of the protein. CaM preferentially inhibits RyR2 at $[Ca^{2+}] < 10\,\mu M$ by binding to a region on RyR2 comprising amino acids 3583–3603 (Yamaguchi et al., 2003). CaM may function to assist closing RyR2 following SR Ca^{2+} release in EC-coupling (Xu & Meissner, 2004). Supporting this hypothesis, cardiomyocytes isolated from mice engineered with RyR2 lacking the CaM-binding site have prolonged Ca^{2+} transients (Yamaguchi et al., 2007).

5. *Sorcin*

Sorcin is a 22 kDa Ca^{2+}-binding protein localized to the dyadic space between RyR2 and $Ca_v1.2$, which associates with RyR2 when $[Ca^{2+}]$ are elevated. In planar lipid bilayers sorcin reduces the Po of RyR2 (Lokuta et al., 1997) and dialyzing sorcin into cardiomyocytes reduces the amplitude of SR Ca^{2+} release without affecting L-type Ca^{2+} channel current (Farrell et al., 2003). These data suggest that sorcin, similar to CaM, may inhibit RyR2 following SR Ca^{2+} release during EC coupling to prevent the formation of positive feedback loops.

6. *Calsequestrin, Triadin, and Junctin*

Calsequestrin, triadin, and junctin have been proposed to form a complex with RyR2 in the SR lumen with triadin and junctin linking calsequestrin to the channel (Gyorke & Terentyev, 2008). Calsequestrin is a low-affinity, high-capacity Ca^{2+}-binding protein which sequesters Ca^{2+} in the SR. According to a current model elevation of luminal Ca^{2+} weakens the interactions between calsequestrin, triadin, and junctin causing an increase in RyR2 Po which normalizes SR Ca^{2+} load (Zhang et al., 1997).

III. Regulation of RyR2 in the Cardiac Response to β-adrenergic Receptor Activation

Early studies using radiolabeled ryanodine to detect channel openings noted that phosphorylation of RyR2 by PKA (Takasago et al., 1989) and

CaMKII (Takasago et al., 1991) increased channel activity. Subsequent studies in planar lipid bilayer demonstrated that PKA phosphorylation of Ser2809 and CaMKII phosphorylation of Ser2815 caused a leftward shift in the sensitivity of RyR2 to cytosolic Ca^{2+} (Wehrens et al., 2004b). Similarly, PKA phosphorylation of RyR2 was reported to increase channel Po to ~1.0 following flash photolysis-induced exposure of the channel to 10 μM cytosolic Ca^{2+} compared to a Po of ~0.75 for nonphosphorylated channels (Valdivia et al., 1995).

Cellular studies analyzing the effect of PKA phosphorylation on RyR2 function have been controversial. In 2004 Bers and colleagues (Ginsburg & Bers, 2004) demonstrated that under conditions where $I_{Cav1.2}$ trigger and SR Ca^{2+} load were constant, addition of the β-adrenergic receptor (AR) agonist Iso enhanced the kinetics of SR Ca^{2+} release but did not induce any changes in peak systolic Ca^{2+} transient amplitude. These findings were challenged by Niggli and colleagues (Ogrodnik & Niggli, 2010) who demonstrated that Iso increased the kinetics and Ca^{2+} transient amplitude in cells with matched Ca^{2+} trigger and SR Ca^{2+} load. Niggli and colleagues used UV-flash photolysis to rapidly uncage Ca^{2+} in order to activate RyR2, while Bers and colleagues used patch clamp to depolarize the sarcolemma to induce SR Ca^{2+} release. Additionally, Bers and colleagues used phospholamban-S16A mice that cannot be phosphorylated by PKA to maintain comparable SR Ca^{2+} loads in the absence and presence of Iso while Niggli and colleagues varied their pre-pacing SR loading protocols. Contrasting these two studies highlights the technical challenge associated with isolating the contribution of RyR2 modulation to SR Ca^{2+} release during β-adrenergic stimulation.

An intriguing possibility has been proposed that PKA phosphorylation of RyR2 contributes to synchronizing SR Ca^{2+} release (Lakatta, 2004; Wang & Wehrens, 2010). PKA phosphorylated RyR2 has a Po of ~1.0 in the moments following a Ca^{2+} trigger compared to ~0.75 for nonphosphorylated channels (Valdivia et al., 1995), which would facilitate the likelihood of two channels opening simultaneously following membrane depolarization. Cellular studies employing a high-affinity Ca^{2+} indicator combined with EGTA (a slow Ca^{2+} buffer) to elicit Ca^{2+} spikes demonstrated that Iso enhances the simultaneous release of Ca^{2+} from multiple RyR2 clusters (Song et al., 2001). PKA phosphorylation of RyR2 may increase the speed and fidelity with which RyR2 releases Ca^{2+} in response to $I_{Cav1.2}$ (Zhou et al., 2009).

Similarly, synchronization studies in SAN cells (SANC) have suggested that PKA phosphorylation of RyR2 during a β-adrenergic response may contribute to heart rate regulation (Lakatta, 2004). Small Ca^{2+} release events have been observed during the diastolic depolarization phase in SANC and are hypothesized to activate inward NCX current and contribute to the rate of depolarization (Vinogradova et al., 2002). Taken together

these studies suggest that PKA phosphorylation of RyR2 may contribute to enhancing cardiac chronotropy and inotropy during a β-adrenergic response.

Two recent studies, however, have challenged the potential role of PKA phosphorylation of RyR2 in the cardiac response to β-adrenergic activation (Benkusky et al., 2007; MacDonnell et al., 2008). In these studies mice engineered with RyR2 that cannot be PKA phosphorylated (RyR2-S2808A) were reported to have a normal inotropic and chronotropic response to Iso treatment using *in vivo* and *ex vivo* cardiac function studies (MacDonnell et al., 2008). Of note, cardiomyocytes isolated from the RyR2-S2808A mice had a blunted enhancement of systolic Ca^{2+} transient amplitude at 3 Hz but not at lower frequencies (Benkusky et al., 2007).

CaMKII activity and subsequent phosphorylation of Ser2815 on RyR2 are increased at faster heart rates (Chelu et al., 2009; Wehrens et al., 2004b) due to the "Ca^{2+} memory" properties of CaMKII, whereby during each contraction a fraction of the CaMKII monomers become activated and that faster frequencies cause a stepwise increase in the number of activated monomers (De Koninck & Schulman, 1998). This led to the proposal that CaMKII-dependent phosphorylation of RyR2 contributes to the force-frequency response, the observation that cardiac contractility increases as a function of heart rate (Li et al., 1997; Wehrens et al., 2004b). This hypothesis has been recently demonstrated in mice engineered lacking the CaMKII phosphorylation site on RyR2 (RyR2-S2814A) which have blunted contractility at faster heart rates (Kushnir et al., 2010b).

Recent studies indicate that β-AR stimulation activates CaMKII, in addition to activating PKA (Ferrero et al., 2007). While the exact mechanism of this phenomenon is unclear it is possible that CaMKII is activated by higher levels $[Ca^{2+}]_{cyto}$ or by activation by another kinase such as PKA or guanine nucleotide exchange protein (Epac) (Pereira et al., 2007). Indeed, transgenic mice with cardiac-specific inhibition of CaMKII have fewer SR Ca^{2+} release events during diastolic depolarization in SANC as well as a blunted heart rate in response to Iso treatment (Wu et al., 2009). This suggests that CaMKII-mediated phosphorylation of RyR2 may contribute to the cardiac response to β-adrenergic activation.

PKA phosphorylation of sorcin relieves its natural inhibition of RyR2. This suggests that phosphorylation of sorcin downstream of β-AR activation may contribute to enhancing RyR2 Ca^{2+} release during EC coupling (Lokuta et al., 1997).

Eisner and colleagues have challenged the hypothesis that modulation of RyR2 Ca^{2+} release contributes to the cardiac "fight or flight" response. Exposing cardiomyocytes to low-dose caffeine, which sensitizes RyR2 to activation by Ca^{2+}, only transiently potentiated SR Ca^{2+} release as SR Ca^{2+} stores are rapidly reduced under these conditions (Trafford et al., 2000). These results, however, also demonstrated that sensitizing RyR2 to

activating Ca^{2+} increases the fractional release of Ca^{2+} from the SR (Ca^{2+} released as a function of SR Ca^{2+} load). Thus, under physiologic conditions the combination of enhanced rate of SR Ca^{2+} uptake (as occurs during a β-adrenergic response) and increased fractional release of Ca^{2+} through RyR2 would result in a sustainable elevation in Ca^{2+} transient amplitude.

IV. RyR2 Dysfunction in Heart Disease

A. RyR2 in Heart Failure

Studies in the 1990s reported alterations in intracellular Ca^{2+} handling in cardiomocytes isolated from hearts at different stages of heart failure (HF). These changes included reduced Ca^{2+} transient amplitude, increased Ca^{2+} transient duration, prolonged Ca^{2+} transient decay time, as well as reduced SR Ca^{2+} load (Hobai & O'Rourke, 2001). This suggested that reduced systolic Ca^{2+} transient amplitude secondary to reduced SR Ca^{2+} stores was responsible for the decreased contractility and reduced cardiac output in HF. Cardiomyocytes isolated from hearts in end-stage HF also had elevated diastolic Ca^{2+} levels, which was not observed in early-stage HF hearts (Zaugg & Buser, 2001). Elevated diastolic Ca^{2+} levels in the cytosol generate transient inward current (I_{ti}) which causes DADs. Additionally, elevated diastolic [Ca^{2+}] induces cell-to-cell uncoupling (Kleber, 1992), which slows the velocity of a propagating impulse, increasing the propensity for reentry circuit formation in the presence of an arrhythmogenic substrate (Fig. 2).

Pathologic reduction in SERCA2a function and expression as well as enhanced NCX activity was originally proposed to explain why SR Ca^{2+} load is lower in HF (Hobai & O'Rourke, 2001; Mercadier et al., 1990; Pogwizd & Bers, 2004). Some suggested that increased NCX activity compensated for the reduced SERCA2a activity in early-stage HF but that continuous uncompensated decline of SERCA2a activity in end-stage HF resulted in elevated baseline Ca^{2+} (Zaugg & Buser, 2001). In fact, overexpressing SERCA2a in cardiomyocytes isolated from patients with failing hearts improves contraction velocity and decreases diastolic [Ca^{2+}] and has been proposed as a possible therapy for HF (del Monte et al., 1999).

Over the past 10 years the role of pathological diastolic Ca^{2+} leak through dysfunctional RyR2 has been recognized as an important contributor to altered Ca^{2+} handling in HF (Marx et al., 2000). The original HF RyR2 Ca^{2+} leak hypothesis was that the chronic hyperadrenergic state observed in HF patients induced chronic PKA hyperphosphorylation of RyR2 at Ser2808, causing depletion of calstabin2 (Marx et al., 2000) from the channel complex. The term hyperphosphorylation describes RyR2 in which 3–4 of the four RyR2 monomers are chronically PKA phosphorylated. PKA hyperphosphorylated/calstabin2 depleted channels

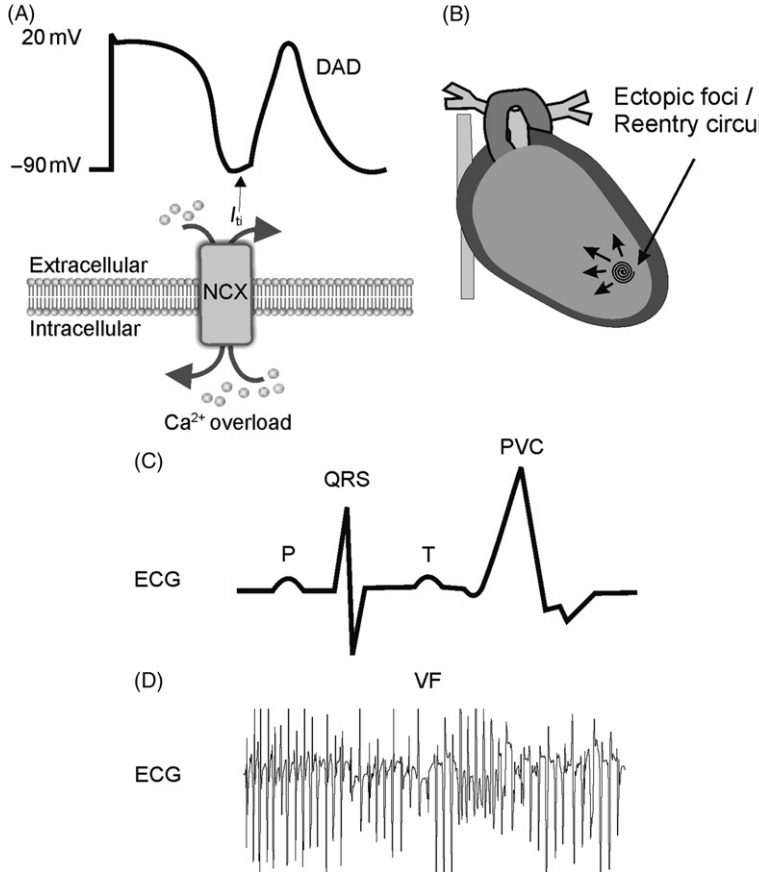

FIGURE 2 Arrhythmias induced by cytosolic Ca^{2+} overload: (A) Elevated cytosolic [Ca^{2+}] causes transient inward current through the Na^{+}/Ca^{2+} exchanger (NCX) which can generate delayed after depolarizations (DADs). (B) Cardiomyocytes eliciting DADs can become ectopic foci of automaticity which can evolve into reentry circuits to cause (C) premature ventricular contractions (PVCs) on the electrocardiogram (ECG). (D) If uncorrected this may deteriorate into ventricular fibrillation (VF) and sudden cardiac death.

are sensitized to cytosolic Ca^{2+} leading to inappropriate Ca^{2+} release during diastole, referred to as a diastolic SR Ca^{2+} leak. Ca^{2+} leak would reduce SR Ca^{2+} stores and activate I_{ti} (Lehnart et al., 2004b).

The HF RyR2 Ca^{2+} leak hypothesis is supported by studies demonstrating that HF patients have PKA hyperphosphorylated and calstabin2 depleted RyR2. Furthermore, patients whose cardiac function was restored by implantable left ventricular assist devices (LVAD) had reduced levels of circulating catecholamines (Estrada-Quintero et al., 1995) and reduced phosphorylation of RyR2 at Ser2809. Normalization of the RyR2 complex

is associated with the improved cardiac function observed during the short period post-LVAD explantation (Marx et al., 2000).

The HF RyR2 Ca^{2+} leak hypothesis has been challenged based on the observation that β-AR density on cardiomyocytes is reduced in HF (Bristow et al., 1982) and that this coincides with a global reduction in cytosolic cAMP levels. Accordingly it is unclear how the presence of a chronic hyperadrenergic state in HF can lead to chronic PKA hyperphosphorylation of RyR2. This paradox can be explained by the general remodeling of the RyR2 macromolecular complex and depletion of phosphatases (Reiken et al., 2001) and PDE4D3 (Lehnart et al., 2005) that occurs in HF. Localized depletion of PDE4D3 and phosphatases can induce discrete microdomains of elevated levels of cAMP in the vicinity of RyR2 (Lehnart et al., 2005) and decreased rate of dephosphorylation of a hyperphosphorylated channel.

The HF RyR2 Ca^{2+} leak model provides a novel mechanism to explain the therapeutic efficacy of β-AR blockers in HF: by preventing stimulation of β-ARs and downstream activation of PKA in failing hearts, thereby reducing phosphorylation at Ser2809 and causing calstabin2 to rebind to the channel and reduce SR Ca^{2+} leak. This proposal is supported by studies showing that HF patients taking β-blockers have reduced phosphorylation of RyR2 at Ser2809 and enhanced calstabin2 binding to RyR2 (Reiken et al., 2003). Furthermore, RyR2 isolated from HF patients which was reconstituted in planar lipid bilayer had elevated Po at diastolic [Ca^{2+}] which was reduced in patients on β-blockers (Reiken et al., 2003).

Evidence from multiple animal models supports a role for PKA hyperphosphorylation of RyR2 in HF progression (Kushnir et al., 2010a). Mice engineered with an RyR2-S2808A mutation, RyR2 that cannot be PKA phosphorylated, were not depleted of calstabin2 4 weeks post-myocardial infarction (MI) and were protected from the deleterious progression of cardiac dysfunction observed in wild-type (WT) mice 4 weeks post-MI (Wehrens et al., 2006). Mice engineered lacking PDE4D3 develop cardiomyopathy and arrhythmias which coincide with progressive PKA hyperphosphorylation of RyR2 and calstabin2 depletion. These mice also had accelerated decline in cardiac function post-MI. A cross between the PDE4D3 deficient mice and the RyR2-S2808A mice results in mice which are protected from the deleterious effects of PDE4D3 deficiency (Lehnart et al., 2005). These PDE4D3 studies provide a novel mechanism to explain the arrhythmogenic propensity of PDE inhibitors such as theophylline. It should be noted that these drugs have also been reported to enhance the sensitivity of RyR2 to luminal Ca^{2+} which could also explain their arrhythmogenic potential (Kong et al., 2008).

Direct evidence that calstabin2 depletion from RyR2 contributes to HF progression comes from transgenic mice overexpressing a mutant D37V-calstabin2, which remains bound to PKA phosphorylated RyR2 channels. Cardiac dysfunction post-MI is ameliorated in these mice (Huang et al.,

2006). Mice engineered lacking calstabin2 suffer from exercise-induced ventricular tachycardia (VT) which parallels the presence of DADs in cardiomyocytes isolated from these mice (Wehrens et al., 2003). Furthermore, RyR2 isolated from calstabin2-deficient mice exhibits relatively normal Po at baseline which is drastically increased when the mice are exercised.

The HF RyR2 Ca^{2+} leak hypothesis introduces novel therapeutic opportunities for HF. JTV-519 (K201), a 1,4-benzothiazepene derivative, was originally discovered based on its cardioprotective effects in a myofibrillar overcontraction model of myocardial injury, possibly related to the drugs ability to block intracellular Ca^{2+} overload (Kaneko, 1994). While early reports lacked a clear mechanism of action for JTV-519 later studies reported that JTV-519 nonspecifically inhibited transmembrane Na^{+}, Ca^{2+}, and K^{+} currents (Kimura et al., 1999) and that the cardioprotective effects of the drug were abolished when hearts were pretreated with inhibitors of protein kinase C (Inagaki et al., 2000; Ito et al., 2000) or nitric oxide synthase (Kawabata et al., 2002).

In 2003 Matsuzaki and colleagues, using microsomes isolated from canine failing hearts, reported that JTV-519 stabilized RyR2 by improving binding of calstabin2 to RyR2 (Kohno et al., 2003). *In vivo* HF studies demonstrated that pretreatment with JTV-519 prevented rapid right ventricular pacing-induced HF in canines (Yano et al., 2003). Studies with homozygous and heterozygous calstabin2-deficient mice provided *in vivo* evidence that the cardioprotective effects of JTV-519 depend on stabilizing the interaction of calstabin2 with RyR2. Exercising homozygous and heterozygous calstabin2-deficient mice induces fatal ventricular arrhythmias due to SR Ca^{2+} leak which can be prevented by pretreating heterozygous calstabin2-deficient mice with JTV-519. JTV-519 does not protect the calstabin2-deficient mice (Wehrens et al., 2004a). Furthermore, treating WT mice with JTV-519 ameliorated the decline in cardiac function post-MI while treating calstabin2-deficient mice with JTV-519 did not (Wehrens et al., 2005). The observation that JTV-519 does not alter the gating properties of non-diseased ryanodine receptor channels and that the drug causes no observable effects in healthy dogs and mice substantiates its therapeutic potential (Lehnart et al., 2008; Wehrens et al., 2004a; Yano et al., 2003).

The HF RyR2 Ca^{2+} leak hypothesis has generated significant controversy with reports supporting (Ono et al., 2000; Yano et al., 2000) and challenging it (Benkusky et al., 2007; Jiang et al., 2002; MacDonnell et al., 2008; Xiao et al., 2007a). Chen and colleagues have published a series of reports challenging this hypothesis. In one study, this group reported that RyR2 isolated from human and canine failing hearts had similar levels of associated calstabin2 and similar Ca^{2+} sensitivity as RyR2 from non-failing hearts (Jiang et al., 2002). The investigators concluded that the reduction in SR Ca^{2+} stores was secondary to a reduction in SERCA2a expression. In another study it was reported that JTV-519 suppresses spontaneous Ca^{2+}

release independent of calstabin2 binding to RyR2 (Hunt et al., 2007). This group also reported that they were unable to induce arrhythmias in calstabin2-deficient mice using combined administration of epinephrine and caffeine (Xiao et al., 2007a). Valdivia and colleagues demonstrated that the RyR2-S2808A mice are not protected from HF induced by thoracic aortic constriction (Benkusky et al., 2007). Of note, in this study the investigators reported that fractional shortening was reduced in WT mice but not in the RyR2-S2808A mice suggesting that the mutation imparted cardioprotection.

Alternative mechanisms have been proposed to explain SR Ca^{2+} leak in HF. Disulfide oxidation of free cysteine residues on RyR2 increases the sensitivity of the channel to luminal Ca^{2+} in lipid bilayer as well as enhances SR Ca^{2+} leak, manifested by reduced SR Ca^{2+} load, in cardiomyocytes (Terentyev et al., 2008). Matsuzaki and colleagues have proposed that redox modification of RyR2 in HF disrupts the interaction between N-terminal (amino acids 1–600) and central domains (2000–2500) of the channel complex, which causes dissociation of CaM from the channel and diastolic Ca^{2+} leak (Oda et al., 2005; Ono et al., 2010; Yano et al., 2009). Furthermore this group has proposed that JTV-519 binds to RyR2 between amino acids 2114 and 2149 and stabilizes the inter-domain interactions of the channel (Yamamoto et al., 2008). These studies also suggest that enhancing binding of CaM to RyR2 may have therapeutic potential in HF.

Chen and colleagues have proposed that RyR2 has increased sensitivity to luminal Ca^{2+} in HF so that when SR Ca^{2+} load is elevated under stressful conditions (i.e., activation of the β-AR cascade) this leads to inappropriate Ca^{2+} "spillover" during diastole (store overload-induced Ca^{2+} release or SOICR) (Xiao et al., 2007). However, it is unclear according to this model how the sensitivity of RyR2 to luminal Ca^{2+} is modified in HF (Fig. 3).

CaMKIIδ levels are elevated in human HF samples (Hoch et al., 1999) suggesting that CaMKII phosphorylation of RyR2 may contribute to the pathogenesis of HF. This hypothesis is supported by reports that there is an increase in CaMKII-dependent phosphorylation of RyR2 in HF, which enhances SR Ca^{2+} leak (Ai et al., 2005). Mice overexpressing CaMKIIδ have enhanced Ca^{2+} spark frequency (Maier et al., 2003) and develop cardiomyopathy (Zhang et al., 2003). Additionally, mice expressing the CaMKII inhibitory peptide AC3-I are cardioprotected from ischemic HF (Zhang et al., 2005) and pressure overload-induced HF (Ling et al., 2009). Based on these studies it has been proposed that CaMKII inhibitors may have therapeutic potential for preventing HF progression. However, mice engineered with RyR2 that cannot be phosphorylated by CaMKII (RyR2-S2814A) are not cardioprotected in a model of ischemic HF suggesting that the pathological consequences of CaMKII activation in HF are not a result of CaMKII phosphorylation of RyR2 (Kushnir et al., 2010b).

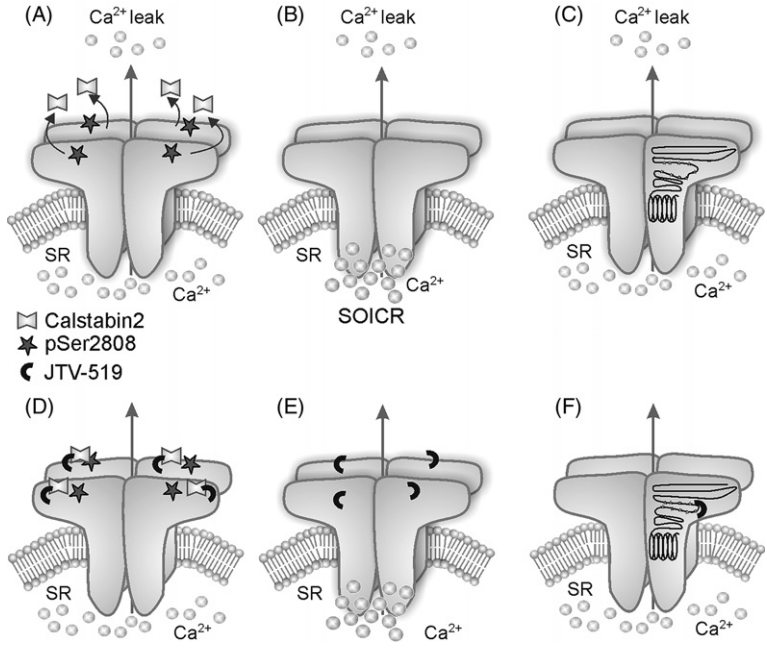

FIGURE 3 Mechanisms of RyR2 Ca^{2+} leak in HF: (A) PKA hyperphosphorylation of RyR2 at Ser2808 and depletion of calstabin2 from the channel causes SR Ca^{2+} leak. (D) The therapeutic efficacy of JTV-519 is based on the ability of the drug to stabilize the interaction between calstabin2 and RyR2. (B) Store overload-induced Ca^{2+} release (SOICR), RyR2 has increased sensitivity to luminal [Ca^{2+}] which causes Ca^{2+} to leak out of the channel. (E) According to this model JTV-519 stabilizes RyR2 independent of calstabin2. (C) Unzipping of the amino and central domains of RyR2 causes the channel to become leaky. (F) JTV-519 stabilizes the zipped state of the channel.

B. RyR2 in Atrial Fibrillation

Atrial fibrillation (AF) is a major cause of morbidity and mortality and the pathophysiology of this disease has not been elucidated. The clinical signs of AF are an irregularly irregular rhythm with a concomitant loss of P-waves (atrial depolarization) and the presence of F-waves in the electrocardiogram. Fibrillating atria lack unifocal pacing and may consist of multiple small reentry circuits or ectopic triggers (Nattel et al., 2008).

Early studies on canine atrial myocytes reported altered Ca^{2+} handling in cells isolated from atria with AF (Sun et al., 1998). RyR2 isolated from canine atria in AF was found to be PKA hyperphosphorylated (Chelu et al., 2009; Vest et al., 2005), depleted of calstabin2, and had increased Po at diastolic [Ca^{2+}] (Vest et al., 2005) suggesting a link between SR Ca^{2+} leak and AF. Furthermore calstabin2-deficient mice have an increased propensity

for developing AF and atrial myocytes isolated from these mice have enhanced SR Ca^{2+} leak (Sood et al., 2008).

Human and goat atria with AF have elevated levels of activated CaMKII and enhanced phosphorylation of RyR2 at Ser2815 suggesting that CaMKII phosphorylation of RyR2 may be involved in the pathogenesis of AF (Chelu et al., 2009; Neef et al. 2010). This is supported by observations that mice with RyR2 that cannot be phosphorylated by CaMKII (RyR2-S2814A) are protected from developing AF (Chelu et al., 2009). Additionally, Ca^{2+} spark frequency and diastolic [Ca^{2+}] are both elevated and SR Ca^{2+} stores are depleted in atrial myocytes isolated from human atria with AF (Neef et al., 2010). Pharmacological inhibition of RyR2 with tetracaine reduces cytosolic Ca^{2+} levels back to baseline confirming that Ca^{2+} leak through RyR2 contributes to the Ca^{2+} abnormalities in these cells (Neef et al., 2010).

The role of RyR2 Ca^{2+} leak in arrhythmogenesis has been challenged based on the observation that increasing RyR2 Po with low-dose caffeine induces Ca^{2+} waves only at high SR Ca^{2+} loads. Accordingly, pathological modification of RyR2 which would induce Ca^{2+} leak would ultimately lead to a reduction in SR Ca^{2+} load to the point where Ca^{2+} waves no longer appear (Venetucci et al., 2007).

C. RyR2 in Catecholaminergic Polymorphic Ventricular Tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare, familial, exercise-induced polymorphic VT that occurs in the absence of gross myocardial morphological abnormalities. Approximately 10 years ago two independent groups identified several RyR2 mutations in families of patients with CPVT (Priori et al., 2001; Swan et al., 1999). To date 71 mutations linked to CPVT have been identified in three "hot spot" regions of RyR2 (a current list is available at http://www.fsm.it/cardmoc).

Three hypotheses have been proposed to explain how mutations in RyR2 lead to SR Ca^{2+} leak and arrhythmias. Marks and colleagues have observed that CPVT mutants RyR2-S2246L, RyR2-R2474S, and RyR2-R4497C all have reduced affinity for calstabin2 (Wehrens et al., 2003). At rest these channels have similar Po compared to WT channels; however, PKA phosphorylation of these mutant channels results in increased Po at diastolic [Ca^{2+}]. This pathological phenotype can be prevented by addition of calstabin2-D37S, a mutant calstabin2 that binds to phosphorylated RyR2 (Wehrens et al., 2003). Furthermore, treating mutant CPVT channels with JTV-519 restores calstabin2 binding to the channels and reduces Po at diastolic [Ca^{2+}] (Lehnart et al., 2004a). Mice engineered with the RyR2-R2474S human CPVT mutation have normal sinus rhythm at baseline and develop ventricular arrhythmias when exercised. These arrhythmias can be

prevented by treating the mice with S107, a drug similar to JTV-519, which rebinds calstabin2 to the CPVT mutant channels and prevents diastolic SR Ca^{2+} leak (Lehnart et al., 2008).

S107 was discovered as part of a project to identify compounds with similar RyR/calstabin stabilizing properties as JTV-519 that did not significantly interact with other ion channels. Other favorable drug properties, such as oral availability and stability, were also taken into account during this screen (Bellinger et al., 2008). The therapeutic efficacy of S107 was originally demonstrated in skeletal muscle where mice treated with S107 had enhanced binding of calstabin to RyR1 in skeletal muscle and improved muscle force generation and exercise capacity (Bellinger et al., 2008). S107 was also able to reduce the incidence of arrhythmias (Fauconnier et al., 2010) and improve muscle function (Bellinger et al., 2009) in mice with Duchenne muscular dystrophy by reducing pathologic SR Ca^{2+} leak in cardiac and skeletal muscle. Incidentally, the ability for S107 to stabilize dysfunctional RyR2 offers therapeutic opportunities for pathologies in other organ systems that are associated with RyR2 dysfunction. Recently, mutations in RyR2 which reduce the affinity of calstabin2 to RyR2 have been linked to seizures in humans (Johnson et al., 2010) and mice and treating these mice with S107 prevented the seizures (Lehnart et al., 2008). While the role of RyR2 in the brain is unknown this study demonstrates the therapeutic potential of drugs targeting dysfunctional RyR2 in other systems other than cardiac and skeletal muscle.

Chen and colleagues have proposed that CPVT mutations in RyR2 sensitize the channel to luminal (SR) Ca^{2+} such that under baseline conditions, where SR load is normal, there is no Ca^{2+} leak. When β-ARs are activated during a sympathetic response SR [Ca^{2+}] is elevated above the reduced threshold, causing Ca^{2+} to leak out of the SR (SOICR). The SOICR-CPVT hypothesis was developed based on the observation that HEK cells expressing recombinant CPVT-mutant-RyR2 had increased sensitivity (manifested as spontaneous Ca^{2+} oscillations) to progressively higher extracellular [Ca^{2+}] compared to cells expressing WT RyR2 (Jiang et al., 2005).

Matsuzaki and colleagues have proposed that CPVT mutations induce unzipping of the N-terminal and central domains of RyR2 which causes the channel to become leaky (Uchinoumi et al., 2010). This hypothesis is supported by experiments using a small peptide, DPc10, which intercalates with the central domain of RyR2 to cause channel unzipping and Ca^{2+} leak in WT cardiomyocytes. In cardiomyocytes isolated from RyR2-R2474S mice, a CPVT mouse model, Ca^{2+} spark frequency is elevated at baseline and is not amplified by addition of the peptide (Uchinoumi et al., 2010). CaM binding to RyR2 is disrupted by channel unzipping in mutant myocytes but not in WT cells suggesting that depletion of CaM from RyR2 secondary to channel unzipping may contribute to the pathogenesis of CPVT (Xu et al., 2010). In all these studies the zipped state of RyR2 is confirmed by changes in the signal from fluorescent markers incorporated into the channel.

Dantrolene, a drug used to prevent malignant hyperthermia in patients with mutations in RyR1 who have been exposed to volatile anesthetics, has been proposed to have therapeutic potential in heart disease by causing "re-zipping" of the amino and central domains of RyR2 (Kobayashi et al., 2009; Uchinoumi et al., 2010). RyR2 isolated from dogs with HF and mice engineered with a CPVT mutation (RyR2-R2474S) had unzipped RyR2 which could be "re-zipped" by treating the defective channels with dantrolene. Furthermore, dantrolene reduced Ca^{2+} spark frequency in cardiomyocytes isolated from these animal models. The authors in these studies have proposed that dantrolene binds to RyR2 between amino acids 601 and 620 (Kobayashi et al., 2009). This hypothesis has been challenged by reports that dantrolene specifically inhibits RyR1 and RyR3 but does not affect the gating properties of RyR2 (Zhao et al., 2001). However, in this report the investigators studied normal channels. Altogether, these data suggest that dantrolene may specifically stabilize dysfunctional RyR2 without affecting the gating properties of normal RyR2 (Kobayashi et al., 2009).

The Na^+ channel antagonist flecainide has recently been demonstrated to prevent lethal ventricular arrhythmias in mice and humans carrying CPVT mutations (Watanabe et al., 2009). Flecainide has been proposed to reduce Ca^{2+} spark amplitude by inducing brief closures of open RyR2 to subconductance states which reduces burst mass without affecting channel closed time (Hilliard et al., 2010).

D. RyR2 in Sudden Infant Death Syndrome

Sudden infant death syndrome (SIDS) describes idiopathic sudden death in infants less then 1 year of age and is a leading cause of postnatal mortality in developed countries. Mutations R2267H and S4565R in RyR2 have been identified in children that have died from SIDS (Tester et al., 2007). These mutations enhance the sensitivity of RyR2 to cytosolic Ca^{2+} during β-adrenergic stress, similar to mutations in RyR2 that cause CPVT. Mice engineered with the R176Q CPVT mutation have a higher rate of postnatal mortality. Death in these animals is due to abnormal Ca^{2+} release and ectopic activity as determined by isochronal Ca^{2+} and voltage mapping of isolated neonatal hearts (Mathur et al., 2009). Mice engineered with the highly arrhythmogenic R2474S CPVT mutation have increased rate of intrauterine lethality, which can be prevented by treating the pregnant mothers with S107 (Lehnart et al., 2008).

V. Conclusion

Many technical approaches have been used to isolate and study RyR2 function including the planar lipid bilayer, Ca^{2+} fluorescence in HEK cells,

permeablized cardiomyocytes, patch clamping, flash photolysis, and *in vivo* and *ex vivo* cardiac function studies on genetically modified mouse models. Despite many advances in our understanding of RyR2, consensus has not achieved on some fundamental questions: What are the PKA and CaMKII phosphorylation sites on RyR2? What is the effect of phosphorylation on the channel macromolecular complex and channel function? What causes RyR2 dysfunction in HF and CPVT? Notwithstanding these issues many studies now agree that diastolic SR Ca^{2+} leak through dysfunctional RyR2 contributes to the pathogenesis of HF and various arrhythmias. The development of novel experimental techniques and additional genetically engineered mouse models will help clarify many discrepancies that currently exist in the field of RyR2 research and will aid in the development of novel therapeutics to target Ca^{2+} leak in patients suffering from various forms of heart disease.

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Conflict of Interest Statement: A.R.M. is a consultant for ARMGO Pharma Inc., a start-up company that is targeting RyR2 for treatment of heart and muscle diseases.

Non standard abbreviations:

Cav1.2	cardiac L-type calcium channels
CPVT	catecholaminergic polymorphic ventricular tachycardia
FKBP	FK506-binding protein
HF	heart failure
RyR	ryanodine receptor
SAN	sinoatrial node
SR	sarcoplasmic reticulum

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Neuregulin-1/ErbB Signaling and Chronic Heart Failure

Abstract

Neuregulin-1 (NRG-1), a cardioactive growth factor released from endothelial cells, is indispensable for cardiac development, structural maintenance, and functional integrity of the heart. In recent years, a growing number of studies have focused on NRG-1 and members of the ErbB family that serve as receptors for NRG-1 in order to better understand the role of this signaling pathway in physiology and pathophysiology of the heart. An essential role for NRG-1 and ErbB in heart development and functionality has been suggested by studies in conditional NRG-1/ErbB-deficient mice and by the cardiac-related side effects of anti-ErbB2 antibody therapies used for treatment of breast cancer. *In vitro* and *in vivo* studies using recombinant human

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neuregulin-1 (rhNRG-1), which contains the epidermal growth factor (EGF)-like domain (necessary for ErbB2/ErbB4 activation), have further supported the hypothesis that NRG-1 plays an important role in heart function. Consistent with other studies, expression of rhNRG-1 not only restored normal cardiomyocytic structure altered by nutritional deficiency in cell cultures, but also improved the pumping function of the heart in several animal models of chronic heart failure (CHF). As a result of these findings, proteins involved in the NRG-1/ErbB-signaling pathway have been explored as potential drug targets for treatment of heart failure. Clinical trials to evaluate the safety and efficacy of rhNRG-1 have been conducted in both China and Australia. As predicted, rhNRG-1 treatment improved both cardiac function and reversed remodeling of the heart. Therefore, rhNRG-1 may represent a new drug for treatment of CHF with a novel therapeutic mechanism.

I. Introduction

Neuregulin-1 (NRG-1) proteins are widely expressed signaling molecules that are involved in cell differentiation, proliferation, growth, survival, and apoptosis. They transmit their signals through interactions with cell membrane receptors of the ErbB family. Activation of ErbB receptors results in the initiation of intracellular signaling cascades that modulate various physiological and etiological processes. Studies in genetically manipulated mice and in cells isolated from adult and neonatal mouse or rat hearts have shown that NRG-1/ErbB signaling not only plays an essential role in heart development, but is also important for maintenance of cardiac function in adult organisms. Various mechanisms are believed to be involved in this process, including promotion of cardiac myocyte survival, improvement of sarcomeric structure and cell–cell adhesion, and maintenance of Ca^{2+} homeostasis. Cross talk between signaling pathways downstream of NRG-1 may be important for these underlying mechanisms, leading to a complicated signaling network dependent upon NRG-1.

Emerging evidence has suggested that NRG-1/ErbB signaling is involved in human disease, including heart failure, which has prompted the development of new therapeutic agents based on this pathway. Clinical studies to evaluate the safety and efficacy of NRG-1 for chronic heart failure (CHF) patients have been conducted, and members of the NRG-1/ErbB-signaling pathway have become important drug targets for treatment of CHF.

II. NRG-1 and Its Receptors

NRG-1 was originally identified in a search for activators of ErbB2 (Holmes et al., 1992). Further characterization revealed that NRG-1

proteins serve as ligands for receptor tyrosine kinases of the ErbB family. The ErbBs are membrane-anchored proteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. All NRG-1 isoforms share the epidermal growth factor (EGF)-like signaling domain, which is sufficient for the activation of ErbB receptors. Currently, more than 30 spliced variants of NRG-1 have been identified, including the α or β variants, which differ in the C-terminus of the EGF domain, leading to changes in activity during receptor activation (Fisahn et al., 2008; Raabe et al., 2004).

Despite the preponderance of structural variability, all isoforms of NRG-1 bind to ErbB receptors. Under physiological conditions, NRG-1 binds to ErbB3 or ErbB4. Binding of NRG-1 to the extracellular domain of ErbB3 or ErbB4 induces a conformational change in the receptor that leads to heterodimer formation with ErbB2 receptors that have no ligand-binding activity. Dimer formation results in phosphorylation of the intracellular C-terminal domain of the receptor (Britsch, 2007). The phosphorylated intracellular domain serves as a docking site for various intracellular adaptor proteins, including Shc, Grb2, and the regulatory subunit of phosphoinositide-3-kinase (PI3-kinase). These adaptor proteins, in turn, activate the corresponding downstream Akt or extracellular-regulated kinase (Erk)-signaling pathway. Interestingly, only ErbB2 and ErbB4 have the intracellular kinase activities, and thus the heterodimer of ErbB4/ErbB2 is distinguished from the dimer of ErbB3/ErbB2 for their intracellular phosphorylated docking sites. These pathways can then induce a number of cellular responses, such as stimulation or inhibition of cell proliferation, cell differentiation, apoptosis, cell migration, or cell adhesion (Baliga et al., 1999; Muthuswamy et al., 1999). For example, NRG-1 treatment of adult rat ventricular myocytes has been shown to stimulate the formation of a multiprotein complex between ErbB2, focal adhesion kinase (FAK), and p130(CAS), which modulates the restoration of cell–cell contacts between isolated myocytes, allowing for synchronous beating. These effects of NRG-1 can be prevented by treatment with either an Src inhibitor or an antibody directed against ErbB2, suggesting a potential role for NRG-1/ErbB2/Src/FAK signaling in the maintenance and repair of electrical and mechanical coupling in cardiomyocytes (Kuramochi et al., 2006).

III. NRG-1/ErbB Signaling in the Heart

NRG-1, together with its receptors, receptor tyrosine kinases of the ErbB family, plays a critical role in both cardiovascular development and maintenance of adult heart function. In the past decades, a preponderance of evidence from both *in vitro* and *in vivo* studies has shown that proteins involved in the NRG-1/ErbB-signaling pathway may represent promising

targets for drugs to restore cardiac function after injury. Various mechanisms are believed to be involved in this restoration process, including promotion of cardiac myocyte survival, counteraction of hyperactivated adrenergic stimulation, improvement of sarcomeric structure and cell–cell adhesion, and maintenance of Ca^{2+} homeostasis.

A. The NRG-1/ErbB Pathway Plays an Important Role in Heart Development

Studies focused on NRG-1 and ErbB knockout mice provided the first evidence for a role of the NRG-1/ErbB pathway in heart development. Mice homozygous for NRG-1, ErbB2, or ErbB4 null mutations die before day E11 and exhibit a similar shortage of cardiac trabeculae and poorly differentiated myocytes (Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995). The EGF domain of NRG-1 and subsequent ErbB receptor activation is essential for this process, as inactivation of NRG-1 via deletion of the EGF domain leads to lack of trabeculation, similar to that observed in ErbB2 and ErbB4 null mice (Meyer & Birchmeier, 1995). In addition to these phenotypes, ErbB3 null mice also exhibit a defective endocardial cushion (Meyer & Birchmeier, 1995). Expression of a kinase-dead form of the ErbB2 receptor in mouse embryos causes a phenotype similar to that of ErbB2 null mice, indicating that the kinase activity of ErbB2 is required for heart development (Chan et al., 2002).

The NRG-1/ErbB pathway also contributes to formation of the conduction system in the heart, as mice with a conditional ErbB2 deletion exhibit both delayed conduction and impaired contractility (Garcia-Rivello et al., 2005). NRG-1 was also shown to play a role in the conversion of embryonic cardiomyocytes into conducting cells in mice (Patel & Kos, 2005; Rentschler et al., 2002). In support of this observation, Ruhparwar et al. (2007) demonstrated that NRG-1 and cyclic adenosine monophosphate (cAMP) share the capacity to transform a mixed population of fetal cardiomyocytes into cardiac pacemaker-like cells.

B. NRG-1/ErbB Signaling Is Important for Maintenance of the Structure of the Postnatal and Adult Heart

In contrast to ErbB3, which is only expressed in prenatal myocytes, ErbB2 and ErbB4 are both expressed in adult ventricular myocytes (Zhao et al., 1998). Although conditional knockout mice deleted for ErbB2 or ErbB4 in myocytes after trabeculation survive, they are characterized by a marked dilated ventricular phenotype that includes chamber dilation, wall thinning, and decreased contractility. All of these defects are associated with abnormal cell structure and sarcomeric

organization (Crone et al., 2002; Garcia-Rivello et al., 2005; Negro et al., 2004). As disclosed by Zhou et al. (patent application published as WO00/37095) and Baliga et al. (1999), NRG-1 treatment stimulates sarcomeric actin reorganization and cardiac contractile unit assembly in myocytes maintained in serum-free cultures that exhibit disrupted sarcomere arrangement and a lack of parallel myofibrillar actin bundles under control conditions. Furthermore, NRG-1 also protects myocytes against structural disarray induced by other stresses, including cytotoxic agents (Sawyer et al., 2002).

Although the mechanisms involved in structural organization of cardiac cells are not completely understood, activation of FAK by NRG-1/ErbB2 signaling may play an important role (Kubalova et al., 2005; Kuramochi et al., 2006). FAK is well known for its essential role in the maintenance of sarcomeric organization, cell survival, and myocyte–myocyte interactions (Boateng et al., 2005; Mansour et al., 2004; Peng et al., 2006). In the presence of LY-294002, a PI3-kinase inhibitor, NRG-1 treatment does not induce reorganization of sarcomere structure, while sarcomere reorganization is observed in the presence of the MEK1 inhibitor PD-098059 (Baliga et al., 1999). These observations suggest that the mitogen-activated protein kinase (MAPK)-signaling pathway is not required for NRG-1-mediated improvement of myocardial structure.

C. NRG-1/ErbB Promotes Survival of Cardiomyocytes under Stress Conditions

In vitro studies have revealed that NRG-1 facilitates proliferation and survival of rat embryonic and neonatal cardiomyocytes cultured in serum-free medium (Baliga et al., 1999; Zhao et al., 1998). Under conditions of stress, including viral infection, treatment with cytotoxic agents, and oxidative stress, activation of NRG-1/ErbB signaling can also protect myocardial cells against apoptosis (Fukazawa et al., 2003; Kuramochi et al., 2004; Liu et al., 2006; Zhao et al., 1998). The PI3-kinase/Akt pathway may be involved in NRG-1 modulation of cardiac myocyte apoptosis, as treatment with the PI3-kinase inhibitor wortmannin or overexpression of a dominant negative form of Akt was shown to inhibit the effects of NRG-1 on myocyte survival (Fujio et al., 2000; Fukazawa et al., 2003; Kuramochi et al., 2004). Enhanced Bcl-2 expression induced by Akt activation may underlie myocyte survival due to NRG-1 signaling (Das et al., 2005; Dhanasekaran et al., 2008).

In contrast to embryonic and neonatal cardiomyocytes, adult myocardial cells are terminally differentiated and have lost the ability to proliferate. Therefore, growth of adult cardiac cells is commonly characterized by hypertrophy and an increased content of contractile proteins

(Chien et al., 1991). To date, there is no *in vivo* evidence suggesting that NRG-1 promotes cardiomyocyte hypertrophy, despite experiments using large concentrations of the protein (Liu et al., 2006). Consistent with these observations, Kenvin et al. (Bersell et al., 2009) recently reported that NRG-1 promotes myocardial regeneration along with decreased hypertrophy surrounding infarcted areas, leading to improved functionality. Thus, instead of triggering hypertrophy, rhNRG-1 treatment may cause inhibition of hypertrophy of the heart during pathological processes. In addition, in NRG-1 or conditional ErbB2 knockout mice, no signs of apoptosis or reduced cell numbers were observed (Crone et al., 2002; Garcia-Rivello et al., 2005; Liu et al., 1998). Furthermore, no significant differences in mitochondrial respiratory function were revealed between the conditional mutants and the controls (Crone et al., 2002; Liu et al., 1998; Negro et al., 2004). These results indicate that NRG-1 is not necessary for cell division and survival under normal physiological conditions.

D. NRG-1/ErbB and Cardiac Homeostasis

In the postnatal heart, NRG-1 is synthesized and released from the cardiac endothelium. Synthesis is controlled dynamically by neurohormonal and biomechanical stimuli, allowing adaptive tuning of ErbB signaling during cardiovascular stress (Lemmens et al., 2006). For example, in the rat cardiac microvascular endothelium, NRG-1 mRNA and protein expression is downregulated by angiotensin II and phenylephrine and upregulated by endothelin-1 and mechanical strain (Pentassuglia & Sawyer, 2009). Recently, a potential new role for NRG-1/ErbB signaling in cardiovascular homeostasis has emerged. In the adult myocardium, NRG-1 appears to be involved in the regulation of cardiac sympathovagal balance through counteraction of adrenergic stimulation via an obligatory interaction with the muscarinic cholinergic system (Lemmens et al., 2004, 2005; Okoshi et al., 2004). Furthermore, during adrenergic stimulation, NRG-1 has a negative inotropic effect on cardiac muscle through activation of NO synthase and desensitization of the myocardium to isoproterenol, resulting in decreased oxygen consumption during stress (Lemmens et al., 2004). NRG-1 is also upregulated in response to pressure overload-induced hypertrophy and acts as an anti-adrenergic factor, although upregulation ceases upon the transition to heart failure (Lemmens et al., 2006). Indeed, Liu et al. (2006) reported that the pharmacological administration of NRG-1 in rats is effective against stress-induced cardiac dilatation, and Hintsanen et al. (2007) demonstrated that the NRG-1 genotype influences job strain-induced atherosclerosis. Since NRG-1 is associated with both cardiac health and the response to stress, it has

been suggested to act as a potential moderator between environmental stress and heart failure (Hintsanen et al., 2007).

IV. NRG-1/ErbB Signaling and Chronic Heart Failure

Decreased expression of ErbB receptors has been observed in human CHF (Rohrbach et al., 2005). A similar decrease is found in mice subjected to chronic pressure overload (Rohrbach et al., 1999). In patients, the inhibitory ErbB2 antibody trastuzumab, used for treatment of mammary carcinomas, increased the risk for development of cardiotoxic cardiomyopathy (Guarneri et al., 2006). As a function of the disease process, NRG-1/ErbB signaling is first activated in the early stages of CHF, together with a profound upregulation of NRG-1 expression in the left ventricle. However, in the later stages of pump failure, both NRG-1 expression and NRG-1/ErbB signaling are inhibited (Lemmens et al., 2006). Together, these observations indicate a potential role for NRG-1/ErbB in the development of heart failure.

A. Preclinical Studies

Enlightened by these findings, we elected to investigate the possibility of utilizing NRG-1 as drug for treatment of heart failure. First, we manufactured a recombinant human neuregulin-1β2 protein (rhNRG-1), which contains 61 amino acids and spans the EGF-like domain necessary for ErbB2/ErbB4 activation (Fig. 1). Treatment of cardiomyocytes in serum-free cultures with rhNRG-1 resulted in the recovery of normal sarcomere arrangement and restoration of cardiac contractile unit assembly (patent application published as WO00/37095, Zensun Co.) (Fig.2). The beneficial effect of NRG-1 on the maintenance of cardiomyocyte structure was also confirmed by Baliga et al. (1999).

Direct evidence for the unique effect of NRG-1 in treatment of CHF was first provided by Liu et al., who explored the results of short-term intravenous administration of rhNRG-1 in different models of CHF. These models included CHF induced by ligation of the left anterior descending coronary artery, by rapid ventricular pacing, by anthracycline, and by myocarditis in dogs and rats. NRG-1 administration in these animal models resulted in improved cardiac performance, reduced pathological changes, and prolonged survival, but did not alter hemodynamics or cardiac contractility in normal animals (Liu et al., 2006). Consistent with these studies, GGF2, another isoform of NRG-1, also exhibited potential to reduce and even reverse the dysfunction of congestive heart failure by strengthening and protecting heart muscle cells (patent application published as US20060019888, Acorda Co.).

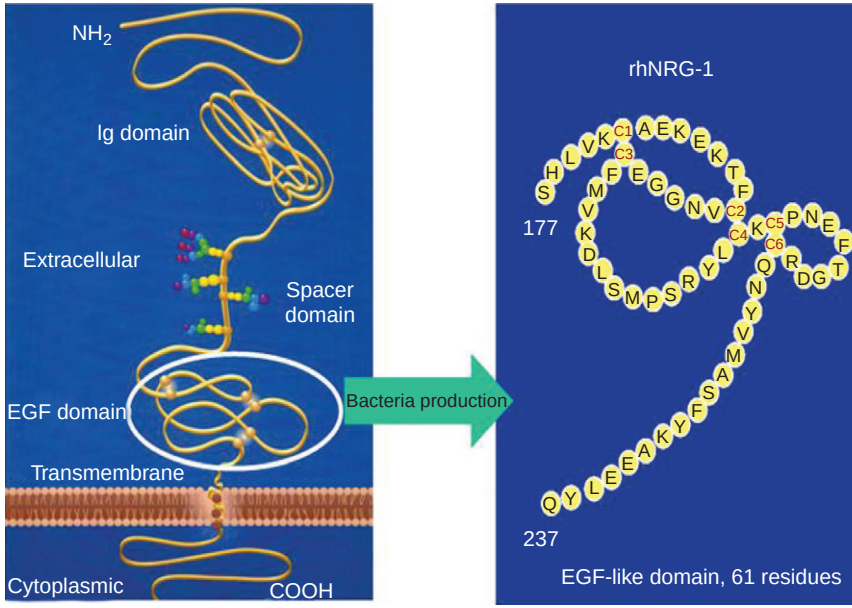

FIGURE 1 Structure of recombinant human neuregulin-1β. The rhNRG-1 sequence, which contains only the EGF-like domain of the wild-type protein, was cloned from the endogenous human neuregulin-1β gene. The recombinant protein was expressed in bacteria, yielding a protein of 61 amino acids, which is necessary and sufficient for ErbB receptor binding and activation.

These observations indicate that NRG-1 is a promising drug candidate for treatment of CHF.

B. Treatment of Chronic Heart Failure with NRG-1 Applied to Clinical Practice

At present, there are no clinically used drugs for treatment of heart failure that directly act on damaged cardiomyocytes and restore heart function. Enlightened by the observation that rhNRG-1 treatment rescued cardiomyocytes from serum-free induced cell structure disarray (patent application published as WO00/37095, Zensun Co.), we speculated that rhNRG-1 may represent a potential drug for treatment of CHF through a novel therapeutic mechanism that directly improves the structure of damaged cardiomyocytes. After the positive impact of rhNRG-1 on the heart was confirmed in both *in vitro* and *in vivo* animal studies and usage

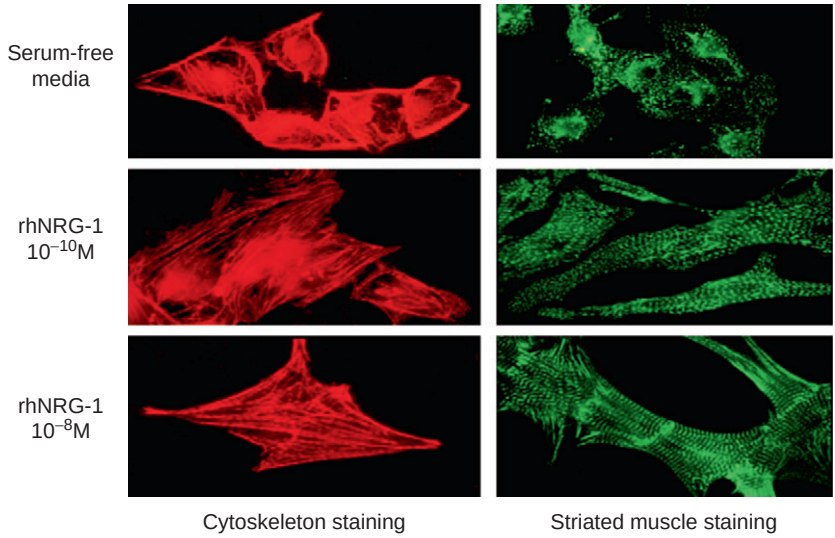

FIGURE 2 Treatment with rhNRG-1 attenuates serum-free, media-induced myofibrillar disorganization in neonatal myocytes. Neonatal myocytes were cultured in serum-free medium with or without the indicated doses of rhNRG-1 for 2 days. The actin cytoskeleton was stained with phalloidin (red/left panels), and striated muscle was stained using an anti-α-actinin antibody conjugated to fluorescein isothiocyanate (FITC) (green/right panels).

of rhNRG-1 was shown to be safe in healthy volunteers in the phase I clinical trial, the effects of rhNRG-1 were assessed in CHF patients.

1. Efficacy Results

The Chinese phase II clinical trial (ZS-01-206) was a double-blind, multicenter, placebo-controlled trial to evaluate the efficacy and safety of rhNRG-1 in patients with chronic systolic heart failure. The primary end points of the trial were changes in left ventricular ejection fraction (LVEF), end-systolic volume (ESV), or end-diastolic volume (EDV), as measured using MRI at baseline, day 11, day 30, and day 90. Secondary efficacy endpoints included cardiac function classification, as defined by the New York Heart Association (NYHA), the 6-min walk (6-MW) test, quality-of-life (QOL) scoring, and plasma levels of the N-terminal pro-brain natriuretic peptide (NT-proBNP). The entire study period was 90 days.

Criteria for participation in the trial included diagnosis of CHF (NYHA class II or III), age between 18 and 65 years old, LVEF ≤ 40%, and relatively stable clinical condition (including clinical signs and symptoms with accepted standard treatment for CHF at the target dose or maximum tolerance dose for over 1 month). Major exclusion criteria included acute myocardial infarction (MI), hypertrophic cardiomyopathy, constrictive

pericarditis, significant valve disease or congenital heart disease, severe pulmonary hypertension, systolic blood pressure <90 or >160 mmHg, severe ventricular arrhythmia, cardiac surgery or a cerebrovascular event within the previous 6 months, claustrophobia, or pregnancy in females. All patients provided witnessed written consent.

A total of 44 patients were randomly assigned to four groups treated with either the placebo or rhNRG-1 (0.3, 0.6, or 1.2 μg/kg/day) for 10 consecutive days. For patients who needed to discontinue the study prematurely due to intolerance, a comprehensive safety evaluation was carried out on the day after the most recent administration. Both the experimental and placebo groups were allowed to continue therapy for CHF with basic standard drugs, including angiotensin-converting enzyme inhibitors (ACEIs) and/or β-blocker or angiotensin II receptor blocker (ARB), diuretics, or digoxin.

a. Cardiac Pumping Function and Remodeling as Measured Using MRI Primary endpoint results showed that a progressive improvement in LVEF and a reduction of EDV and ESV occurred in the 0.6 μg/kg dosage group after treatment. However, between groups, changes in LVEF, ESV, and EDV were not statistically significant, likely due to the small number of patients enrolled in the trial. Encouragingly, in contrast to other clinical drugs that favor cardiac remodeling, rhNRG-1 administration resulted in a sustained beneficial effect on the heart after only 10 days of treatment. Comparison of ESV and EDV levels on day 90 and day 30 showed that both endpoints decreased with time in a dose-dependent manner for dosages between 0.3 and 0.6 μg/kg. Together, these results suggest that rhNRG-1 could effectively enhance heart-pumping functionality and likely activate processes involved in reverse remodeling of the ventricular chamber.

b. The Prognosis of CHF Patients Trial ZS-01-207, another phase II trial conducted in China, utilized the same protocol as trial ZS-01-206, but assessed a different primary end point. This trial also had a larger sample size, consisting of 195 patients. In support of ZS-01-206, this trial provided greater universal safety information and longer term prognosis data, through plasma NT-proBNP testing and telephone follow-up.

An increase in NT-proBNP levels was observed in the placebo group at day 90 (39.4 $\pm$ 247%) in comparison to the baseline, likely due to progression of CHF. In contrast, although an increase in NT-proBNP levels occurred immediately after treatment with rhNRG-1, NT-proBNP levels were significantly reduced, particularly in the 0.6 μg/kg group, at day 30 (3.22 $\pm$ 73%) and day 90 (8.8 $\pm$ 58%) in comparison with the baseline ($p = 0.069$ and $p = 0.029$, respectively). These results indicated that short-term treatment with rhNRG-1 leads to a favorable prognosis for CHF patients. The transient increase in NT-proBNP levels immediately after

rhNRG-1 administration may be due to direct stimulation of cellular secretion, as no changes in ventricular load were observed in preclinical studies.

A telephone interview to analyze rehospitalization was conducted 15 months after treatment. Of the 62 patients with available rehospitalization data in the placebo group, 11 (17.74%) were rehospitalized for worsening CHF at least once. For the 0.6 μg/kg group, only 6 (9.68%) of the 62 patients were readmitted to the hospital. The rehopitalization rate of the 1.2 μg/kg group was 15.38% (10/65). The average number of times of rehospitalization was 0.355 (22/62) per patient for the placebo group, 0.177 (11/62) per patient for the 0.6 μg/kg group, and 0.292 (19/65) per patient for the 1.2 μg/kg group. Rehospitalization was reduced by 50.0 and 17.7% for the 0.6 and 1.2 μg/kg groups, respectively, in comparison with the placebo group. Thus, short-term treatment with rhNRG-1 could effectively reduce rehospitalization due to worsening of CHF, providing a better prognosis for CHF patients.

2. *Safety Results*

All adverse events (AEs) were recorded during the studies. The most commonly occurring AEs were gastrointestinal disorders, such as nausea, vomiting, poor appetite, dyspepsia, diarrhea, and abdominal discomfort, which were well tolerated in most cases. Other AEs included headache, dizziness, fatigue, and palpitations, none of which worsened over the period of administration. There was no significant difference in AE incidence between the rhNRG-1 (≤0.6 μg/kg) treatment groups and the placebo group, and 0.6 μg/kg was shown to be the most effective dosage. Although an increased incidence of gastrointestinal disorders occurred in the high-dosage group, no changes in biochemical markers, including markers for myocardium evaluation, were observed after treatment. Therefore, even in the high-dosage group, rhNRG-1 did not cause organ damage or increase the risk of serious adverse events (SAEs).

C. Discussion

Both the NT-proBNP and rehospitalization results indicate that rhNRG-1 administration could benefit the prognosis of CHF. It was very interesting that, despite the short period of administration, patients obtained long-term benefits from treatment with rhNRG-1. Although the mechanism involved in this process is still under investigation, results from trial ZS-01-206 indicate that short-term treatment with rhNRG-1 may not only increase the LVEF, but also inhibit and even reverse cardiac remodeling. These changes in cardiac structure might improve the long-term prognosis of CHF. While other stresses that might accelerate the development of CHF were controlled by standard CHF therapies, these treatments did not repair injured cardiac muscles. In contrast, rhNRG-1 treatment caused a

fundamental change in cardiomyocytes, which, when accompanied by reduced stress due to standard therapies, might lead to sustained improvement of both left ventricular function and remodeling in the late phase.

A phase II clinical trial was also conducted independently in Australia in order to evaluate the safety and efficacy of rhNRG-1. This trial reached the conclusion that short-term administration of rhNRG-1 results in acute and sustained improvement in cardiac function. Thus, rhNRG-1 was effective for treatment of heart failure in a different ethnic population.

In the phase II trial conducted in China, an unexpected result was that no improvement in cardiac functionality occurred in the high-dosage (1.2 μg/kg) group. This observation is likely due to the side effects caused by a high dosage of the drug. *In vitro* studies have shown that NRG-1 balances β-adrenergic activation through the stimulation of a parasympathetic-like activity (Lemmens et al., 2004). This observation was consistent with the most predominately observed side effects that occurred during infusion of high-dose rhNRG-1 in our trial, such as nausea and vomiting. Furthermore, the activation of parasympathetic-like activity had a negative inotropic effect on the cardiac muscle, which might further inhibit cardiac pumping function.

V. The Molecular Mechanisms Underlying the Effects of rhNRG-1 Treatment on Heart Failure

It will be very interesting to understand how NRG-1 enhances heart contractility and coupling between contraction and compensation, resulting in reverse remodeling. Improvement of myocardial structure is believed to be the general basis for the therapeutic effects of NRG-1 due to the known causative role of myofiber and sarcomere disorganization in heart failure.

Previous reports have suggested that NRG-1/ErbB signaling triggers activation of FAK and PI3-kinase, favoring intracellular connection formation and organized sarcomere structure. However, the precise mechanisms and signaling pathways involved in this process have not yet been fully elucidated. To better understand the mechanism of NRG-1, two experiments were performed. First, microarray technology was used to compare gene expression patterns in NRG-1-treated and NRG-1-untreated rats with failing hearts induced by 8 weeks of coronary ligation in order to provide clues as to which proteins may be regulated by NRG-1 stimulation. Microarray experiments, confirmed by Northern and Western blot analysis, revealed that cardiac-specific myosin light chain kinase (cMLCK) expression was upregulated approximately 120% in NRG-1-treated animals. Second, cellular calcium cycling, which is directly correlated with pumping function, was analyzed in the myocardial cells of these rats. This experiment

demonstrated an increase in Ca^{2+}-ATPase (SERCA2a) activity of approximately 30% as a result of NRG-1 treatment.

A. cMLCK Is Involved in Restoration of Cardiomyocyte Structure as a Result of rhNRG-1 Treatment

The cMLCK protein has been shown to be an important regulator of sarcomere assembly through activation of the myosin regulatory light chain (MLC2v) and plays a role in heart contractility (Chan et al., 2008; Seguchi et al., 2007). In contrast to smooth and skeletal muscle MLCKs, cMLCK expression is restricted to cardiac myocytes (Seguchi et al., 2007). Abnormal cMLCK expression has been reported in models of cardiac disease. Specifically, one laboratory found that cMLCK is downregulated (Chan et al., 2008), while another found that cMLCK is upregulated in rats treated with coronary ligation for 3–4 weeks (Seguchi et al., 2007). We found that in rats treated with 3–4 weeks of coronary ligation, cMLCK expression levels were unchanged, but after 8 weeks of ligation, which is associated with more stable heart failure in rats, cMLCK expression was downregulated by approximately 30% (unpublished observations). These divergent findings may illustrate a shift of the failing myocardium from a compensated to a decompensated stage. In addition to these observations, overexpression of cMLCK using a viral vector in cultured myocardial cells has been shown to result in increased cell contractility (Chan et al., 2008). In our study, treatment with rhNRG-1 resulted in significant upregulation of cMLCK in the CHF rat model, together with an improvement in both cardiomyocyte structure and pumping function. Furthermore, although rhNRG-1 treatment led to restoration of the normal structure of neonatal cardiomyocytes cultured in serum-free medium, this protective effect of rhNRG-1 was completely inhibited by an inhibitor of cMLCK (Fig. 3). Therefore, cMLCK is likely to be one of the downstream proteins regulated by NRG-1/ErbB signaling and to play a role in rhNRG-1-mediated effects on CHF.

B. SERCA2 Plays a Role in rhNRG-1-Mediated Improvements in Heart Pumping

SERCA2 is an exciting drug target for treatment of heart failure. During the last decade, altered calcium homeostasis has been suggested to play a role in the development of heart failure, and SERCA2 is believed to be responsible for changes in calcium levels. Modulated by phospholamban (PLB), SERCA2 regulates uptake of Ca^{2+} into the sarcoplasmic reticulum (SR) from the cytoplasm and contributes to the relaxation of cardiomyocytes (Bassani et al., 1995; Go et al., 1995; Jiang et al., 2002; Kubalova et al., 2005; Pathak et al., 2005). This process is also important for

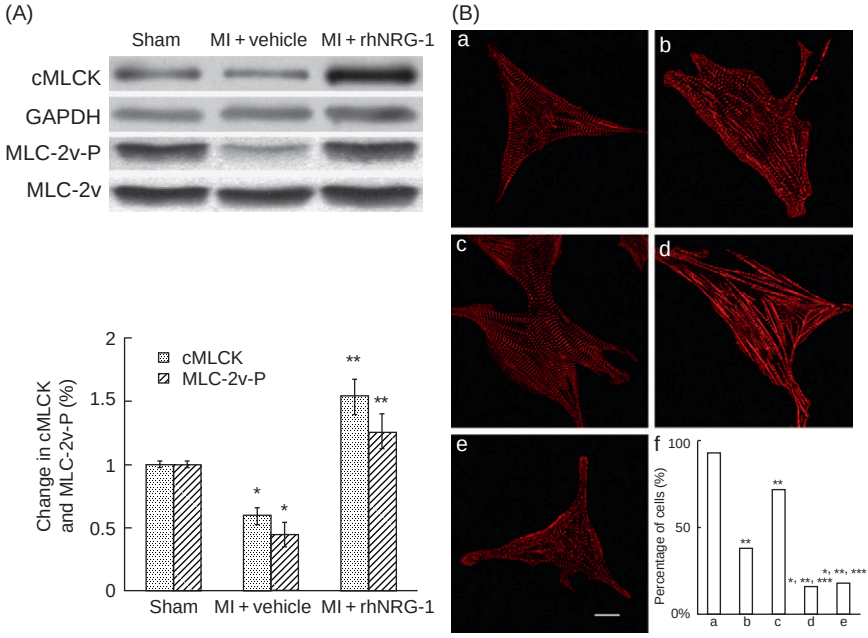

FIGURE 3 rhNRG-1 plays a role in maintenance of cardiomyocyte structure through upregulation of cMLCK. (A) MI rats were generated by coronary artery ligation. Sham-operated animals were treated similarly, with the exception that the suture around the left anterior descending artery was not tied. Eight weeks after the operation, MI rats were treated with 0.2% albumin/phosphate-buffered saline (MI + vehicle) or rhNRG-1 (MI + rhNRG-1) for 1 week. Tissues isolated from the left ventricle were prepared for Western blot analysis. *Upper panel*: Expression of cMLCK and phosphorylation of MLC-2v were analyzed using Western blot and immunoprecipitation. *Lower panel*: Expression of cMLCK and MLC-2v-P was normalized to expression of GAPDH and MLC-2v, respectively. $*p < 0.01$ versus sham, $**p < 0.01$ versus vehicle. (B) Neonatal rat cardiomyocytes cultured in DMEM medium with (a) 10% FBS; (b) in serum-free medium; (c) in serum-free medium containing rhNRG-1; (d) in serum-free medium containing ML-7, an inhibitor of MLCK; (e) and in serum-free medium containing both ML-7 and rhNRG-1. Graph (f) shows the relative percentages of cells with well-organized areas ($\geq 2/3$) in cultures treated as described in panels a–e. Scale bar represents 10 μm. $*p < 0.01$ versus a, $**p < 0.01$ versus b, $***p < 0.01$ versus c.

determining the SR Ca^{2+} load after relaxation and, thus, impacts on contractility (Bassani et al., 1995; Verboomen et al., 1992). Protein phosphatase 1 (PP1) contributes to the dephosphorylation of PLB, and dephosphorylated PLB is an inhibitor of SERCA2 (MacDougall et al., 1991). In the failing heart, PP1 expression is upregulated, resulting in increased PLB dephosphorylation and decreased SERCA2 activity (El-Armouche et al., 2003; Gupta et al., 2003). Our recent preliminary studies in post-MI rats revealed that rhNRG-1 restored altered intracellular Ca^{2+} transport and enhanced

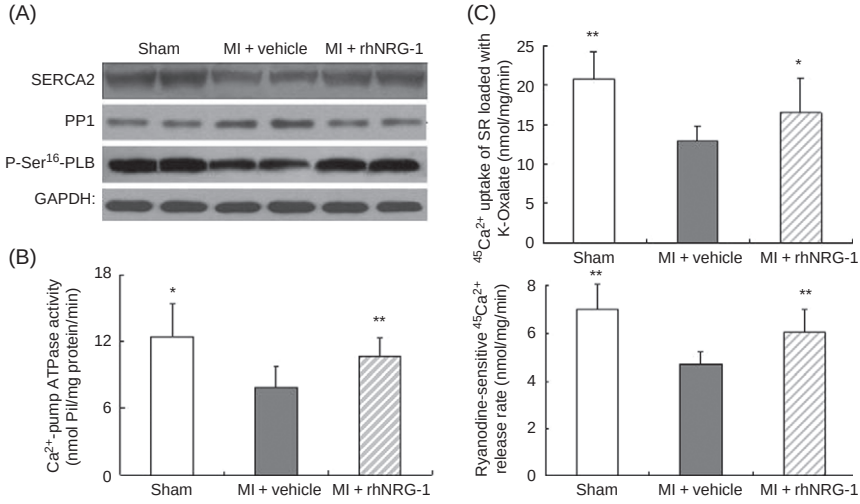

FIGURE 4 rhNRG-1 modulates the expression and function of calcium regulatory proteins. Four weeks after coronary artery ligation, MI rats were treated with 0.2% albumin/phosphate-buffered saline (MI + vehicle) or rhNRG-1 (MI + rhNRG-1) for 1 week. For sham-operated animals, the suture around the left anterior descending artery was not tied and no treatment was applied. Five weeks after the operation, cardiomyocytes were isolated from the left ventricle for further analysis. (A) Western blot analysis of SERCA2, PP1, and P-Ser^{16}-PLB expression. GAPDH is shown as a loading control. (B) Activity of SR Ca^{2+} pump ATPase (SERCA2) in left ventricular myocytes isolated from sham ($n = 6$), MI + vehicle ($n = 6$), and MI + rhNRG-1 ($n = 6$) animals. $*p < 0.05$ and $**p < 0.01$ versus MI + vehicle group. Error bars represent mean $\pm$ SD. (C) SR Ca^{2+}-uptake and Ca^{2+}-release activities in left ventricular myocytes isolated from sham ($n = 6$), MI + vehicle ($n = 6$), and MI + rhNRG-1 ($n = 6$) animals. Upper panel: changes in SERCA2 $^{45}Ca^{2+}$-uptake activity. Lower panel: Changes in ryanodine-sensitive $^{45}Ca^{2+}$-release rates from the SR. $*p < 0.05$ and $**p < 0.01$ versus MI + vehicle group. Error bars represent mean $\pm$ SD.

myocardial contractility through the suppression of PP1 expression, which led to increased PLB phosphorylation and activation of SERCA2 (Fig. 4).

C. Summary

As described above, NRG-1/ErbB likely plays a beneficial role in treatment of CHF by promoting survival of cardiac myocytes, improving sarcomeric structure, balancing Ca^{2+} homeostasis, and enhancing pumping function. Downstream effectors of NRG-1/ErbB, including cMLCK, PP1, SERCA2, and FAK, may contribute to the positive therapeutic effects of rhNRG-1 (Fig. 5). However, determination of the precise molecular factors involved in this signaling pathway requires further investigation. In addition to rhNRG-1, downstream effectors in the NRG-1/ErbB-signaling pathway, such as cMLCK and SERCA2a, could also represent possible therapeutic

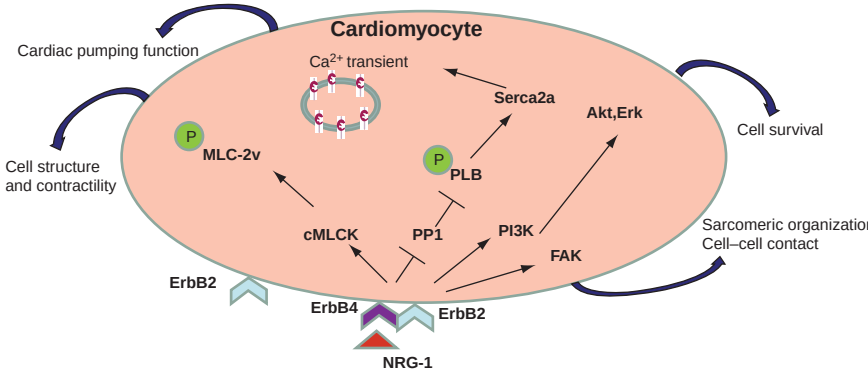

FIGURE 5 NRG-1/ErbB signaling in the adult heart. NRG-1, neuregulin-1; cMLCK, cardiac-specific myosin light chain kinase; MLC-2v, myosin regulatory light chain; PP1, protein phosphatase 1; PLB, phospholamban; FAK, focal adhesion kinase; PI3K, phosphoinositide-3 kinase; Erk, extracellular-regulated kinase.

targets for the treatment of heart failure. The successful outcome of trials using rhNRG-1 as a CHF treatment encourages us to search for more candidates in this pathway for use as potential drug targets.

VI. Conclusion

Heart failure affects approximately five million Americans, and more than 550,000 new patients are diagnosed with the condition each year. The last three decades have seen major advances in the treatment of heart failure, with an extensive list of proposed medical drug therapies, including β-blockers, ACEIs, ARB, and brain natriuretic peptide (BNP). At the same time, despite these substantial clinical advances, heart failure has become a major cause of human cardiovascular morbidity and mortality worldwide. It, therefore, remains a challenge to develop novel drugs with new mechanisms of action for the treatment of heart failure.

NRG-1, together with its receptors of the ErbB family, plays an important role in multiple cellular functions, including cell growth, differentiation, and survival. A growing number of studies have highlighted the critical roles of the NRG-1/ErbB pathway in cardiovascular development and in maintenance of adult heart function and structure. The profound effects of NRG-1/ErbB on heart development and pumping function, as well as its unique role in maintaining the structure of cardiac cells, justify the investigation of

the possible application of molecules involved in this pathway for use as novel drugs for treating heart failure.

Indeed, our studies in different animal models of CHF strongly suggest a beneficial therapeutic effect of rhNRG-1 in treatment of heart failure. Treatment with rhNRG-1 results in upregulation of pumping function, inhibition of ventricular enlargement, and prolonged survival. Unlike drugs currently used for treatment of heart failure, rhNRG-1 appears to act directly on cardiomyocytes by helping to repair the damaged cells. Furthermore, clinical trials conducted in China and Australia indicate that short-term administration of rhNRG-1 to CHF patients resulted in sustained improvement of cardiac function and structure and a better prognosis, consistent with preclinical studies. The available animal and human data for rhNRG-1 suggest that drugs targeting this pathway show great promise for development of an effective therapy for CHF with a novel mechanism of action. Further studies are needed to explore the underlying mechanisms of NRG-1/ErbB signaling in treatment of CHF, as other possible therapeutic targets downstream of NRG-1/ErbB, such as cMLCK and SERCA2a, may also be candidates for the treatment of heart failure.

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Abbreviations

AE	adverse event
CHF	chronic heart failure
EDV	end-diastolic volume
LVEF	left ventricular ejection fraction
ESV	end-systolic volume
MI	myocardial infarction
NRG-1	Neuregulin-1
NT-proBNP	N-terminal pro-brain natriuretic peptide
NYHA	New York Heart Association
QOL	quality of life
rhNRG-1	recombinant human neuregulin-1
SAE	serious adverse event

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I_f Inhibition in Cardiovascular Diseases

Abstract

Heart rate (HR) is determined by the pacemaker activity of cells from the sinoatrial node (SAN), located in the right atria. Spontaneous electrical activity of SAN cells results from a diastolic depolarization (DD). Despite controversy in the exact contribution of funny current (I_f) in pacemaking, it is a major contributor of DD. I_f is an inward Na^+/K^+ current, activated upon hyperpolarization and directly modulated by cyclic adenosine monophosphate. The f-proteins are hyperpolarization-activated cyclic nucleotide-gated channels, HCN4 being the main isoform of SAN. Ivabradine (IVA) decreases DD and inhibits I_f in a use-dependent

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manner. Under normal conditions IVA selectively reduces HR and limits exercise-induced tachycardia, in animals and young volunteers. Reduction in HR with IVA both decreases myocardial oxygen consumption and increases its supply due to prolongation of diastolic perfusion time. In animal models and in human with coronary artery disease (CAD), IVA has anti-anginal and anti-ischemic efficacy, equipotent to classical treatments, β-blockers, or calcium channel blockers. As expected from its selectivity for I_f, the drug is safe and well tolerated with minor visual side effects. As a consequence, IVA is the first inhibitor of I_f approved for the treatment of stable angina.

Available clinical data indicate that IVA could improve the management of stable angina in all patients including those treated with β-blockers. As chronic elevation of resting HR is an independent predictor of mortality, pure HR reduction by inhibition of I_f could, beyond the control of anti-anginal symptoms, improve the prognosis of CAD and heart failure; this therapeutic potential is currently under evaluation with IVA.

I. Introduction

There is strong evidence that elevated heart rate (HR) is an important predictor of total and cardiovascular mortality. Epidemiological studies have clearly demonstrated a relationship between high resting HR and increased risk of cardiovascular morbidity and mortality in the general population (Levine, 1997; Mensink & Hoffmeister, 1997); in patients with coronary artery disease (CAD), hypertension, metabolic syndrome; and in the elderly (Arnold et al., 2008; Cook et al., 2006; Diaz et al., 2005; Flannery et al., 2008; Fosbol et al., 2010; Fox et al., 2007; Gillman et al., 1993; Palatini, 2007; Palatini et al., 1999).

In the context of CAD, reduction of HR is a well-known strategy to treat patients. It directly minimizes the myocardial oxygen demand, as HR is the major determinant of oxygen consumption and also enhances oxygen supply by increasing diastolic perfusion period. The anti-anginal and anti-ischemic effects of rate-limiting drugs, as calcium channel blockers (CCBs) or β-adrenoceptor blockers (BBs), are well documented, but the clinical use of these traditional drugs is commonly associated with inadequate control, poor tolerance, and adverse effects. For these reasons innovative drugs, as pure HR-reducing agents, have emerged as a new strategy for the treatment of stable angina pectoris. This review summarizes the pharmacology, preclinical, and clinical results of HR-lowering agents that selectively inhibit the pacemaker I_f current in the sinoatrial node (SAN).

II. Role of I_f in Cardiac Pacemaking

A. I_f Current

SAN is the natural pacemaker region of the heart (Anderson et al., 2009; Boyett et al., 2000; Chandler et al., 2009; Dobrzunski et al., 2005; Mangoni & Nargeot, 2008). Cells from this region are responsible for initiating spontaneous activity and controlling HR. Spontaneous action potentials (APs) originating from the central region of the SAN first propagate through the atria and then, after a slow transition across the atrioventricular node, spread through the ventricles via the specialized conduction tissue, His bundles, and Purkinje fibers (Fig. 1). The generation of spontaneous APs is an intrinsic properties of SAN cells related to the presence of the diastolic depolarization (DD) (phase 4) that brings the membrane potential from the maximal diastolic potential reached after repolarization of an AP to the threshold for firing of the next AP.

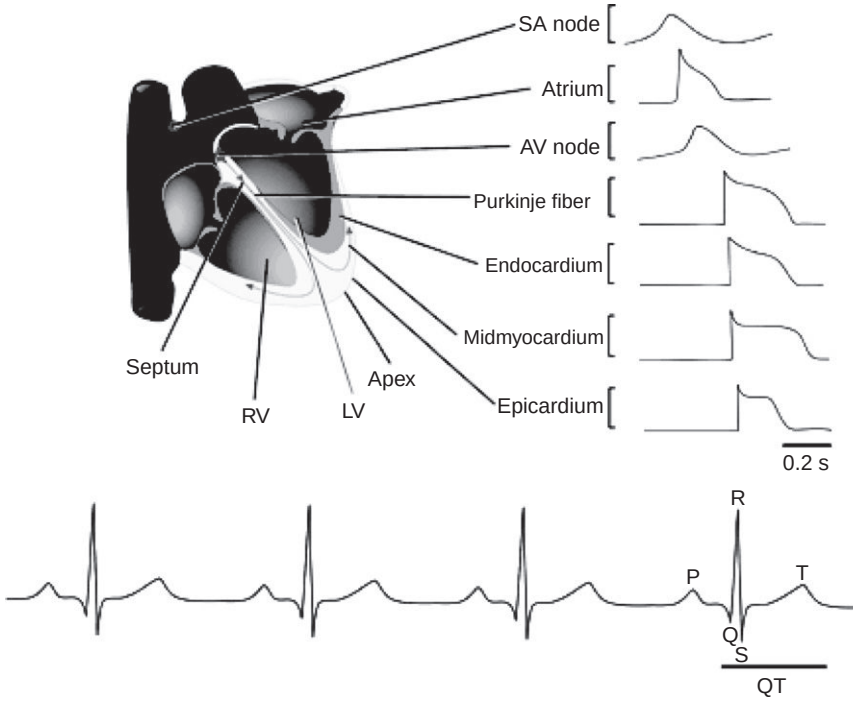

FIGURE 1 Electrical activity in the myocardium. *Top*: schematic of a human heart with illustration of typical action potential waveforms recorded in different regions. *Bottom*: schematic of a surface electrocardiogram (from Nerbonne & Kass (2005) with permission).

The exact mechanism underlying the generation of automaticity in pacemaker cells has not been completely clarified, with contribution of voltage-dependent ionic currents ("ion channels clock") or sodium–calcium exchanger (NCX) and intracellular diastolic calcium release from the sarcoplasmic reticulum ("calcium clock") or both (DiFrancesco, 1993, 2010a; Irisawa et al., 1993; Maltsev & Lakatta, 2008; Vinogradova & Lakatta, 2009; Vinogradova et al., 2005). Although there is still debate about the precise involvement of these different cellular processes contributing to the pacemaker depolarization (for revue on "point/counterpoint," see Lakatta & DiFrancesco, 2009), there is now general agreement that a major role in the generation and control of this phase is played by the pacemaker I_f current (Baruscotti et al., 2005; DiFrancesco, 2005, 2010a; Mangoni & Nargeot, 2008).

The I_f current was first described in SAN cells in 1979 (Brown et al., 1979) and was named "funny" because of its unusual electrophysiological properties for a voltage-gated channel (for review, Baruscotti et al., 2005, DiFrancesco, 1993, 2010a). It is a mixed cationic current, with reversal potential of about –10/–20 mV, carried by both Na^+ and K^+ with a predominant permeability to Na^+ and an activation of its conductance by K^+. On the contrary to other voltage-dependent channels, it is activated upon hyperpolarization of the membrane at a threshold of about –45 mV and fully activated at around –100 mV. Thus, in the pacemaker range of voltages, it is a slowly activating inward depolarizing current, which suits for the generation of DD (Fig. 2). Furthermore, it is rapidly deactivated upon depolarization to voltages within the plateau range.

The pacemaker activity in the SAN is highly controlled by the autonomic nervous system with acceleration or slowing under sympathetic or parasympathetic stimulation, respectively. As illustrated in Fig. 3, this autonomic modulation of rate occurs via changes in the slope of DD, increased through activation of β1 adrenergic receptors (e.g., with isoproterenol), and decreased through stimulation of M2 muscarinic receptors (e.g., with acetylcholine), with no change in the firing threshold (Fig. 3A). This autonomic regulation is directly related to another unusual property of I_f channel, i.e., its modulation by intracellular cyclic adenosine monophosphate (cAMP), which acts as a second messenger and favors channel opening by direct binding to the c-terminus of the channel, leading to a shift of its activation curve (Fig. 3B and C) with no change in its unitary conductance. In other words, in this example, for a physiological diastolic membrane potential, for example –65 mV, under control conditions, about 35% of I_f channels are open (Fig. 3C). Adrenergic stimulation with isoprenaline, via increasing internal cAMP, induces a depolarizing shift of the activation curve leading to an elevated number of open channels for the same voltage, about 65%. As a consequence, this increase in I_f current availability promotes a deeper slope of DD (Fig. 3A and B), a reduced diastolic duration and thus an increase in HR (see recent review, DiFrancesco, 2010a).

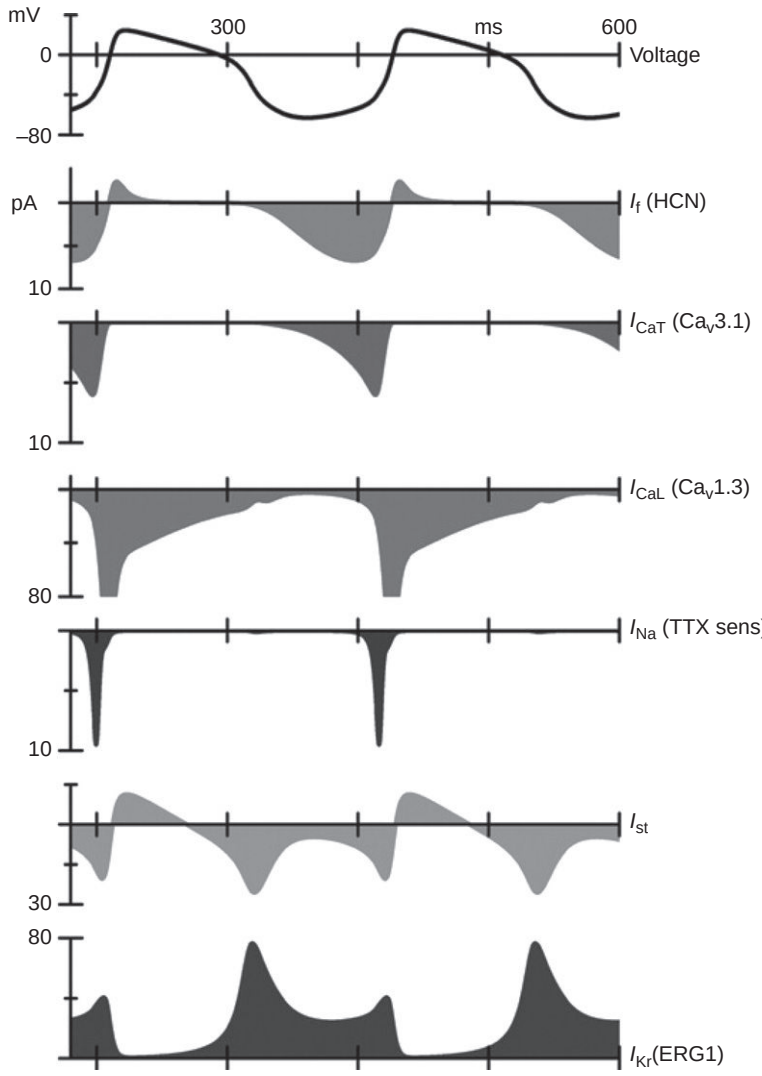

FIGURE 2 Voltage-dependent "ion channels clock" generating automaticity. The "clock" has been represented by using a numerical computation of the mouse SAN automaticity. For each ionic current considered, the underlying ion channel or channel gene family has been indicated [from Mangoni & Nargeot (2008) with permission].

Another characteristic of this channel is its tiny conductance, with a current density of about 8 pA/pF in human SAN cells (Verkerk et al., 2007), that leads, once again, to debate whether the funny current is more important than "calcium clock" in DD (DiFrancesco, 2010b; Maltsev & Lakatta,

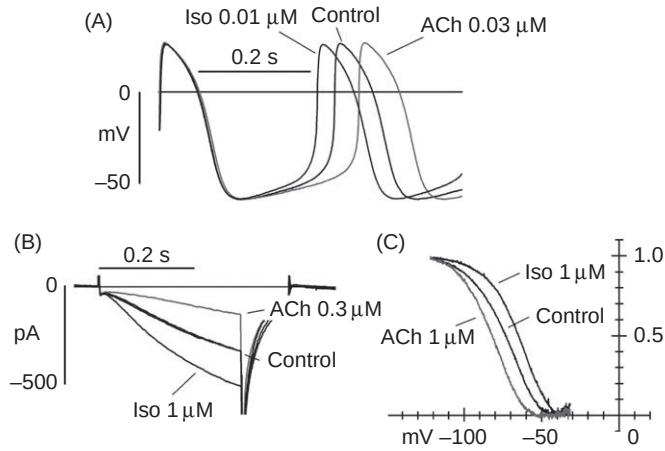

FIGURE 3 Rate modulation by autonomic nervous system. Low doses of isoproterenol and acetylcholine selectively modulate the slope of the diastolic depolarization in rabbit SAN cells (A) and shift the I_f voltage dependence (B, C). [(A) from DiFrancesco (1993) and (B) and (C) from Accili et al. (1997) with permission].

2010; Verkerk & Wilders, 2010). Despite this controversial debate not being under the scope of the present review, it is important to underline that the net amount of current (I_{net}) required during DD to pace a cell is very low and thus not incompatible with a major role of funny current. I_{net} is proportional to the voltage derivative, according to the well-known equation: $I_{net} = -C_m \, dV_m/dt$ (where C_m is cell capacitance and V_m membrane voltage). DiFrancesco has calculated that in a human pacemaker cell with a capacitance of about 60 pF, an I_{net} of only 1.2 pA is required for DD (DiFrancesco, 2010b).

B. HCN Channels

A gene family coding for I_f channels, also named I_h or I_q, with "h" for hyperpolarization-activated (Biel et al., 2009; Ludwig et al., 1999a) and "q" for queer (Pape, 1996) in some tissues, has been cloned from the mouse (Ludwig et al., 1998; Santoro et al., 1998), rabbit (Ishii et al., 1999), and human (Ludwig et al., 1999b) tissue. This family, called HCN for Hyperpolarization-activated Cyclic Nucleotide-gated channels, is composed of four members, HCN1-4 that are expressed in heart and nervous system (for review, Accili et al., 2002; Baruscotti et al., 2005; Biel et al., 2009; Wahl-Schott & Biel, 2009). HCN channels have a tetrameric structure with each subunit composed of six transmembrane domains (S1–S6) and the cytosolic C- and N-termini domains (Fig. 4). Thus, HCN channels show structural analogies with voltage-gated K^+ channels with S1–S6 domains,

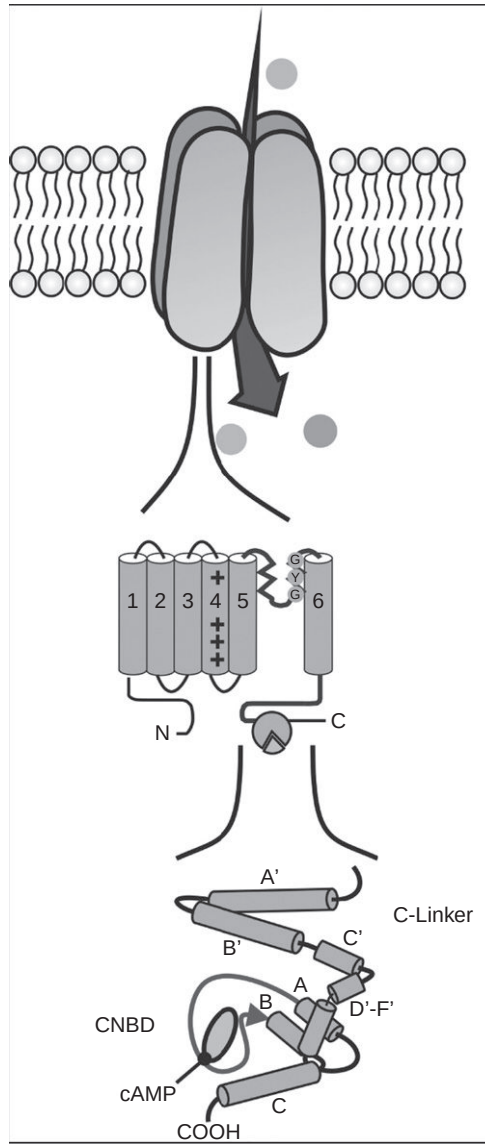

FIGURE 4 Structure of HCN channels. *Top*: HCN channels are tetramers. One monomer is composed of six transmembrane segments including the voltage sensor (S4), the selectivity filter, and the pore region between S5 and S6. The C-terminal channel domain contains the cyclic nucleotide-binding domain (CNBD; *middle*). *Bottom*: the C-terminal channel domain is composed of two domains. The C-linker domain consists of six α-helices, designated A′ to F′. The CNBD follows the C-linker domain and consists of α-helices A–C with a β-roll between the A and B helices (arrow) [from Wahl-Schott & Biel (2009) with permission].

a positively charged S4, as voltage sensor, and the GYG (glycine-tyrosine-glycine) motif, selective filter of potassium channels, in the pore between S5 and S6 domains. As cyclic nucleotide-gated channels, HCN channels also possess a cyclic nucleotide binding domain (CNBD) in their cytoplasmic C-terminus that confers to the channel its sensitivity to cAMP. The S4–S5 linker of the transmembrane core and C-linker of the proximal C-terminus interact allosterically with each other during channel gating (for more details, see Wahl-Schott & Biel, 2009).

Amino acid sequence alignment of the four human HCN isoforms (Baruscotti et al., 2005) indicates a high degree of sequence conservation within the HCN channel family (sequence identity of around 80–90% between HCN1 and HCN4). In contrast, cytosolic NH2 termini and the sequence downstream of the CNBD vary considerably in their length and share only modest to low homology between various HCN channels.

When expressed in heterologous systems, all four HCN channel types (HCN1–4) display the principal biophysical properties of native I_f /I_h/I_q: cationic inward current carried by Na^+ and K^+, activated upon hyperpolarization and regulated by internal cyclic nucleotides. Nevertheless, they differ quantitatively from each other with respect to their voltage dependence, activation/deactivation kinetics, and the extent of cAMP sensitivity. The biophysical properties of each channel type will not be detailed as they were extensively reviewed in excellent papers (Baruscotti et al., 2005; Biel et al., 2009). Furthermore, I_f /I_h measurements are very sensitive to experimental parameters and also differ depending on the expression system or cell type, further increasing heterogeneity. Briefly, the kinetics of HCN1 are faster than those of others isoforms with time constant of HCN4 > HCN3 (subtype less investigated) > HCN2 > HCN1. HCN2 has more negative activation threshold than HCN1 and HCN4. HCN4 is the more sensitive to the shift of its activation curve by cAMP with an order rank for sensitivity as follows: HCN4 (about 10–25 mV) ≥ HCN2 > HCN1 (2–7 mV) > HCN3 (no shift).

Although mRNA for HCN channels has been detected at low levels of expression in various tissues, I_f /I_h and HCN channels have been mainly identified in heart and the nervous system. Tissue distribution of HCN1–4 is not under the scope of this review; readers who are interested in a more complete description of the expression pattern are referred to review from Wahl-Schott and Biel (2009). Briefly, all four HCN isoforms are expressed in the brain, with HCN1 and HCN2 showing the highest expression levels. While HCN2 is distributed nearly ubiquitously throughout most brain regions, HCN1 is expressed in specialized regions as neocortex, hippocampus, and cerebellar cortex. The expression of HCN4 is lower except in thalamic nucleus and the olfactory bulb. In the dorsal root ganglions of the peripheral nervous system, all four subtypes have been reported with HCN1, the most abundant one. In the retina, HCN1 is also highly expressed

in all cellular layers. All four HCN channel isoforms have been detected in the heart. The expression levels strongly depend on the cardiac region with low levels in normal heart muscle compared to the spontaneously active cells of the conduction system (SAN, AVN, and Purkinje fibers). In atria and ventricular myocytes, HCN2 is the predominant isoform, displaying a rather ubiquitous distribution. In these contractile myocytes, with no DD, HCN channels activate at highly hyperpolarized membrane voltage under physiological conditions; thus, not expected to play a functional role. In the SAN, HCN4 is the major isoform accounting for about 80% of I_f (Shi et al., 1999) with HCN4 expression that correlates with pacemaker activity. This conclusion was supported by different studies that are summarized in Table I. While HCN4 can be considered as a marker of SAN pacemaker tissue, its functional properties do not fulfil exactly with those of I_f (Baruscotti et al., 2005), probably as a result of contribution of several mechanisms, as heteromerization of different isoforms (HCN4 and HCN1), regulation by modulatory factors (as the minK-related protein 1, MiRP1, phosphatidylinositol 4,5-bisphosphate, PIP_2, and caveolin 3), and a "context"-dependent intracellular environment (Barbuti et al., 2007; Pian et al., 2006; Yu et al., 2001).

The importance of HCN4 in cardiac pacemaking and control of HR is also supported by information obtained from HCN4-knockout mice and from patients with HCN mutations. Inactivation of HCN4 in mice is associated with embryo lethality with slow HR, quite complete abolition of I_f, and insensitivity to cAMP regulation of HR (Stieber et al., 2003). A direct correlation between a heterozygous mutation of HCN4 channels (S672R mutation) and a form of asymptomatic sinus bradycardia was shown in 27 patients from a large family (Milanesi et al., 2006).

III. Pharmacology of I_f Inhibitors

A. Chemistry

The interest on I_f inhibitors really emerged at the end of 1980s with the proposition of a new pharmacological class of compounds, "specific bradycardic agents, SBAs" (Kobinger & Lillie, 1987), which were characterized by a slowing of the sinus rate within physiological limits as the prominent cardiovascular effect, with exclusion of involvement of α-adrenergic, β-adrenergic, and cholinergic receptors. The potential beneficial effect in the context of myocardial ischemia was based on a reduction of myocardial oxygen consumption and a pronounced increase in diastolic period, which improves blood supply.

Among the first described SBAs, Alinidine (ST-567, *N*-allyl-clonidine) and falipamil (AQ-A-39, derived from verapamil) are representative of two

TABLE I Presence of Hyperpolarization-Activated Cyclic Nucleotide-Gated (HCN) Channels mRNA and Protein in the Sinoatrial Node from Different Species

Species	*Method*	*HCN1*	*HCN2*	*HCN3*	*HCN4*	*References*
Rabbit	RNAse protection assay	18%	1%	–	81%	Shi et al. (1999)
	N blot	ND	ND	ND	++	Ishii et al. (1999)
	N blot/*in situ* H	ND	ND	ND	+++	Tellez et al. (2006)
	N blot/*in situ* H	ND	ND	ND	+++ (central zone)	Brioschi et al. (2009)
Mouse	*in situ* H	+/–	+	–	+++	Moosmang et al. (2001)
	RT-PCR	+	+/–	–	+++	Marionneau et al. (2005)
	in situ H/IF	–	–	–	+++ (central zone)	Liu et al. (2007)
Rat	Q-PCR/*in situ* H/IF	ND	ND	ND	+++	Yanni et al. (2010)
Dog	RT-PCR/*in situ* H/IF/W blot	–	+	ND	+++	Zicha et al. (2005)
Human	RT-PCR	+/–	+	–	+++	Thollon et al. (2007)
	Q-PCR/*in situ* H/IF	++	+	–	+++	Chandler et al. (2009)

N blot, Northern blot; IF, immunofluorescence; *in situ* H, *in situ* hybridization; Q-PCR, quantitative polymerase chain reaction; RT-PCR, nonquantitative polymerase chain reaction; W blot, Western blot; ND, not determined.

Expression levels: below detection level (–), just above background (+/–), low (+), moderate (++), high (+++).

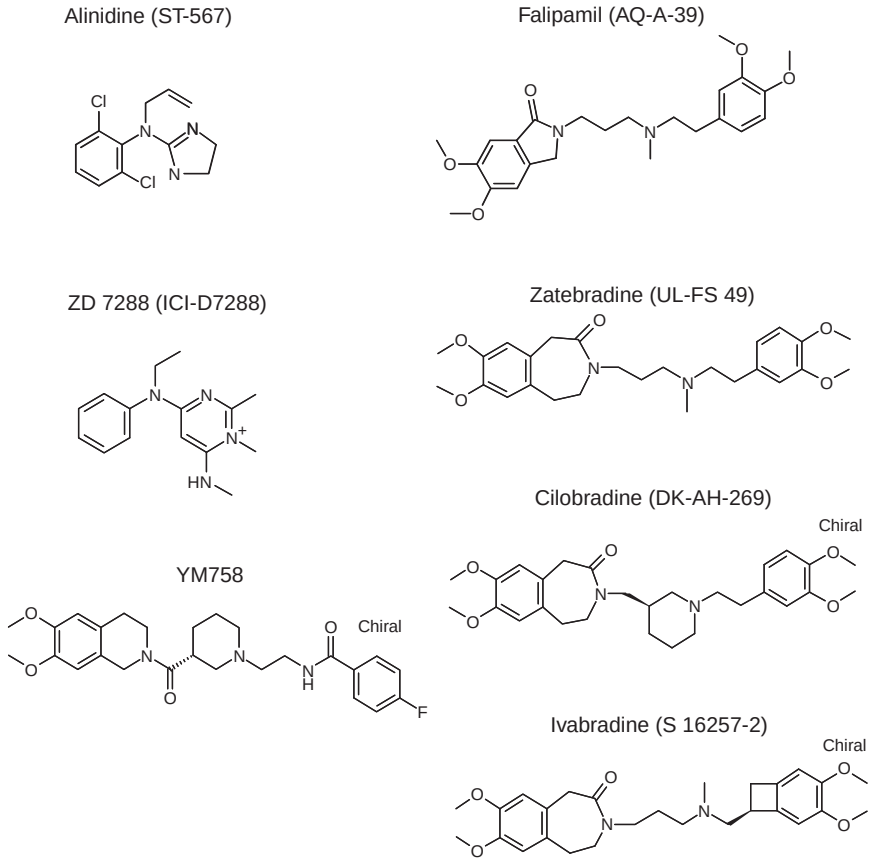

FIGURE 5 Chemical structures of "heart-rate-lowering" agents targeting the pacemaker activity of the sinus node.

different chemical groups of I_f /I_h inhibitors (for more details about preclinical and clinical results with these two compounds, readers are referred to *Supplement L of the European Heart Journal* in 1987).

Among more recent molecules, the benzazepinone derivative zatebradine (UL-FS 49) is a close analogue of falipamil (Fig. 5). Cilobradine (DK-AH-269) and ivabradine (S 16257-2) are also benzazepinone derivatives while ZD 7288 (ICI-D7288) could be viewed as a distant analogue of alinidine (Fig. 5). Except ivabradine (Procoralan®, Coralan®, Corlantor®) which was recently approved for the treatment of stable angina, all clinical trials for the other compounds were discontinued (Table II). The more recently described drug, YM758 [(–)-*N*-{2-[(*R*)-3-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-2-carbonyl) piperidino]ethyl}-4-fluorobenzamide monophosphate] from Astellas Pharma,

TABLE II Sinus Node Inhibitors: Main Effects on I_f Properties

Generic name (Originator)	*IC_{50} or K_d*	*Voltage shift*	*G_{max}*	*Use-dependence*	*Status (Clinical trials)*	*References*
Alinidine (Boehringer Ingelheim)	$K_d = 23.7\,\mu M$ (PF)	Negative 7.8 mV at 28 μM	–27%	No	Discontinued 1997 (Phase III 1989)	Snyders and Van Bogaert (1987)
ZD 7288 (AstraZeneca)	Range tested: 0.1–100 μM (gpSAN)	Negative 16 mV at 0.3 μM	–52% at 0.3 μM	No	Discontinued 1995 (Phase II 1993)	BoSmith et al. (1993)
Zatebradine (Boehringer IngelheimPharma KG)	$K_d = 5.6$ nM (PF) at 0.4 Hz	No	Reduced	Yes	Discontinued 1997 (Phase III 1992)	Van Bogaert and Pittors (2003)
	480 nM (rbSAN) at 0.5 Hz					Van Bogaert et al. (1990)
Cilobradine (Boehringer Ingelheim)	$K_d = 21.4$ nM (PF) at 0.4 Hz	No	Reduced	Yes	Discontinued 1996 (Preclinical)	Van Bogaert and Pittors (2003)
Ivabradine (Servier)	$IC_{50} = 2.8\,\mu M$ 1.5 μM (rbSAN) at 1/6 Hz	No	–50.4% at 3 μM	Yes	Launched 2005 (Stable angina)	Bois et al. (1996) Bucchi et al. (2002)
YM-758 (Astellas Pharma)	ND	ND	ND	ND	Discontinued 2007 (Phase II 2006)	ND

PF, Purkinje Fibers; rbSAN, rabbit sinoatrial node; gpSAN, guinea pig sinoatrial node; ND, not determined.
Modified from Bois et al. (2007) with permission.

under clinical development for stable angina and atrial fibrillation, was recently discontinued (PharmaProjects source).

B. Mechanism of Action

The hallmark of all specific "HR-lowering" agents is their ability to reduce the slope of DD of pacemaker AP, thus increasing the diastolic period, without altering other phases of AP (Baruscotti et al., 2005; Bois et al., 2007). In isolated rabbit SAN, 0.3 μg/ml alinidine (ST-567) decreased the spontaneous firing rate but a prolongation of AP duration was also noted suggesting an inhibition of potassium channels involved in cardiac repolarization (Satoh & Hashimoto, 1986). Briggs et al. (1994) using isolated guinea pig SAN and ventricular preparations, also demonstrated that alinidine and zatebradine (UL-FS 49) reduced the DD rate of SAN cells but were less specific than ZD 7288 for a same bradycardic effect. Alinidine and zatebradine prolonged AP duration in concentrations similar to those with bradycardic action. Furthermore, alinidine had negative inotropic effects. The direct action of zatebradine on cardiac repolarization was also confirmed by Thollon et al. (1994) when comparing ivabradine (S 16257) to zatebradine in different isolated cardiac preparations. Similar to ivabradine, zatebradine reduced the pacemaker activity in isolated SAN tissue by slowing the slope of DD but was less specific. As reported in a recent review from Baruscotti et al. (2010), ZD 7288 and ivabradine are among the more specific I_f inhibitors described to date with quite no effect on calcium and potassium channels at concentrations highly effective on I_f current (Table III).

As illustrated in Fig. 6, selective inhibition of I_f with ivabradine lowers the pacemaker firing rate by decreasing the DD rate with no significant changes in other parameters of AP even at a high concentration (3 μM).

All the specific "HR-lowering" drugs are effective inhibitors of I_f /I_h current (Baruscotti et al., 2005; Bois et al., 2007), thus reducing the maximal conductance (G_{max}) of the channels (Table II). Interestingly, two mechanisms of block have been described: (i) shift of the activation curve to more negative voltage (left shift on the voltage axis) and (ii) reduction of the maximal conductance in a use- and frequency-dependent way, with no change in the position of the activation curve. These two mechanisms were first shown by Van Bogaert and Goethals (1987), using voltage-clamp technique in cardiac Purkinje fibers. These authors have demonstrated that falipamil, UL-FS 49 (zatebradine), and alinidine all reduced the amount of pacemaker current I_f activated during the DD phase. The reduction of I_f conductance showed use-dependence with falipamil and UL-FS 49, as less I_f was activated at each successive voltage-clamp pulse of a train, with little recovery of block after a rest interval. On the contrary, no use-dependent block was observed with alinidine, which caused reduction of I_f by shifting its activation range to more negative potential (Van Bogaert & Goethals, 1987). ZD 7288 inhibited I_f in

TABLE III Effects of "Heart Rate-Lowering" Agents on Electrical Properties of Cardiac Pacemaker Cells

	Alinidine	*Zatebradine*	*Cilobradine*	*ZD 7288*	*Ivabradine*
I_f reduction	≈80% 30 μM (rbSAN cells)	≈65% 1 μM (rbSAN cells)	≈60% 1 μM (mSAN cells)	78% 1 μM (gpSAN cells)	≈60% 3 μM (rbSAN cells)
I_K reduction	80% 30 μM (rbSAN cells)	≈20% 1 μM (rbSAN cells)	≈22% 5 μM (mSAN cells)	No effect 1 μM (gpSAN cells)	No effect 3 μM (rbSAN cells)
I_{Ca} reduction	80% 30 μM (rbSAN cells)	No effect 1 μM (rbSAN cells)	Not tested	14% 1 μM (gpSAN cells)	No effect 3 μM (rbSAN cells)
DDS	↓ 30 μM (rbSAN cells)	–42% 3 μM (rbSAN tissue)	–70% 1 μM (mSAN cells)	–54% 1 μM (gpSAN tissue)	–67% 3 μM (rbSAN tissue)
APD_{50}	+23% 30 μM (rbSAN cells)	+29% 3 μM (rbSAN tissue)	+60% 1 μM (mSAN cells)	+8% 1 μM (gpSAN tissue)	+9% 3 μM (rbSAN tissue)
Rate reduction	22% 30 μM (rbSAN cells)	28% 3 μM (rbSAN tissue)	≈55% 1 μM (mSAN cells)	≈35% 1 μM (gp right atrium)	≈24% 3 μM (rbSAN tissue)
References	Satoh and Hashimoto (1986)	Goethals et al. (1993) Thollon et al. (1994) Bois et al. (1996)	Stieber et al. (2006)	BoSmith et al. (1993) Marshall et al. (1993)	Thollon et al. (1994) Bois et al. (1996) Bucchi et al. (2002)

APD, action potential duration; DDS, diastolic depolarization slope; gpSAN, guinea pig sinoatrial node; rbSAN, rabbit sinoatrial node; mSAN, mice sinoatrial node

From Baruscotti et al. (2010) with permission.

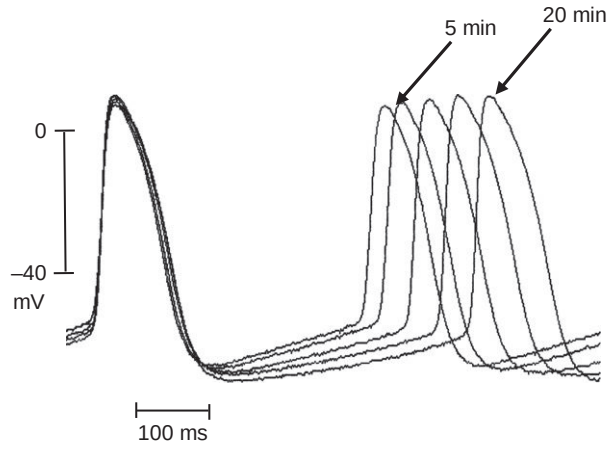

FIGURE 6 Experimental traces of action potentials in isolated SAN tissue, showing reduction of pacemaker firing rate by 3 μM ivabradine (0, 5, 10, 15, 20 min).

guinea pig isolated SAN cells (BoSmith et al., 1993) by shifting the current activation curve in the negative direction on the voltage axis and reducing the activation curve amplitude. On the contrary to UL-FS 49, ZD 7288 did not display use-dependent block of I_f (BoSmith et al., 1993). Both zatebradine and its congener cilobradine reduced concentration-dependently the amplitude of I_f without modifying either the voltage dependence or the kinetics of channel activation. Both drugs induced a use-dependent block of I_f that was stronger and faster for cilobradine. Recovery from blockade during prolonged hyperpolarization in the presence of the drug was shown for both molecules, but was faster with zatebradine (Van Bogaert & Pittoors, 2003). Investigation in isolated rabbit SAN cells showed that inhibition of I_f by ivabradine was concentration dependent with an IC_{50} of around 3 μM (Fig. 7) and was due to use-dependent intracellular blockade of the channels (Bois et al., 1996). The overall effect of ivabradine is similar to those of zatebradine and cilobradine as all drugs are open channel blockers; they affect I_f in a use-dependent way with no change in the voltage dependence of its activation. In the study from Bois et al. (1996), 3 μM ivabradine did not affect $I_{Ca,L}$, $I_{Ca,T}$, and I_{Kr} currents while I_f was inhibited by about 60%, indicating a good selectivity for pacemaker current over other channels (Table III). More recently, Bucchi et al. (2002) also confirmed that ivabradine, as zatebradine and cilobradine, inhibited more efficiently f-channels at depolarized voltages and blocks them from the intracellular side of the membrane. These authors also demonstrated that the block of I_f was dependent on the current flowing across the pore. Additionally, block removal during prolonged hyperpolarization also required inward ionic flow. Thus, the action of ivabradine displays both a marked use-dependence and current-dependence since the direction of current

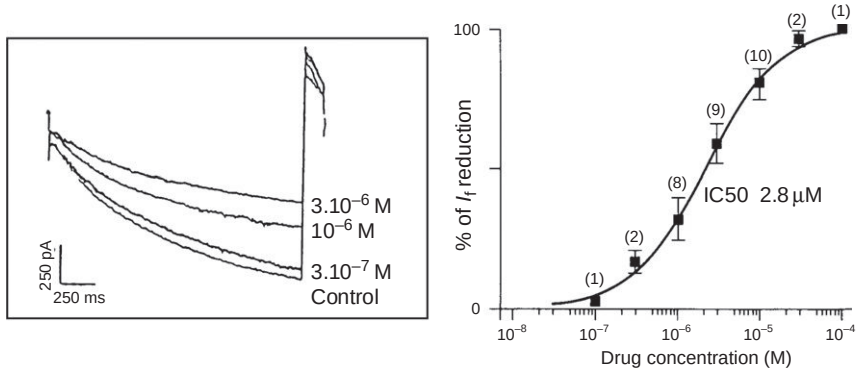

FIGURE 7 Concentration-dependent inhibition of I_f current recorded in isolated rabbit SAN cells by S 16257 (ivabradine). I_f current was elicited by hyperpolarizing steps from –30 to –I00 mV at 1/6 Hz. *Left panel*: traces of I_f current recorded in sequence just before (control) and during cell perfusion with S 16257 at 0.3, 1, or 3 µM. *Right panel*: concentration-dependent effect of S 16257 on I_f. The data were fitted with the expression $f(x) = 100/[1 + (IC_{50}/\times)nH]$. $IC_{50} = 2.8\,\mu M$. The Hill coefficient nH = 0.96 [modified from Bois et al. (1996) with permission].

affects the stability of its binding in the pore of the channel. The efficacy and mechanism of block of the new HR-lowering agent YM-758 from Astellas Pharma are not published yet. For other compounds, mechanisms of block are summarized in Table II (from Bois et al., 2007).

As HCN4 is the main isoform of pacemaker channels in the SAN, the effect of ivabradine on human HCN4 current has been evaluated (Bucchi et al., 2006; Thollon et al., 2007). The drug inhibits hHCN4 current in a concentration- and use-dependent way as for native I_f. Bucchi et al. (2006) have also reported that ivabradine blocking behavior on HCN4 channels is similar to that observed previously for native I_f channels in isolated SAN cells (Bucchi et al., 2002). As use-dependence is the mechanism responsible for block accumulation during repetitive channel opening-closing pulses (Fig. 8), one possible consequence is that effect of ivabradine will be more effective at higher HR (Thollon et al., 2007). Another consequence of pure inhibition of I_f is that even a total inhibition of the current with ivabradine will never stop pacemaking as it is not the only contributor to DD. As a result full blockade of I_f will only lead to HR reduction with a plateau extent depending on the initial resting HR (Mangoni & Nargeot, 2008).

C. Selectivity

The selectivity of "HR-lowering" agents for native f-channels among other voltage-gated channels is critical for their safety. In particular,

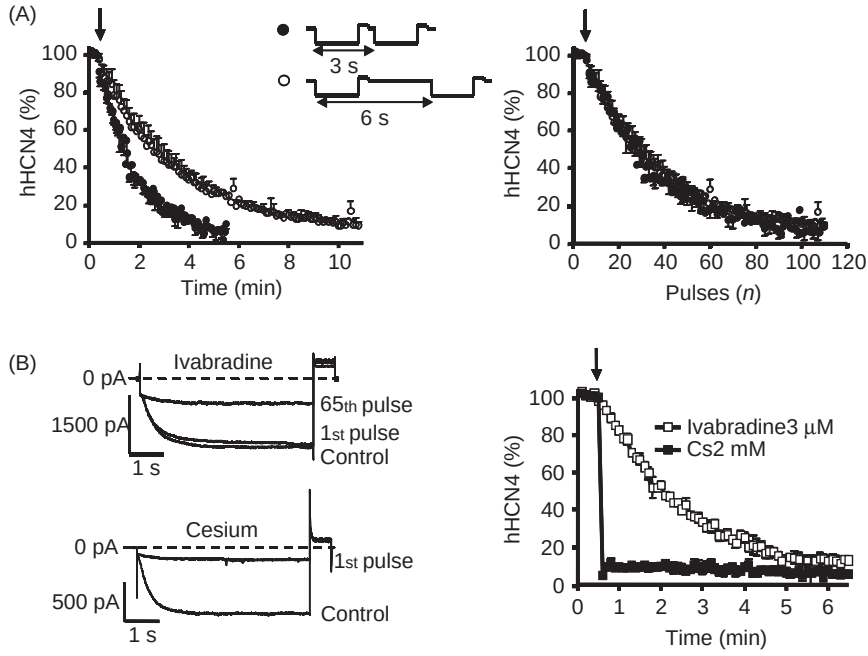

FIGURE 8 Use-dependent inhibition of hHCN4 current in CHO cells by 3 µM ivabradine. (A) Same protocol (–110 mV for 2 s followed by a 0.6 s pulse at +20 mV at 35 ± 1° C) is applied every 3 s ($n = 2$) or 6 s ($n = 2$). The kinetics of block are expressed as a function of time (left) or pulse number (right), showing same relationship to the pulse number with both protocols. The arrows indicated the beginning of the application of drug. (B) Comparison of the time course of inhibition of hHCN4 by ivabradine 3 µM and Cesium 2 mM. Pulses at –110 mV for 5 s followed by +20 mV for 0.6 s at 1/6 Hz. Use-dependent inhibition by ivabradine ($n = 4$) and time-independent by Cesium ($n = 3$) leading to reduction of about 90% of hHCN4 at steady-state for both compounds. The steady-state block was obtained at the first pulse for Cesium while 65 pulses were needed for ivabradine (traces on left panels) (from Thollon et al., 2007).

structural analogies between HCN and ERG channels involved in cardiac repolarization lead to extreme difficulties to obtain selective blockers of pacemaker channels. An illustration of these difficulties is given in Table III that reports the effects of developed drugs on others channels at concentrations that block I_f (from Baruscotti et al., 2005). At the beginning of 1990s, ivabradine (S 16257) was selected for clinical development on the basis of this selectivity among many other active molecules. Furthermore, the *S* enantiomer ivabradine (*S* 16257) was shown to be more selective than the *R* enantiomer (*S* 16260) and than the racemate (*S* 15544) for a same HR-lowering effect, both *in vitro* and *in vivo* (Thollon et al., 1997).

Experiments investigating the affinity of ivabradine for homomeric HCN channels did not demonstrate any isoform specificity when using classical patch-clamp pulse protocols (Bucchi et al., 2006; Stieber et al., 2006).

Nevertheless, it was interestingly shown that, on the contrary to HCN4 and native I_f, ivabradine is able to block HCN1 in the closed state (Bucchi et al., 2006). For more details concerning the electrophysiological profile of ivabradine, readers are referred to a recent review from Savelieva and Camm (2008).

IV. *In Vivo* Preclinical Studies

A. Selective HR Reduction in Normal Animals

As ivabradine is the only member of the family of HR-reducing agents having completed clinical development for chronic stable angina, this review will focus on preclinical and clinical studies performed with this drug.

1. Resting Conditions

A compound that inhibits pacemaker I_f is expected to reduced HR *in vivo* with no other hemodynamic and electrophysiological effects. This has been extensively investigated for ivabradine in different species during intravenous (*IV*) or oral (*PO*) administration, for both single dose and chronic treatments (Table IV). In small species, ivabradine significantly reduces HR with a long-lasting reduction for 7–12 h at doses of about 2 and 3 mg/kg for *IV* and *PO* administration, respectively. In pig and dog, the effective doses for HR reduction are around 0.1 and 5 mg/kg for *IV* and *PO* administration, respectively. Taking into account that small animals have a higher basal HR, the reduction obtained with ivabradine, expressed in beats per minute (bpm), is more important with elevated HR: about –125 bpm for mice and –45 bpm for rat compared with –16 to –18 bpm for dog and pig after oral administration. Chronic oral treatment in conscious rats for 6 days with ivabradine 3 mg/kg/day was shown to reduce HR and limit significantly the nocturnal tachycardia up to 12 h after administration, with no change in mean arterial pressure (Vilaine, 2006; Vilaine et al., 2003b). Efficacy of ivabradine was maintained similarly over the 6 days of treatment with no rebound effect on treatment cessation.

As expected from the *in vitro* studies, it was demonstrated in the pig that ivabradine induced selective HR reduction without significant prolongation of the corrected QT interval and the PR interval, thus preserving ventricular repolarization and atrioventricular conduction even at a high dose (1 mg/kg *IV*) inducing more than 35% reduction in HR (Thollon et al., 1997).

2. Reduction of Exercise-Induced Tachycardia

Coronary blood flow occurs mostly during diastole because the coronary vasculature has one particular property: it is compressed by the contracting myocardium such that no flow occurs during systole. In normal myocardium, tachycardia increases myocardial oxygen demand

TABLE IV Efficacy of Ivabradine for Selective Heart Rate (HR) Reduction in Different Species

Species		N	*Administration*	*Doses*	*Bas. HR (bpm)*	*Max HRR*	*HRR duration*	*Reference method*
Mice	Conscious	5	*PO* gavage	3 mg/kg	650	19%	12 h	Arterial catheter[a]
	Conscious	8/gr	*PO* drinking water	10 mg/kg/day (1 week)	≈760	≈10–15%		Du et al. (2004)
	Anesthetized				≈460	≈30%		
Rat	Conscious	8	*IV*	2 mg/kg	350	37%	≥7 h	Telemetry[a]
	Conscious	12	*PO* gavage	3 mg/kg	300	16%	12 h	Vilaine et al. (2003b)
		6/gr	*PO* gavage	1.5 & 6 mg/kg	320	14 & 22%	≥6 h	Vilaine et al. (2003b)
Guinea pig	Conscious	6	*PO* gavage	3 mg/kg	250	44%	12 h	Telemetry[a]
Rabbit	Anesthetized	6/gr	*IV*	1, 2, & 4 mg/kg	180	19, 21, & 21%		Arterial catheter[a]
	Conscious	4	*IV*	2 mg/kg	210	26%	≥7 h	Arterial catheter[a]
	Conscious	4	*PO* gavage	6 mg/kg	200	≈20%	≥8 h	Arterial catheter[a]
Pig (Large white)	Anesthetized	10	*IV*	0.1, 0.3, & 1 mg/kg (30 min/dose)	104	15, 26, & 35%	≥30 min	Thollon et al. (1997)
(Göttingen)	Conscious	7	*IV*	1 mg/kg	86	21%	≥5 h	Hemodynamic[a]
(Yucatan)	Conscious	5	*PO* gavage	5 mg/kg	82	19%	≥5 h	Hemodynamic[a]
Dog	Conscious	1	*IV*	1 mg/kg	54	33%	≥3 h	ECG[a]
	Conscious	5	*IV*	0.1–1 mg/kg	94	19% (0.5 mk)		Simon et al. (1995)
					90	22% (1 mk)		
	Conscious	1	*PO* gavage	5 mg/kg	58	31%	6 h	ECG[a]

[a] Unpublished results from Servier Research Center; bas., basal; bpm, beats per minute; ECG, electrocardiogram; HRR, heart rate reduction; max, maximal; mk, mg/kg.

(Sonnenblick et al., 1968), and the resulting augmentation in myocardial oxygen consumption is matched by increased coronary blood flow through metabolic coronary vasodilatation. Nevertheless, coronary blood flow per cardiac cycle is reduced, because the duration of diastole is decreased over proportionately at high HR (Colin et al., 2004). Under physiological conditions, metabolic coronary vasodilatation is so powerful that it largely overcompensates for decreased diastolic duration and a slight increase in myocardial oxygen extraction also contributes at very high HR. Therefore, under normal circumstances, coronary blood flow and oxygen delivery are always matched to myocardial performances and oxygen consumption (for review, Heusch, 2008). Hemodynamic studies, performed in instrumented dog or pig, have demonstrated that ivabradine limits the tachycardia induced by exercise (Table V). For equipotent reduction of HR at rest and during exercise, leading to a decrease in oxygen consumption, ivabradine clearly distinguished over β-blockers (BBs). Both propranolol (Simon et al., 1995; Vilaine et al., 2003a) and atenolol (Colin et al., 2003; Lucats et al., 2007a) showed negative inotropic effects at rest and during adaptation of left ventricular (LV) contractility to exercise, whereas ivabradine did not change LV dP/dt_{max} at rest and did not limit its increase induced by exercise. This important difference between ivabradine and BBs explains not only why ivabradine preserved better LV performance but also why for the same reduction in HR during exercise in dogs, it increased more the diastolic perfusion time than a BB which, due to its negative inotropic and lusitropic effects, increased the duration of ejection and relaxation at the expense of the diastolic perfusion time (Colin et al., 2003), a difference that can be crucial at the ischemic threshold. Furthermore, β-blockade unmasked α-adrenergic coronary vasoconstriction which limited the coronary arteries adaptation in diameter during the exercise (Simon et al., 1995), a deleterious effect that was not observed with the pure HR-reducing drug ivabradine.

B. Pathological Models

1. Myocardial Ischemia

The anti-ischemic efficacy of ivabradine was initially described in a model of exercise-induced regional myocardial ischemia in the pig: ivabradine, in comparison with the BB propranolol, induced equipotent HR reduction at rest and during exercise, similar limitation of regional myocardial ischemia but, unlike the BB, preserved global LV function and improved better regional myocardial function (Vilaine et al., 2003a).

In a model of exercise-induced myocardial ischemia in the dog (Monnet et al., 2004) ivabradine and the BB atenolol, at doses inducing similar reductions in HR, induced beneficial redistribution of regional myocardial blood flow to the subendocardium and reduced myocardial dysfunction in

TABLE V Beneficial Effects of IVA during Exercise-Induced Tachycardia in Different Species

Species	*Treatment: dose*	*Treatment*	*Change in HR*	*Beneficial effects*	*Reference*
Dog	IVA 0.5 mg/kg *IV* [vs vehicle and propranolol 1 mg/kg *IV*]	Exercise 10 min after treatment	–14% at rest –19% during exercise	– ↓ Exercise-induced tachycardia – IVA differs from BB with no negative inotropic and vasoconstrictor effects	Simon et al. (1995)
Yucatan micropig	IVA [vs vehicle and propranolol] Both 5 mg/kg *PO*	Exercise: 1, 3, & 5 h after treatment	– at rest: –11, –19, & –18% – during exercise: –17, –22, & –26%	– ↓ Exercise-induced tachycardia (~ cf. BB) – IVA differs from BB with no change in the exercise-induced increase in LV dP/dt_{max}	Vilaine et al. (2003b)
Dog	IVA [vs vehicle and atenolol] Both 1 mg/kg *IV*	Bolus before exercise	–16% at rest –30% during exercise	– ↑ Diastolic perfusion time – ↓ MVO2 – ↓ LV dP/dt_{max} only with BB	Colin et al. (2003)
Dog	IVA 0.25, 0.5, & 1 mg/kg *IV* vs vehicle	Bolus before exercise	–17, –21, & –32% during exercise	– Dose-dependent effects (suppressed by pacing) – Linear relationship between HR and MVO2	Colin et al. (2004)
Dog	IVA [vs vehicle and atenolol] Both 1 mg/kg *IV*	Bolus before exercise	–10% to –20% at rest About –30% during exercise	On the contrary to atenolol, IVA does not change dP/dt_{max}, and PSWT	Lucats et al. (2007a)

BB, β-blocker; HR, heart rate; IVA, ivabradine; LV dP/dt_{max}, maximal rate of rise of left ventricular pressure; MVO2, myocardial oxygen consumption; PSWT, post-systolic wall thickening.

the ischemic area. Only ivabradine reduced the severity and duration of myocardial stunning, when atenolol worsened this phenomenon. This important difference was observed when the treatments were administrated either before or after ischemia. The beneficial effect of ivabradine on stunning was dependent on HR reduction, whereas the deleterious effect of the BB, mainly dependent on its negative inotropic and lusitropic effects, was aggravated when its HR reduction effect was prevented by atrial pacing. In a model of exercise-induced ischemia and stunning in the dog, ivabradine, in addition to attenuate myocardial stunning, reduced the increase in post-systolic wall thickening (PSWT) in the stunned myocardium, a paradoxical diastolic wall motion that alters early ventricular relaxation (Lucats et al., 2007b), thus converting PSWT into ejectional thickening (Table VI). On the contrary, the BB atenolol further depressed systolic wall thickening and did not reduce PSWT. The same authors have demonstrated that for a similar HR reduction at rest and during exercise in normal dog, ivabradine did not change dP/dt_{max} and PSWT and preserved the part of ventricular wall thickening contributing to ejection, on the contrary to atenolol (Lucats et al., 2007a). Ivabradine (0.6 mg/kg *IV*) improved both myocardial blood flow and contractile function and reduced the infarct size (Table VII) in a model of ischemia–reperfusion in the pig (Heusch et al., 2008). Couvreur et al. (2010) has recently demonstrated that ivabradine administrated during 3 weeks of reperfusion following 20 min of coronary occlusion in the rabbit improved ejection fraction and myocardial contractility and that these changes were associated by an increase in FKBP12/12.6 indicating a beneficial adaptation in calcium handling (Table VII).

2. *Post-ischemic Heart Failure*

A long-term treatment (3 months) with ivabradine, inducing chronic HR reduction, in rat with post-ischemic congestive heart failure (HF), preserved stroke volume and cardiac output with a decrease in LV systolic diameter and no change in LV diastolic diameter (Mulder et al., 2004). Preservation of LV function was associated with improvement of LV structure with decrease in perivascular collagen and increase in capillary density and decrease in plasma norepinephrine (Table VIII).

More recently, Dedkov et al. (2007) and Milliez et al. (2009) also demonstrated the beneficial effect of chronic treatment with ivabradine using also a severe model of post- myocardial infarction (MI) HF in rat (Table VIII).

3. *Atherosclerosis*

Several epidemiological studies have reported that elevated HR is associated with coronary atherosclerosis independently of other risk factors, probably by increasing magnitude and frequency of the mechanical load imposed to the arterial wall (for review Giannoglou et al., 2008;

TABLE VI Beneficial Effects of HRR with IVA During Treadmill Exercise-Induced Ischemia and Stunning After Partial Coronary Artery Stenosis

Species	*Treatment: dose*	*Treatment duration*	*Change in HR*	*Beneficial effects*	*Reference*
Pig	IVA [vs propranolol] Both 5 mg/kg *PO*	Treatment 3 h before exercise	–14% at rest –23% during exercise –23% during exercise	– Reduction of exercise-induced tachycardia and exercise-induced regional ischemia – No reduction in LV contractility with IVA (≠BB) – No reduction in LV contractility with IVA (≠BB)	Vilaine et al. (2003a)
Dog	1 mg/kg *IV* bolus + 0.5 mg/kg/h 6 h [vs atenolol 1 mg/kg *IV*]	Treatment before exercise (+6 h reperfusion)	–21% at rest –29% during exercise	– Anti-ischemic effects – ↑Regional myocardial blood flow – ↑Diastilic perfusion time – ↑ Contractility in the stunned myocardium (≠BB)	Monnet et al. (2004)
Dog	IVA [vs atenolol] Both 1 mg/kg *IV*	Bolus (beginning of reperfusion)	–24% after 6h reperfusion	– Opposition to depressed systolic wall thickening and increased PSWT in the stunned myocardium – IVA converts PSWT into ejectional thickening (≠BB)	Lucats et al. (2007b)

BB, β-blocker; HF, heart failure; HR, heart rate; HRR, heart rate reduction; IVA, ivabradine; LV, left ventricular; PSWT, post-systolic wall thickening.

TABLE VII Beneficial Effects of Heart Rate Reduction (HRR) with Ivabradine (IVA) during Ischemia-Reperfusion in Different Species

Experimental model	*Species*	*Surgical procedure*	*Treatment: dose*	*Treatment duration*	*HR*	*Beneficial effects*	*Reference*
MI-R	Pig	Coronary hypoperfusion (90 min) reperfusion (120 min)	IVA 0.6 mg/kg *IV*	Treatment before or during ischemia, or before reperfusion	–23%	–Improved regional myocardial blood flow and contractile function –Reduction of infarct size –Elimination of benefit on flow and function by pacing	Heusch et al. (2008)
Acute MI	Pig	Total 1 min coronary occlusion	IVA 0.5 mg/kg *IV*	Treatment after occlusion	–31%	– Increase in ventricular fibrillation threshold – Prevention of ischemia-induced action potential shortening	Vaillant et al. (2008)
MI-R	Rabbit	Coronary occlusion (20 min) reperfusion (3 w)	IVA 10 mg/kg/day *PO*	Treatment on reperfusion	–20%	–Improved ejection fraction (+35%) and systolic displacement (+26%) – × 2 increase in FKBP12/12.6 (adaptation in Ca^{2+} handling)	Couvreur et al. (2010)

HR, heart rate; MI-R, myocardial ischemia-reperfusion.

TABLE VIII Beneficial Effects of HRR with IVA in a Model of Post-MI HF in Rat Following Coronary Artery Ligation

Treatment	*Treatment duration*	*HR*	*Beneficial effects*	*Reference*
10 mg/kg/day *PO* in food	3 months starting 7 days after ligation	–18%	↓ LV end-systolic but not end-diastolic diameter (preserved cardiac output); ↓ LV collagen and ↑ capillary density. Three days after interruption of treatment: improvement of LV function preserved.	Mulder et al. (2004)
10.5 mg/kg/day *IP* osmopump	4 weeks starting after ligation	≈–25%	Improved maximal myocardial perfusion and coronary reserve by reduced periarteriolar collagen.	Dedkov et al. (2007)
10 mg/kg/day *PO* drinking water	3 months starting 2 months after ligation	–10%	–Improved LV ejection fraction and LV end-diastolic pressure. –Reduced interstitial fibrosis. –Inhibition of ↑ gene and protein expression of ACE and AT1.	Milliez et al. (2009)

HF, heart failure; HR, heart rate; HRR, heart rate reduction; IVA, ivabradine; LV, left ventricular; MI, myocardial ischemia.

Tardif, 2009), thus damaging the vascular endothelium (Thorin & Thorin-Trescases, 2009). Consequently, reducing HR could protect the endothelium and slow the onset of atherosclerosis. Using dyslipidemic mice (expressing ApoB-100), Drouin et al. (2008) demonstrated that a 3-months treatment with ivabradine prevented endothelium dysfunction in renal and cerebral arteries. Furthermore, Custodis et al. (2008) and Baumhäkel et al. (2010) showed that long-term treatment of ApoE–/– mice by ivabradine improved endothelial function and reduced atherosclerotic plaque formation. All these results are in favor of a protective effect mediated by a reduction of vascular oxidative stress.

V. Clinical Studies

A. Chronic Stable Angina

Clinical studies in patients with chronic stable angina have shown that ivabradine acts as a pure HR-reducing agent, has an anti-ischemic and anti-anginal efficacy at least as potent as that of BBs and CCBs, and presents a good safety profile during long-term treatment. A brief overview of main clinical trials performed with ivabradine is given in this issue.

1. Anti-anginal Efficacy and Safety of Ivabradine

The clinical efficacy and safety of ivabradine was initially evaluated in comparison with placebo in 360 patients with stable angina pectoris and documented CAD (Borer et al. 2003). The description of the study is detailed in Table 9. This study has shown a dose-dependent reduction in HR at rest and during exercise, improvement in exercise tolerance and time to development of ischemia during exercise tolerance test, a reduction of angina attacks and consumption of short-acting nitrates, and no rebound phenomena at treatment cessation (Borer et al., 2003). The incidence of adverse effects was low, the only effects linked to treatment were visual symptoms, mostly phosphenes.

2. Comparison of Ivabradine with Other Anti-anginal Agents

The anti-anginal and anti-ischemic effects of ivabradine (Table IX) were compared to those of active anti-anginal drugs: atenolol (BB) and amlodipine (CCB).

a. Ivabradine Versus BBs The 4-month INITIATIVE randomized trial (INternatIonal TrIal of the AnTi-anginal effects of IvabradinE), comparing ivabradine (5 mg bid + 12 additional weeks at 7.5 or 10 mg bid) and atenolol (50 mg od + 12 additional weeks at 100 mg od) in 939 patients with stable angina and documented CAD, demonstrated the noninferiority of ivabradine compared with atenolol at all doses and for all exercise parameters such

TABLE IX Randomized Clinical Trials with Ivabradine (IVA), Comparing it Either with Placebo or Active Anti-anginal Drugs, i.e., Atenolol (At) or Amlodipine (Amlo), β-blocker (BB) or calcium channels blocker (CCB), respectively

Study/Objective/Reference	*Study design*	*Population*	*Patients*	*Treatment*	*Conclusion*
Evaluation of anti-anginal and anti-ischemic effects of IVA	Randomized	Stable angina	360	IVA 2.5/5/10 mg bid	IVA improves the exercise tolerance and increase the time to development of ischemia.
Borer et al. (2003)	Double-blind Placebo controlled study	Documented CAD			
INITIATIVE:	Randomized	Stable angina	939	IVA 5→10 mg bid	IVA as atenolol is effective in patients with stable angina pectoris.
Comparison of anti-anginal and anti-ischemic effects. IVA vs BB. Tardif et al. (2005)	Double-blind	CAD > 3 months		At 50→100 mg	
	Parallel group non-inferiority			4 w→12 weeks	
Comparison of anti-anginal and anti-ischemic effects. IVA vs CCB. Ruzyllo et al. (2007)	Randomized	Chronic and stable angina >3 months	1195	IVA 7.5 or 10 mg bid	IVA as amlodipine is effective in patients with stable angina pectoris.
	Double-blind	Effort-induced angina		Amlo 10 mg	
	Parallel group non-inferiority			3 months	

(*Continued*)

Table IX (*Continued*)

Study/Objective/Reference	*Study design*	*Population*	*Patients*	*Treatment*	*Conclusion*
ASSOCIATE:	Randomized	Documented CAD	889	IVA 7.5 mg bid	Combination of IVA + Atenolol well tolerated. Despite standard BB treatment, IVA improves exercise capacity.
Evaluation of further anti-anginal and anti-ischemic effects of IVA under BB. Tardif et al. (2008b)	Double-blind	Stable angina		At 50 mg	
	Parallel group	Atenolol 50 mg		4 months	
BEAUTI$_f$UL:	Randomized	Documented CAD and LVEF < 40%	10,917	IVA 7.5 mg bid	Reduction in HR does not improve cardiac outcome in all patients. IVA could be used to reduce CAD outcomes in patients with HR>70 bpm.
Primary endpoint: composite CV death, hospitalization for AMI, or worsening of HF. Fox et al. (2008a)	Double-blind			Follow-up 19 months	
	Placebo controlled study				
	Parallel group study				

bpm, beats per minute; CAD, coronary artery disease; HR, heart rate; LVEF, left ventricular ejection fraction.

as increase in total exercise duration and increase in time to exercise-induced ischemia. In comparison with atenolol, a similar or greater improvement in exercise capacity was observed with ivabradine for smaller reduction in HR that could reflect the advantage of the selective hemodynamic effect of ivabradine on HR without the negative inotropic and indirect vasoconstrictor effects of the β-blocker that are potentially limiting (Tardif et al., 2005). Both drugs induced similar decreases in the number of anginal attacks and use of short-acting nitrates.

b. Ivabradine Versus CCBs A 3-month randomized trial, in 1195 patients with stable angina and documented CAD, demonstrated the noninferiority of twice daily ivabradine (7.5 and 10 mg) with respect to amlodipine 10 mg once daily in improving exercise capacity (Ruzyllo et al., 2007). Both drugs induced similar improvements in exercise tolerance test parameters and in symptom control with reduction in angina attacks frequency and in use of short-acting nitrates.

All these results indicated that the sole reduction of HR induced by ivabradine, resulting from selective inhibition of I_f, is sufficient to induce a major anti-anginal and anti-ischemic effect.

3. Long-Term Efficacy and Safety of Ivabradine

The long-term efficacy and safety of ivabradine at two oral doses (5 and 7.5 mg bid) was assessed, on top of standard therapy, in a 12-month study in 386 patients with chronic stable angina and documented CAD. Ivabradine, at these two recommended doses, was effective in reducing resting HR and the number of angina attacks per week. The drug was well tolerated, the most frequently adverse events were phosphene-like mild transient visual symptoms, leading to treatment withdrawal in only four cases. Sinus bradycardia caused treatment cessation in three patients and there was no increase in the QTc (Bazett) interval (Lopez-Bescos et al., 2007). The safety of ivabradine was confirmed throughout its clinical development in a large population of patients with stable angina and CAD. The most frequent adverse drug reaction was confirmed to be visual symptoms, mostly phosphenes, that are transient and well tolerated, leading to less than 1% treatment withdrawal. These effects can be attributed to the inhibition of HCN channels in the retina (Cervetto et al., 2007; Demontis et al., 2009; Santina et al., 2010). All visual symptoms resolved spontaneously during treatment or after drug cessation. Ophthalmic investigation has found no deleterious effect of ivabradine on retinal function and morphology (European Medicines Agency, 2006).

Excessive bradycardia was reported in only 2.2% of patients treated with ivabradine 7.5 mg bid, compared with 4.4% of patients treated with atenolol 100 mg od. The absence of excessive bradycardia was predicted by preclinical data and expected from the selective inhibition of only one of the

currents, the I_f current, contributing to the regulation of pacemaker activity. The selective electrophysiological profile of ivabradine was confirmed in patients. QT intervals from all patients enrolled in the development of ivabradine were evaluated using a population-corrected formula (QTcP); the absence of change of corrected QT interval throughout the follow-up period confirmed the absence of significant direct effect of ivabradine on the duration of ventricular repolarization (Savelieva & Camm, 2005). Electrophysiological exploration in patients with normal electrophysiology showed that ivabradine increased only corrected sinus node recovery time, but had no effect on atrioventricular conduction and QRS duration (Camm & Lau, 2003).

Ivabradine was also well tolerated in asthmatic patients without alteration of lung function and symptoms (Babu et al., 2006), and did not have adverse effects in diabetic patients on blood glucose and glycated hemoglobin levels (Borer, 2006).

4. *Combination with Anti-anginal Drugs*

Recently, the 4-month ASSOCIATE trial (Table IX), performed in 889 patients with stable angina pectoris, receiving the BB atenolol at 50 mg/day, a commonly used dosage in these patients, showed that ivabradine at the initial dose of 5 mg bid, increased to 7.5 mg for the two last months, in comparison with placebo, induced additional efficacy with a significant improvement in total exercise duration and all exercise test criteria, accompanied by reductions in HR. The combination was shown to be safe, with only 1.1% of treatment withdrawal due to sinus bradycardia (Tardif et al., 2009). These data with others, demonstrating the benefit and the safety of the combination of ivabradine with BBs, will permit to extend the initial therapeutic indications: "treatment of chronic stable angina in patients with normal sinus rhythm who have a contraindication or intolerance to BBs" by adding "or in combination with BBs in patients inadequately controlled with an optimal BB dose and whose HR is >60 bpm."

B. CAD with LV Dysfunction

The main objective of the randomized BEAUTI$_f$UL (morBidity-mortality EvAlUaTion of the I_f inhibitor ivabradine in patients with coronary disease and left ventricULar dysfunction) study (Fox et al., 2008a), conducted in 781 centers with 10,917 patients, was to examine the effects of ivabradine (target dose of 7.5 mg bid) on cardiovascular events in patients with stable CAD and LV dysfunction (ejection fraction < 40%). 5438 patients received placebo on top of conventional CV treatment and the other arm received ivabradine. Optimal therapy in the ivabradine group included antithrombotic agent (94%), statins (74%), ACE inhibitor or ARB (90%), and BB (87%). The mean HR at baseline was 72 bpm and the mean ejection fraction

32%. The primary endpoint was a composite of cardiovascular death, admission to hospital for MI, and admission to hospital for a new onset or worsening of HF during a mean follow-up of 19 months (Table IX). The results of the BEAUTI$_f$UL study showed that ivabradine did not affect the primary endpoint with similar results in the two groups (Fox et al., 2008a). However, the baseline HR was rather low in these patients leading to limiting the HR reduction induced with ivabradine by inhibition of the I_f current. In a prespecified subgroup of patients with HR ≥70 bpm, ivabradine did decrease the incidence of secondary endpoints related to CAD, such as admission to hospital for fatal and non-fatal MI (–36%) and reduced coronary revascularization (–30%). The analysis of the placebo arm of the study demonstrated that a basal resting HR of 70 bpm or greater compared with less than 70 bpm was associated with an increase in risk for all outcomes assessed (cardiovascular death, admission to hospital for HF, admission to hospital for fatal and non-fatal MI and coronary revascularization) in this population with CAD and LV dysfunction (Fox et al., 2008b).

C. Following Clinical Program

The clinical program of evaluation of ivabradine in heart diseases does not end with BEAUTI$_f$UL. Two other large trials follow: SHI_fT (Systolic HF treatment with the I_f inhibitor ivabradine Trial) and SIGNI$_f$Y (Study assessInG the morbidity-mortality beNefits of the I_f inhibitor ivabradine in patients with CAD). The goal of the ongoing SHI$_f$T study is to further determine the role of HR reduction in patients with moderate to severe HF and LV systolic dysfunction (EF < 35%), with a primary endpoint of CV death and hospitalization for HF (Swedberg et al., 2010). SIGNI$_f$Y is a logical extension of the BEAUTI$_f$UL study as it will enroll CAD patients with a resting HR ≥ 70 bpm and an ejection fraction > 40%, with no clinical symptoms of HF (Ferrari, 2009). VIVI$_f$Y (eValuation of the IintraVenous I_f inhibitor ivabradine after ST segment elevation mYocardial infarction) will be the first clinical experience of intravenous ivabradine in the context of acute coronary syndrome, evaluating the effects of ivabradine *IV*, compared with placebo, on HR and LV dimensions and function, in the setting of primary catheter-based mechanical therapy.

These ongoing trials should provide important evidence and insights that will enhance the care and management of cardiovascular patients.

VI. Conclusion

HR reduction is a key factor for the management of stable angina pectoris. Ivabradine is the first selective inhibitor of I_f pacemaker current in the SAN that has been approved for clinical application. The largest

clinical development ever dedicated to an anti-anginal drug has established that ivabradine has anti-anginal and anti-ischemic efficacy at least as potent as that of conventional therapies, such as BBs or CCBs, demonstrating the therapeutic relevance of pure HR reduction. The efficacy is maintained with long-term treatment, with no development of tolerance, and no rebound phenomenon after drug cessation. It is safe and well tolerated, the magnitude of its effect on HR is proportional to the baseline HR, greater decreases are observed in patients with the higher HR and more limited effects in patients with low basal HR, and there is low incidence of bradycardia. It has no negative inotropic properties, no impact on corrected QT interval, and atrioventricular conduction. It has no negative impact on respiratory, sexual function, and on lipid or glucose metabolism.

The use of ivabradine in combination with a BB is not only safe but can also induce clear additional activity.

The present data indicate that ivabradine could improve the management of patients with stable angina, not only in those intolerant or having contraindications to BBs but in all patients not controlled by previous anti-anginal drugs, including BBs.

Far beyond the sole control of anginal symptoms, long-term reduction in HR with ivabradine could improve the prognosis of CAD and HF. Experimental data obtained with ivabradine support the hypothesis of beneficial effects on systolic HF, on progression and complications of atherosclerosis lesions. The inhibition of the I_f current per se, in addition to decrease HR, could represent an interesting antiarrhythmic mechanism in pathological conditions, in particular HF, where over-expression of I_f/HCN channels, as part as electrophysiological remodeling, could be arrhythmogenic (Fernandez-Velasco et al., 2003, 2006; Sartiani et al., 2006, 2009; Stillitano et al., 2008; Zicha et al., 2005). The HR-lowering effect of ivabradine, without negative inotropic and lusitropic effects, could be beneficial in HF with preserved ejection fraction by improving impaired LV diastolic filling and decreasing inappropriate high arterial elastance (Reil et al., 2009).

More and more major clinical trials including BEAUTI$_f$UL (Fosbol et al., 2010; Fox et al., 2008b; Ho et al., 2010) indicate the deleterious effect of chronic elevation in HR on the prognosis of cardiovascular diseases and permit to better understand the magnitude of increase and reduction of HR that could impact the prognosis depending on the underlying diseases.

The BEAUTI$_f$UL trial has already confirmed the deleterious role of HR higher than 70 bpm on cardiovascular outcome in patients with CAD and LV dysfunction and suggests that in these patients with higher HR than 70 bpm, ivabradine on top of BBs can improve CADs outcomes.

The ongoing other trials with ivabradine, SHI$_f$T, SIGNI$_f$Y, and VIVI$_f$Y will permit to further precise its therapeutic potential in HF, in CAD, and in acute coronary syndromes.

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Conflict of Interest Statement: The authors are employees of Servier Research company that has discovered and developed the I_f inhibitor Ivabradine.

Abbreviations

ACE	angiotensin converting enzyme
ApoE	apolipoprotein E
ARB	angiotensin receptor blocker
BB	β-adrenoceptor blocker
Bid	twice daily
CAD	coronary artery disease
cAMP	cyclic adenosine 3′, 5′-monophosphate
CCB	calcium channel blocker
Cm	cell capacitance
CNBD	cyclic nucleotide binding domain
CV	cardiovascular
DD	diastolic depolarization
DDR	diastolic depolarization rate
ECG	electrocardiogram
EF	ejection fraction
FKBP12/12.6	12/12.6 kDa FK506-binding protein
G_{max}	maximal conductance
HCN	hyperpolarization-activated cyclic nucleotide-gated
HR	heart rate
I_f	funny current
I_h	hyperpolarization-activated current (similar to I_f)
IVA	ivabradine
KO	knockout
LV dP/dt_{max}	left ventricular maximal pressure rise (LV contractility)
LV	left ventricular
PSWT	post-systolic wall thickening
SAN	sinoatrial node
SBAs	specific bradycardic agents

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Pharmacology of Cardiac Potassium Channels

Abstract

Cardiac K^+ channels are cardiomyocyte membrane proteins that regulate K^+ ion flow across the cell membrane on the electrochemical gradient and determine the resting membrane potential and the cardiac action potential morphology and duration. Several K^+ channels have been well studied in the human heart. They include the transient outward K^+ current I_{to1}, the ultra-rapidly activating delayed rectifier current I_{Kur}, the rapidly and slowly activating delayed rectifier currents I_{Kr} and I_{Ks}, the inward rectifier K^+ current I_{K1}, and ligand-gated K^+ channels, including adenosine-5'-triphosphate (ATP)-sensitive K^+ current (I_{KATP}) and acetylcholine-activated current (I_{KACh}). Regional differences of K^+ channel expression contribute to the variable morphologies

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and durations of cardiac action potentials from sinus node and atrial to ventricular myocytes, and different ventricular layers from endocardium and midmyocardium to epicardium. They also show different responses to endogenous regulators and/or pharmacological agents. K^+ channels are well-known targets for developing novel anti-arrhythmic drugs that can effectively prevent/inhibit cardiac arrhythmias. Especially, atrial-specific K^+ channel currents (I_{Kur} and I_{KACh}) are the targets for developing atrial-selective anti-atrial fibrillation drugs, which has been greatly progressed in recent years. This chapter concentrates on recent advances in intracellular signaling regulation and pharmacology of cardiac K^+ channels under physiological and pathophysiological conditions.

I. Introduction

Cardiac K^+ channels play a pivotal role in maintaining normal cardiac electrical activity. They regulate the resting membrane potential and excitability, participate in the repolarization, and determine the shape and duration of cardiac action potential. Malfunction of K^+ channels, due to either congenital encoded gene mutations or drug blockade, alters not only the cardiomyocyte excitability, but also the electrical balance of depolarization and repolarization, and thus causes a long QT interval or short QT interval of the electrocardiogram (ECG) and underlies different types of cardiac arrhythmias (Kannankeril & Roden, 2007; Zareba & Cygankiewicz, 2008). Therefore, cardiac K^+ channels are important targets of anti-arrhythmic drugs.

It is well recognized that the shape and duration of cardiac action potential are determined by a balance (i.e., sequential activation and inactivation) of inward currents and outward currents (Nerbonne & Kass, 2005). Fig. 1 schematically illustrates the time course of different current contribution to the action potentials of human atrial and ventricular myocytes. The inward currents include voltage-gated Na^+ current (I_{Na}) responsible for the phase 0 depolarization and L-type Ca^{2+} current ($I_{Ca.L}$) responsible for maintaining plateau (phase 2) of the action potential. The inward component of electrogenic Na^+–Ca^{2+} exchanger may also contribute to the phase 2 of the action potential.

The outward currents are mainly carried by different K^+ channels in human cardiac myocytes. They contribute to repolarization of different phases of the action potential. These K^+ currents include the inward rectifier K^+ current I_{K1}, the transient outward K^+ current I_{to1}, the ultra-rapidly activating delayed rectifier K^+ current I_{Kur}, the rapidly and slowly activating delayed rectifier K^+ currents (I_{Kr} and I_{Ks}), acetylcholine-regulated K^+ current (I_{KACh}), and ATP-sensitive K^+ current ($I_{K.ATP}$).

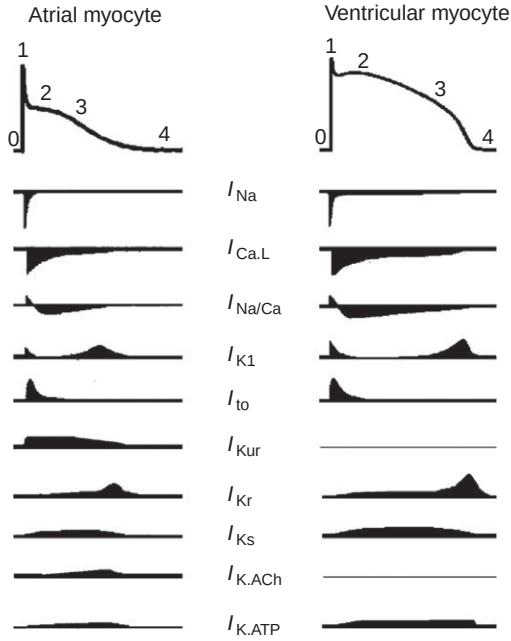

FIGURE 1 Ionic current contribution to human atrial and ventricular action potentials. The graph schematically shows that the major ionic currents contribute to the action potential waveforms at different phases. The action potentials are recorded from a human atrial myocyte and ventricular myocyte. The depolarizing inward (downward) currents and repolarizing outward (upward) currents represent the potential contribution to the action potentials.

II. K^+ Channel Classification

A. Diversity of K^+ Channels

K^+ channels are the most widely distributed type of ion channels composed of α- and β-subunits (Snyders, 1999). The α-subunits of K^+ channels are characterized by (i) an ion-conducting pore through which K^+ ions pass through the plasma membrane; (ii) a K^+ selectivity filter in the P loop from each subunit which have their electronegative carbonyl oxygen atoms aligned toward the center of the filter pore and form an anti-prism similar to a water-solvating shell around each K^+-binding site; and (iii) a gating machinery that controls the switch between open and close states in response to either changes in membrane potential or a ligand (Snyders, 1999). The cytoplasmic N- and C-termini of α-subunits are also important functional domains. The pore-forming subunits homogenously form functional channels (MacKinnon, 1995). K^+ channels have a tetrameric structure

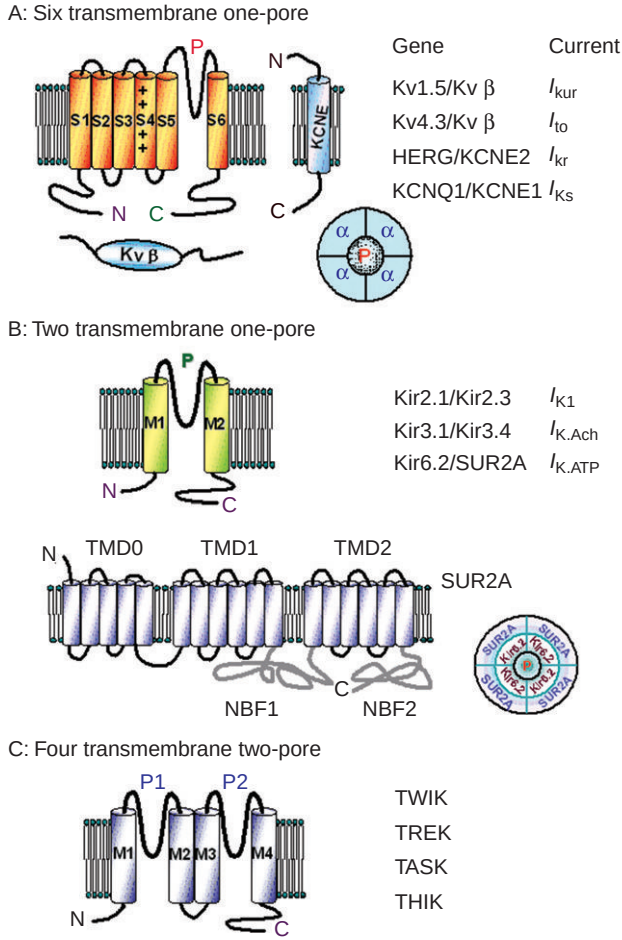

FIGURE 2 Schematic topology of the potassium channel subunits. (A) 6-TM subunits. The Kv channels are composed of four subunits each containing six transmembrane segments (S1–S6) and one conducting pore (P) domain between S5 and S6 with a positively charged voltage sensor S4. The auxiliary β-subunit may be cytoplasmic protein (Kvβ, for I_{Kur} and I_{to1}) or single transmembrane protein (KCNE, for I_{Kr} and I_{Ks}). The inset shows the general assembly of K^+ channels. The channel tetramer may be homogenous (with four identical α-subunits) or heterogeneous (with four different α-subunits). (B) 2-TM subunits. The α-subunits of the inward rectifier K^+ channels (Kir2.x, Kir 3.x, and Kir6.x) contain four subunits each containing two transmembrane segments (M1 and M2) with one pore. Cardiac I_{KATP} channels are composed of four α-subunits (Kir6.2) and four β-subunits (sulfonylurea receptor, SUR2A) containing three transmembrane domains (TMD0–TMD2). (C) 4-TM subunits. K_{2P} channels have four transmembrane segments (M1–M4) with two pores.

in which four identical α-subunits associate to form a symmetric complex arranged around a central ion-conducting pore (MacKinnon, 1995). Alternatively, four related but not identical α-subunits may associate to form heterotetrameric complexes.

The classification of K^+ channels is based upon the primary amino acid sequence of the pore-containing α-subunits. Three groups (Fig. 2) with six, two, or four putative transmembrane segments are recognized, including (i) voltage-gated K^+ (Kv) channels containing six transmembrane regions (S1–S6) with a single pore loop formed by the S5 and S6 segments and S5–S6 linker. The S4 segment is rich in positively charged residues and serves as the voltage sensor (e.g., I_{to1}, I_{Kur}, I_{Kr}, and I_{Ks}); (ii) inward rectifier K^+ (Kir) channels containing two transmembrane domains (M1–M2) with intracellular N- and C-termini, and a single pore loop formed by the M1 and M2 domains (e.g., I_{K1}, I_{KACh}, and I_{KATP}); and (iii) two-pore K^+ (K_{P2}) channels containing four transmembrane domains (M1–M4), intracellular N- and C-termini, and two pore regions related to background or leak channels, such as TWIK (two pore weak inward rectifier K^+ channels), TASK (TWIK-related acid sensitive K^+ channels), TREK (TWIK-related K^+ channels), and THIK (two-pore halothane inhibited K^+ channel) (Gurney & Manoury, 2009; Snyders, 1999).

B. β-Subunits of K^+ Channels

Most K^+ channels heterogeneously assemble with the auxiliary β-subunits in native cells (Fig. 2). The β-subunits are either cytoplasmic proteins (e.g., Kvβ1–4, KChIP) or transmembrane proteins, such as minK and minK-related proteins (MiRPs) encoded by KCNE gene family, and large ATP-binding cassette (ABC) transport-related proteins, such as the sulfonylurea receptors (SURs) for the inward rectifiers Kir6.1–6.2 (Snyders, 1999; Stephan et al., 2006). Most β-subunits assemble with α-subunits and act as a molecular chaperone of the α-subunits in regulating the channel-gating kinetics, pharmacology, folding/coassembly, trafficking, or cell surface expression (Bett & Rasmusson, 2008; Martens et al., 1999; Xu et al., 2009). Both α- and β-subunits are pharmacological targets. Cardiac K^+ channels are also regulated by numerous endogenous molecules and signals. The complex interaction between α-subunits, β-subunits, and endogenous modulators represents diversity of native cardiac potassium currents (Snyders, 1999).

III. Cardiac Voltage-Gated K^+ Channels

A. The Transient Outward K^+ Current I_{to1}

The transient outward current, I_{to}, is composed of two components, i.e., 4-aminopyridine (4-AP)-sensitive transient outward K^+ current (I_{to1}) and

Ca^{2+}-activated transient outward Cl^- current (I_{to2}) in some species (Li et al., 1995). I_{to2} is not present in human cardiac myocytes and therefore is not discussed here. I_{to1} is a voltage-gated and Ca^{2+}-independent K^+ current that is rapidly activated and inactivated in response to depolarization, and contributes to the early repolarization (phase 1) of the action potential in human cardiac myocytes (Fig. 1). Density of I_{to1} is greater in atria than in ventricles and is 3–4-fold greater in ventricular midmyocardial (M) cells and/or subepicardial cells than in subendocardial cells (Li et al., 1995, 1998); therefore, I_{to1} contributes significantly to the phase 1 of the action potentials in these regional myocytes to maintain normal cardiac heterogeneous electrophysiology.

1. Molecular Identification of I_{to1}

Kv4.3 (KCND3) is the dominant candidate coding for cardiac I_{to1} in human hearts (Dixon et al., 1996). Kv4.3 channel mRNA and/or protein is significantly expressed in human atrium and ventricle (Gaborit et al., 2007; Kaab et al., 1998) and in canine hearts (Zicha et al., 2004) and displays a gradient expression in human and canine ventricular wall from endocardium to epicardium (Zicha et al., 2004), comparable to the region-dependent I_{to1} (Li et al., 1998a; Liu et al., 1993; Nabauer et al., 1996). Kv4.3 assembles with cytoplasmic KChIPs to form I_{to1} channels (Zicha et al., 2004). In addition, DPP6 (dipeptidyl aminopeptidase-like protein 6) is another putative subunit of cardiac Kv4.3 (Radicke et al., 2005). Moreover, the potential contribution of Kv1.4 channels to human ventricular I_{to1} cannot be excluded (Po et al., 1992; Snyders, 1999).

The downregulation of cardiac I_{to1} frequently occurs in failing hearts associated with a prolonged action potential duration (APD) (Li et al., 2002, 2004a). The reduction of I_{to1} likely contributes to the diminished phase 1 amplitude of action potential and also partially to the prolonged APD and early afterdepolarizations (EADs) in failing hearts (Li et al., 2002, 2004a). In patient with chronic atrial fibrillation (AF) and canine with experimental AF, atrial I_{to1} and/or Kv4.3 gene and/or protein are also downregulated (Brundel et al., 2001; Grammer et al., 2000; Yue et al., 1999b).

2. Signaling Regulation of I_{to1}

The gating properties and kinetics of cardiac I_{to1} are modulated by various endogenous molecules and signal pathways including serine/threonine and tyrosine phosphorylation. α-Adrenergic stimulation inhibits cardiac I_{to1} in rabbit ventricular myocytes (Fedida et al., 1990) and in human cardiac myocytes and cloned hKv4.3 current expressed in mammalian cells (Po et al., 2001), which may be mediated by protein kinase C (PKC), since the PKC activator phorbol 12-myristate 13-acetate (PMA) exhibits a similar inhibition of rat cardiac I_{to1} and Kv4.2/Kv4.3 currents (Nakamura et al., 1997). In addition, *c*-Src tyrosine kinase increases hKv4.3 current by

phosphorylating the channels through the associated macromolecular complex, which is mediated by the SH2 and SH3 domains of *c*-Src kinases (Gomes et al., 2008).

Activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) slows the inactivation and accelerates the recovery from inactivation of Kv4.3 channels via directly phosphorylating S550 located in C-terminus (Sergeant et al., 2005). SAP97, one of the membrane-associated guanylate kinase proteins, participates in CaMKII-dependent regulation of cardiac Kv4.3/Kv4.2 channels (El-Haou et al., 2009). Nitric oxide is a diffusible messenger that inhibits Kv4.3 channels and human I_{to1} mediated by activating adenylate cyclase and the subsequent activation of PKA and the serine/threonine phosphatase 2A (Gomez et al., 2008). The increase of the bioavailability of atrial nitric oxide could partially restore the duration of the plateau phase of remodeled action potential by inhibiting I_{to1}. Angiotensin II regulates the membrane distribution and gating properties of Kv4.3 channels (Doronin et al., 2004).

3. *Pharmacology of* I_{to1}

4-AP is the first compound used by Kenyon and Gibbon to separate I_{to1} from I_{to2} (or $I_{Ca.Cl}$) in sheep Purkinje fibers (Kenyon & Gibbons, 1979). It is then widely employed as a pharmacological tool to separate I_{to1} from other currents (Li et al., 1995). I_{to1} is a major repolarization current in human atrium. The blockade of I_{to1} is supposed to prolong atrial APD and the refractory period and therefore exerts anti-arrhythmic effect. Several anti-arrhythmic drugs inhibit I_{to1} in human atrial myocytes (see review by Tamargo et al., 2004). The class I anti-arrhythmic drug flecainide blocks inactivated I_{to1} in a frequency-independent manner (Wang et al., 1995), and quinidine shows an open-channel blocker of I_{to1} in a frequency-dependent manner. Propafenone blocks human atrial I_{to1} in a voltage- and use-independent fashion (Seki et al., 1999). Ambasilide inhibits I_{to1} without affecting voltage dependence of activation, inactivation, or recovery from inactivation, but accelerates the inactivation of I_{to1}, suggesting an open-channel block (Feng et al., 1997).

In addition, Ca^{2+} channel antagonists (diltiazem and nifedipine) inhibit I_{to1} in human atrial myocytes (Gao et al., 2005). Raloxifene, a selective estrogen receptor modulator, directly inhibits human atrial I_{to1} (Liu et al., 2007). The antifungal antibiotic clotrimazole inhibits I_{to1} by accelerating the inactivation and slowing the recovery from inactivation of the channels (Tian et al., 2006). The antihistamine drug loratadine rate dependently inhibits I_{to1} at a therapeutic concentration (10 nM) (Crumb, 1999). The omega-3 ($n-3$) polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oil inhibit human atrial I_{to1} in a concentration-dependent manner (Li et al., 2009) (Table 1).

TABLE 1 Pharmacological Blockers and Activators of Cardiac Potassium Channels

Drug	I_{to1}/*Kv4.3*	I_{Kur}/*Kv1.5*	I_{Kr}/*hERG*	I_{Ks}	I_{KACh}	I_{KATP}	I_{K1}	*Species*	*References*
Blocker									
Acacetin	9.3 μM	3.2 μM			4 μM			Human/GP atrial myocytes	Li et al. (2008)
Adenosine							20.7 μM	Rat/GP cardiac myocytes	Wang et al. (2006)
AMP579							4.1 μM	Rat/GP atrial myocytes	Wang et al. (2006)
AVE0118	3.4 μM	6.2 μM	10 μM					CHO	Gogelein et al. (2004)
AVE1231	5.9 μM	3.6 μM			8.4 μM			Pig atrial myocytes/CHO	Wirth et al. (2007)
$BaCl_2$							19.6 μM	hIRK/oocytes	Wible et al. (1995)
Cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate		5.7 μM						CHO	Gong et al. (2008)
Citalopram		2.8 μM						CHO	Lee et al. (2010)
Clomiphene			180 nM					HEK293 cells	Yuill et al. (2004)
Clotrimazole	29.5 μM	7.6 μM	3.6 μM	15.1 μM				Human atrial myocytes/HEK293	Tian et al. (2006)
Diltiazen	29.2 μM	11.2 μM						Human atrial myocytes	Gao et al. (2005)
DPO-1		20 nM						Human atrial myocytes/CHO	Lagrutta et al. (2006)
Docosahexaenoic acid	4.1 μM	4.3 μM						Human atrial myocytes	Li et al. (2009)
Eicosapentaenoic acid	6.2 μM	17.5 μM						Human atrial myocytes	Li et al. (2009)
JTV-519(K201)					<2.4 μM			GP atrial myocytes	Nakaya et al. (2000)
KB130015					0.6 μM			GP atrial myocytes	Brandts et al. (2004)
Ketanserin			110 nM					HEK293	Tang et al. (2008)

Ketoconazole			1.9 μM				HEK293	Takemasa et al. (2008)
LY294002		7.9 μM					CHO cells	Wu et al. (2009)
Nifendipine	26.8 μM	8.2 μM					Human atrial myocytes	Gao et al. (2005)
NIP-151					1.6 nM		HEK293 cells	Hashimoto et al. (2008)
Nordihydroguaiaretic acid		16.4 μM					CHO cells	Gong et al. (2008)
Quinidine			0.6 μM				CHO cells	McPate et al. (2006)
Raloxifene	0.9 μM	0.7 μM					Human atrial myocytes	Liu et al. (2007)
S0100176		0.7 μM					Oocytes	Decher et al. (2004)
SD-3212					<0.4 μM		GP atrial myovytes	Hara and Nakaya (1995)
Spironolactone	27%/1 μM	23%/1 μM	104 nM	38%/1 μM			CHO/*Ltk*$^-$	Gomez et al. (2005)
Canrenoic acid	27%/1 nM	19%/1 nM		223 nM				Caballero et al. (2003)
Tertiapin/Tertiapin Q					10 nM		Oocytes	Jin and Lu (1998)
U50488H	12.4 μM	3.3 μM					Human atrial myocytes	Xiao et al. (2003)
U73122/U73343					0.5 μM		HEK293 cells	Klose et al. (2008)
Verapamil		3.2 μM					Human atrial myocytes	Gao et al. (2004)
Vernakalant	30 μM	13 μM	21 μM				HEK293 cells	Fedida et al. (2005)
Verrucotoxin						16 μg/ml	GP atrial myocytes	Wang et al. (2007)
Ziprasidone			120 nM				HEK293 cells	Su et al. (2006)
Activator								
A-312110						4.3 nM	GP cells	Davis-Taber et al. (2003)
A-935142			60 μM				HEK293 cells	Su et al. (2009)
Adenosine	2.3 mM						Rat cardiac myocytes	Wang et al. (2006)
AMP579	8.3 mM						Rat cardiac myocytes	Wang et al. (2006)

(*Continued*)

TABLE I (*Continued*)

Drug	I_{to1}/*Kv4.3*	I_{Kur}/*Kv1.5*	I_{Kr}/*hERG*	I_{Ks}	I_{KACh}	I_{KATP}	I_{K1}	*Species*	*References*
BMS-191095						83 nM		Reconstituted sarcI_{KATP}	Grover et al. (2001)
Atpenin A5						1 nM		Rat mitoI_{KATP}	Wojtovich and Brookes (2009)
Ephedrine				50 nM				HEK293 cells	Jing et al. (2009)
Ginsenoside Re				3 μM				GP ventricular myocytes	Bai et al. (2004)
ICA-105574			0.5 μM					HEK293 cells	Gerlach et al. (2010)
KB130015			12.2 μM					HEK293 cells	Gessner et al. (2010)
NS1643			10.5 μM					HEK293 cells	Hansen et al. (2006)
NS3623			79.4 μM					Oocytes	Hansen et al. (2006)
P1705						68 nM		Rabbit mitoI_{KATP}	Oldenburg et al. (2003)
PD-118057			3 μM					HEK293 cells	Zhou et al. (2005)
PD-307243			<1 μM					CHO cells	Gordon et al. (2008)
Phenylboronic acid				1.6 mM				Oocytes	Mruk and Kobertz (2009)
Resveratrol				10 μM				GP ventricular myocytes	Zhang et al. (2006)
R-L3				<1 μM				GP ventricular myocytes/Oocytes	Salata et al. (1998)
RPR260243			<3 μM					HEK293 cells	Kang et al. (2005)
Tanshinone IIA				64.5 μM				HEK293 cells	Sun et al. (2008)
TEA					2.5 mM			GP atrial myocytes	Wang and Armstrong (2000)

Data are expressed as IC_{50} (for blockers) or EC_{50} (for activators) values (concentrations producing 50% inhibition or enhancement of the current or half-maximal effects), and some values are estimated according to the reference, which are mainly from the reports after 2003. Please refer to the review by Tamargo et al. (2004) for the information before 2003. CHO, Chinese hamster ovary; GP, guinea pig; TEA, tetraethylammonium.

B. The Ultra-Rapidly Activating Delayed Rectifier K^+ Current I_{Kur}

I_{Kur} is a rapid activation and slow inactivation K^+ current (Snyders et al., 1993; Wang et al., 1993). It is functionally present in the atria but not in the ventricles of human heart (Li et al., 1996b). I_{Kur} is therefore thought to be a promising molecular target for developing selective anti-atrial arrhythmic drugs (Ford & Milnes, 2008; Kozlowski et al., 2009; Tamargo et al., 2009). I_{Kur} is also observed in canine (Fedida et al., 2003; Yue et al., 1996), pig (Li et al., 2004b), and rat (Boyle & Nerbonne, 1992) atrial myocytes.

1. Molecular Identification of I_{Kur}

Kv1.5 (KCNA5) encodes the pore-forming subunit of human atrial I_{Kur} (Fedida et al., 1993; Snyders, 1999), which is supported by the evidence that Kv1.5 antisense oligodeoxynucleotides specifically inhibit I_{Kur} density in human atrial myocytes (Feng et al., 1997), and Kv1.5 gene is significantly expressed in human atrium (Gaborit et al., 2007). In canine atrium, I_{Kur} is initially found to be encoded by Kv3.1 (Yue et al., 1996); however, a later study confirms that Kv1.5 does encode the I_{Kur} in canine atrial myocytes (Fedida et al., 2003). The finding of the later study makes it possible to use canine model in evaluating I_{Kur}/Kv1.5 blockers for the treatment of AF (Li et al., 2008). In addition, Kv1.5 mRNA and protein, responsible for I_{Kur}, are present in mouse and rat hearts (Nerbonne, 2000). Moreover, Kv1.5 transcripts are also detected in extracardiac tissues, e.g., pituitary gland, brain, pancreas, etc. (Takimoto et al., 1993).

Multiple members of a Kvβ1-subunit family for Kv1.5 have been cloned and multiple N-terminal splice isoforms were detected (Snyders, 1999). Kvβ1 encodes the ancillary subunit (Martens et al., 1999) of I_{Kur} channels. Coexpression of accessory Kvβ-subunits (Kvβ-1.2/1.3) increases the inactivation of Kv1.5 channels and the sensitivity to channel phosphorylation by PKA and/or PKC (Kwak et al., 1999).

In chronic AF, I_{Kur} density, Kv1.5 mRNA and protein (like other ion currents, including I_{to1}, $I_{Ca.L}$, and I_{Na}) are downregulated (Brundel et al., 2001; Grammer et al., 2000). Loss of function of mutant Kv1.5 channels may lead to inheritable AF (Olson et al., 2006). Loss-of-function mutations of KCNA5 gene located at the N-terminus (A115V), S1 (A251T), S1–S2 linker (P307S, P310L), and C-terminus (P532L and R578K) have been reported in patients with idiopathic AF (Simard et al., 2005). These findings suggest that defective Kv1.5 channels may predispose the atria to electrical instability and arrhythmogenesis.

2. Signaling Regulation of I_{Kur}

β-Adrenergic stimulation enhances, whereas α-adrenergic stimulation inhibits I_{Kur} in human atrial myocytes mediated by PKA and PKC, respectively (Li et al., 1996a). However, in canine atrial myocytes, both β- and

α-adrenergic stimulation increase I_{Kur} by activating PKA and PKC (Yue et al., 1999a). Whether the different regulation of I_{Kur} by PKC is related to the nonuniform coding (Kv1.5 and Kv3.1) (Fedida et al., 2003; Yue et al., 1996) remains to be studied in canine atrial myocytes. PKA activation reduces the Kvβ-induced fast inactivation of Kv1.5 channels by phosphorylating Kvβ-subunit itself (Kwak et al., 1999). Nitric oxide inhibits hKv1.5 channels by a cyclic guanosine monophosphate (cGMP)-dependent mechanism and by S-nitrosylating the channel protein (Nunez et al., 2006). Pyridine nucleotides modulate Kv1.5 channels by regulating the cellular redox state (NADPH-to-$NADP^+$ ratio) (Tipparaju et al., 2007). Moreover, phosphatidylinositol 4,5-bisphosphate (PIP2) regulates the inactivation of Kv1.5 by an equilibrium binding of the N-terminus of Kvβ1.3 between phosphoinositides and the inner pore region of the channels (Decher et al., 2008).

3. *Pharmacology of I_{Kur}*

Human cardiac I_{Kur} and/or Kv1.5 channels are sensitive to inhibition by 4-AP (Snyders et al., 1993; Wang et al., 1993), but not by tetraethylammonium (TEA) or Ba^{2+} (Wang et al., 1993). Low concentration of 4-AP ($<50\,\mu M$) is used to separate I_{Kur} from I_{to1} in human atrial myocytes (Feng et al., 1997; Li et al., 1996b).

The compounds capable of I_{Kur}/Kv1.5 channel block are shown in Table I. Several anti-arrhythmic drugs (see review, Tamargo et al., 2004), including quinidine, flecainide, and ambasilide, inhibit I_{Kur} in isolated human atrial myocytes (Feng et al., 1997; Wang et al., 1995) and/or Kv1.5 channel expressed in mammalian cells (Snyders & Yeola, 1995). The L-type Ca^{2+} channel antagonists verapamil, diltiazem, and nifedipine reversibly inhibit human atrial I_{Kur} (Gao et al., 2004, 2005). The local anesthetic drugs benzocaine and bupivacaine block Kv1.5 channels in a concentration-dependent manner (Caballero et al., 2002). The omega-3 polyunsaturated fatty acids also inhibit human atrial I_{Kur} (Li et al., 2009) (Table I).

The binding of these drugs is determined by an electrostatic component reflecting the electrical binding distance. The critical residues for hydrophobic binding of quinidine in hKv1.5 channels are located in the putative S6 of pore-lining region and are important in determining drug affinity and specificity (Snyders & Yeola, 1995). Stereoselective bupivacaine block of hKv1.5 channels is determined by a polar interaction with T507 and two hydrophobic interactions at positions L510 and V514 (Franqueza et al., 1997). The binding sites for quinidine and bupivacaine in the residues of the S6 segment also determine the binding of neutral and acid drugs (e.g., benzocaine) (Caballero et al., 2002; Tamargo et al., 2004). Nonetheless, the external mouth of the channel pore formed by the P-loop and adjacent S5–S6 segments is the potential binding sites for some drugs (Lin et al., 2001; Tamargo et al., 2004). Long-chain polyunsaturated fatty acids bind to an external site to block Kv1.5 channels (Honore et al., 1994).

AVE0118 is one of the most studied novel atrial-selective compounds (Table I). It blocks human atrial I_{Kur} and Kv1.5 channels (Gogelein et al., 2004; Wettwer et al., 2004) by binding to T479, T480, V505, I508, V512, and V516 within the S6 domain and pore helix region (Decher et al., 2006), and prolongs the APD in atrial myocytes from patients with AF (Wettwer et al., 2004). AVE0118 also inhibits hKv4.3 channels expressed in Chinese hamster ovary (CHO) cells and I_{KACh} in guinea-pig atrial myocytes (Gogelein et al., 2004), which may contribute to the atrial-selective effects on APD and effective refractory period (ERP) (Christ et al., 2008). Importantly, AVE0118 terminates AF in a goat model with an intravenous administration (Blaauw et al., 2004; de Haan et al., 2006). However, AVE0118 has a low solubility and undergoes rapid first-pass hepatic metabolism with a short half-life in canine and pig (intravenous administration, $T_{1/2} = 0.2–0.4$ h) (Ford & Milnes, 2008; Wirth et al., 2007); therefore, it has not progressed into phase 3 clinical trial for anti-AF (Ford & Milnes, 2008). AVE1231 is a chemically different compound with similar effects to those of AVE0118 (Ehrlich et al., 2008) on I_{Kur}/Kv1.5, I_{to}, and I_{KACh}. It is orally effective and prolongs atrial ERP in pig and goat models with rapid pacing (Wirth et al., 2007). AVE1231 is undergoing phase 1 clinical trial (Ford & Milnes, 2008).

Diphenyl phosphine oxide (DPO) compounds selectively inhibit human atrial I_{Kur} and hKv1.5 current without affecting I_{to1} or prolonging cardiac APD in human ventricular myocytes (Lagrutta et al., 2006). DPO-1 preferentially binds to the open channels of Kv1.5 with putative binding sites T480, L499, L506, I508, L510, and V514 (Du et al., 2010). DPO-1 significantly prolongs atrial ERP in African green monkeys (Regan et al., 2006) and terminates atrial arrhythmia in a canine atrial flutter model without increasing ventricular ERP or PR, QRS or QT interval of ECG (Stump et al., 2005).

ISQ-1 is an isoquinoline Kv1.5 channel blocker, prolongs atrial ERP in African green monkey and dogs, and terminates dog atrial flutter and/or AF (Regan et al., 2007, 2008). Vernakalant (RSD1235) blocks I_{Kur}/Kv1.5 current with a high affinity and binding to T479, T480, I502, V505, and V508 residues (Eldstrom et al., 2007; Fedida et al., 2005). It also inhibits the cardiac I_{to1}, I_{KACh}, I_{Kr}, and I_{Na} at higher concentrations (Fedida, 2007; Fedida et al., 2005). Clinical trial has demonstrated that vernakalant converts AF rapidly without prolonging QT interval of ECG in patients (Fedida, 2007; Roy et al., 2004); it is under consideration by the Food and Drug Administration (FDA) of the United States for clinical use in treating patients with AF.

Acacetin is a natural flavone compound initially isolated from Chinese traditional medicine Xuelianhua (*Saussurea tridactyla*). It suppresses not only atrial I_{Kur}, but also I_{to1} and I_{KACh}. Acacetin prolongs canine atrial ERP without prolonging QTc interval and effectively prevents AF in a canine model after duodenal administration (Li et al., 2008), suggesting that acacetin may be orally effective.

C. The Rapidly Delayed Rectifier K^+ Current I_{Kr}

Sanguinetti and Jurkiewicz demonstrate that the class III anti-arrhythmic drugs notably E-4031 and sotalol selectively block I_{Kr} and that the drug-sensitive (I_{Kr}) and drug-resistant (I_{Ks}) components of I_K differ in terms of voltage dependence, kinetics, rectification properties, and pharmacological sensitivity (Sanguinetti & Jurkiewicz, 1990). I_{Kr} and I_{Ks} play an important role in cardiac repolarization in different species including human (Li et al., 1996b). I_{Kr} channels open rapidly upon depolarization of the action potential, but quickly inactivate. The channel inactivation is released following repolarization with a slow deactivation (Sanguinetti & Jurkiewicz, 1990). Due to this inward rectification property, I_{Kr} contributes a little during the plateau of cardiac action potential and progressively increases at phase 3 repolarization of the action potential (Fig. 1) (Jost et al., 2005). Therefore, I_{Kr} plays a pivotal role in cardiac repolarization, especially in the later phases of the action potential due to its unique kinetics (Fig. 1).

1. Molecular Identification of I_{Kr}

The human *ether-à-go-go*-related gene (hERG or Kv11.1, or KCNH2) coding for the channels carrying the current resembling I_{Kr} has been identified in human heart (Sanguinetti et al., 1995), encodes the α-subunit underlying human cardiac I_{Kr}, and is expressed in both atria and ventricles of human heart (Gaborit et al., 2007). Coassembly of the regulatory β-subunit MiRP1 (minK-related peptide 1 encoded by KCNE2 gene) with hERG α-subunits is required to form native I_{Kr} (Gordon et al., 2008b).

Suppression of I_{Kr}/hERG channels by medications may cause acquired long QT syndrome (LQTS) (Farkas & Nattel, 2010; Perrin et al., 2008). Therefore, potential hERG channel blockade has been a necessary screening procedure at the early stage of developing compounds (Dennis et al., 2007; Farkas & Nattel, 2010). Loss-of-function mutations of hERG and KCNE2 gene induce congenital LQT2 and LQT6, respectively, which are characterized in patients with prolonged QT interval of ECG, abnormal T waves, and a risk of fatal ventricular arrhythmias named Torsades de Pointes (TdPs) (Perrin et al., 2008; Thomas et al., 2006). However, on the other hand, gain-of-function mutations of hERG channels (McPate et al., 2009) are responsible for inherited short QT syndrome (SQT1), which is characterized in patients with abnormally short QT interval, and a high risk of atrial/ventricular fibrillation and sudden death (Brugada et al., 2004; Charpentier et al., 2010).

2. Signaling Regulation of I_{Kr}

Although it is generally accepted that I_{Kr} and/hERG channels are relatively insensitive to signaling modulation, a list of signaling molecules have been reported to regulate I_{Kr}/hERG channels, including cAMP, PKA, PKC,

PIP2, etc. (see review, Charpentier et al., 2010). Activation of β- or α-adrenergic receptor suppresses hERG channels by increasing intracellular cAMP levels. PKA decreases the current amplitude, accelerates the deactivation, and positively shifts the activation conductance via phosphorylating the hERG channel protein. On the other hand, intracellular cAMP may increase the current by directly binding to the cyclic nucleotide-binding domain in C-terminus of hERG channels and negatively shifting the activation conductance (Thomas et al., 2006).

PIP2 upregulates hERG current by accelerating the activation and slowing the inactivation of the channels (Bian et al., 2004). The polycationic region in C-terminus of hERG channels is the potential electrostatic interaction sites of PIP2 (Bian et al., 2004). Stimulation of α-1_A adrenoceptor decreases hERG current and positively shifts the activation conductance, which is related to the consumption of endogenous PIP2 induced by phospholipase C (PLC) activation (Bian et al., 2004). In addition, hERG channels are also constitutively activated by Src family kinases and epidermal growth factor receptor (EGFR) kinase via phosphorylating the tyrosine residues Y475 and/or Y611 (Zhang et al., 2008). Moreover, nitric oxide inhibits hERG current via an interaction with free radical oxygen species in a cGMP-independent manner (Taglialatela et al., 1999).

A recent study demonstrates that chronic hypokalemia rabbits exhibit a prolonged QT interval of ECG, and a downregulated I_{Kr} without significantly affecting other membrane current in ventricular myocytes isolated from the hypokalemia rabbits. Further investigation indicates that a reduced extracellular K^+ decreases the current amplitude, plasma membrane stability, and expression of hERG channel protein by accelerating internalization and degradation of hERG channels (Guo et al., 2009). This study reveals the potential mechanism why hypokalemia patients are susceptible to LQTs and TdPs tachyarrhythmias (Roden et al., 1986).

3. *Pharmacology of I_{Kr}*

I_{Kr}/hERG channels are extraordinarily sensitive to block by a large number of structurally diverse drugs/chemicals (Farkas & Nattel, 2010; Raschi et al., 2009). I_{Kr}/hERG blockers such as quinidine, *d*-sotalol, and dofetilide, have been used for many years as class III anti-arrhythmic drugs, and they are efficacious in preventing and terminating AF and flutter, but their intrinsic arrhythmogenic activity largely restricts their use due to the well-known major effect of inducing LQTs and TdPs, a substrate of life-threatening ventricular arrhythmia. Therefore, the class III anti-arrhythmic drugs with I_{Kr}/hERG channel block are not an ideal medication used for treating AF.

In addition to class III anti-arrhymic drugs, many other commonly used clinical medications also block human I_{Kr} and hERG channels (see review, Tamargo et al., 2004) (Table I), e.g., the antihistamine drugs terfenadine and

loratadine (Crumb, 2000), the anti-estrogenic drugs tamoxifen (Thomas et al., 2003a) and clomiphene (Yuill et al., 2004), the antifungal drug ketoconazole (Takemasa et al., 2008), the antihypertensive drug ketanserin (Tang et al., 2008), and the antipsychotic drug ziprasidone (Su et al., 2006). These drugs preferentially block the open hERG channels, but also exhibit low affinity for closed and inactivated channels (Tristani-Firouzi & Sanguinetti, 2003). The hERG channel inhibition of the non-anti-arrhythmic drugs is the major cause of life-threatening proarrhythmia. This has directly prompted the withdrawal of several clinical drugs from the market (Thomas et al., 2006). Regulatory agencies therefore request that all new drug candidates be tested with I_{Kr}/hERG channels for this possibility.

The binding sites of hERG channel blockers are located within the inner cavity of hERG channels. The individual residues that form high-affinity and lower affinity binding sites have been identified by site-directed mutagenesis approaches, and the gating processes have an important influence on the drug-binding sites (Mitcheson, 2008). The S620 in pore helix of hERG channels is the binding site of dofetilide and verapamil, and an intact C-type inactivation process is also crucial for high-affinity drug binding (Ficker et al., 1998; Zhang et al., 1999). The binding sites of high-affinity hERG blocker MK-499 are located on S6 transmembrane domain (G648, Y652, and F656) and pore helix (T623 and V625) of the hERG channels that face the vestibule of the channels. Compounds structurally unrelated to MK-499, such as terfenadine and cisapride, also interact with Y652 and F656, but not with V625 (Mitcheson et al., 2005). However, the binding site of propafenone (a lower affinity hERG blocker) is located in the F656 residue alone (Witchel et al., 2004). When the drug binds within the inner cavity of the channels, they are trapped in the vestibule by closure of the activation gate upon repolarization. So hERG channel blockers such as the methanesulfonanilides MK-499 show a slow recovery from blockade (Mitcheson et al., 2005).

In addition to the direct channel block by binding to the hERG channels, some drugs suppress I_{Kr}/hERG channels and induce acquired LQTs in patients by interrupting the trafficking of hERG channels and reducing the expression of membrane hERG channel protein. The antiprotozoal drug pentamidine is one of the examples (Kuryshev et al., 2005). To rescue LQTs, a number of compounds have been developed for activating I_{Kr}/hERG channels (Table I).

RPR260243 is the first small molecule that activates I_{Kr}/hERG channels. It dramatically slows the deactivation of hERG tail current in a CHO cell line expressing hERG gene. RPR260243 reverses the action potential-prolonging effect by dofetilide in guinea-pig ventricular myocytes and reduced QT interval of ECG in perfused guinea-pig hearts (Kang et al., 2005).

Several potent hERG channel activators developed by Pfizer (Groton, CT, USA) are reported with a distinct mechanism (Zhou et al., 2005).

PD-118057 enhances hERG step and tail currents in a human embronic kidney (HEK) 293 stable cell line in a concentration-dependent manner and is able to prevent and reverse QT prolongation and arrhythmias induced by a selective I_{Kr} blocker (i.e., dofetilide) in the arterially perfused rabbit ventricular wedge preparation (Zhou et al., 2005).

NS1643 and NS3623 developed by NeuroSearch A/S (Ballerup, Denmark) increase wild type (WT) hERG current by reducing channel inactivation, while reducing the activation in inactivation-deficient hERG mutants (S620T, S631A) (Hansen et al., 2006a). Interestingly, mutation of the high-affinity binding site F656 for hERG channel blocker strengthens the agonist activity of NS1643 and NS3623, suggesting a dual mode of action, being both an activator and an inhibitor of hERG channels (Hansen et al., 2006b).

A-935142 is a recently developed compound by Abbott Laboratories (Abbott Park, IL, USA) with significant hERG enhancement by facilitating activation, reducing inactivation, and slowing deactivation of the channels. It shortens cardiac APD in guinea-pig atrial myocytes and canine Purkinje fibers (Su et al., 2009).

The putative binding sites for RPR260243 are located in S5 (L553 and F557) and an adjacent region of S6 (N658, V659) (Perry et al., 2007), while the binding sites of PD-118057 are located in the pore helix (F619) or the S6 segment (L646). Interestingly, the mutants of nearby residues in the S6 segment (C643, M645) enhance the drug activity of PD-118057 (Perry et al., 2009). In contrast, PD-307243, an analog of PD-118057, increases hERG current by remarkably slowing hERG channel deactivation and inactivation and holding the channels in a constitutively open state via binding to the extracellular region of the pore (Gordon et al., 2008a).

3-Nitro-*N*-(4-phenoxyphenyl) benzamide (ICA-105574) is a unique and potent hERG channel activator more recently developed by Icagen (Durham, NC, USA) (Gerlach et al., 2010). It steeply increases the hERG current amplitudes by removing the inwardly rectifying inactivation of the channels, negatively shifting the voltage dependence of channel activation, and slowing channel deactivation. It shortens cardiac APD in ventricular myocytes from guinea-pig hearts (Gerlach et al., 2010).

D. The Slowly Activating Delayed Rectifier K^+ Current I_{Ks}

I_{Ks} activates slowly with almost no inactivation after activation (Charpentier et al., 2010; Sanguinetti & Jurkiewicz, 1990) and contributes to the phase 2 slow repolarization of cardiac action potential (Fig. 1). I_{Ks} has been demonstrated in cardiac tissues/myocytes from different species including human (Charpentier et al., 2010; Jost et al., 2005; Li et al., 1996b). The physiological contribution of I_{Ks} to the human ventricular action potential is limited; however, during tonic sympathetic stimulation or when cardiac

repolarization reserve is attenuated, I_{Ks} plays an important role in limiting APD prolongation due to its slow deactivation (Jost et al., 2005).

Heterogeneous expression of I_{Ks} is present in different regions of the heart. In the canine ventricle, I_{Ks} density is greater in epicardial and endocardial cells than in the M cells (Li et al., 2002; Liu & Antzelevitch, 1995). The lower I_{Ks} density in the M cells is considered to be related to the steeper APD-rate relations and their greater tendency to display longer APD and to develop EADs at slow heart rates or in response to QT prolonging drugs (Liu & Antzelevitch, 1995).

1. I_{Ks} Molecular Identification

I_{Ks} channels are comprised of four pore-forming α-subunits and two accessory β-subunits, which are encoded by KCNQ1 (Kv7.1 or KvLQT1) and KCNE1 (minK) genes (Barhanin et al., 1996; Sanguinetti et al., 1996). The cytoplasmic C-terminus of KCNQ1 subunit contains a tetramer assembly domain, in which mutations lead to tetramerization deficiency. The KCNE1 subunit dynamically engages in I_{Ks} gating and is critical for the biophysical properties of the native cardiac I_{Ks} (Charpentier et al., 2010; Sanguinetti et al., 1996).

I_{Ks} density is downregulated in failing canine (Li et al., 2002) and human (Li et al., 2004a) hearts. A decreased I_{Ks} and KCNQ1/KCNE1 mRNA levels are found in cardiac myocytes from infarcted canine ventricle (Jiang et al., 2000). Loss-of-function mutations in KCNQ1 gene and KCNE1 gene are associated with congenital LQT1 and LQT5, respectively (Charpentier et al., 2010; Splawski et al., 1997). However, gain-of-function mutations in KCNQ1 gene cause congenital SQT2 and familial AF (Charpentier et al., 2010; Lundby et al., 2007).

2. Signaling Regulation of I_{Ks}

It is well established that catecholamines shorten cardiac APD by enhancing I_{Ks} amplitude mediated by cAMP/PKA and/or PKC via β- and/or α-adrenoceptor stimulation (Sanguinetti et al., 1991; Walsh & Kass, 1991). Activation of PKA by cAMP or phosphodiesterase inhibition increases I_{Ks} current and enhances the rate-dependent shortening of cardiac APD (Lo & Numann, 1998; Terrenoire et al., 2005). I_{Ks} is also modulated by EGFR kinase (Dong et al., 2010). In addition, I_{Ks} or KCNQ1/KCNE1 channels are regulated by CaMKII, nitric oxide, and PIP2. Readers are recommended to refer to the recent review by Charpentier et al. (2010) for the detailed information of I_{Ks} regulation.

3. Pharmacology of I_{Ks}

Chromanols (e.g., 293B, HMR-1556) are the first group of relatively selective I_{Ks} blockers (Busch et al., 1996; Thomas et al., 2003b) (Table I) and show stereospecific block on I_{Ks} (Yang et al., 2000). Indapamide (Turgeon

et al., 1994) and benzodiazepine compounds (L-735821, L-761334, etc.) (Lengyel et al., 2001; Seebohm et al., 2003; Stump et al., 2003) also remarkably block I_{Ks}. Most of them act as open-channel blockers. Bepridil, a Ca^{2+} channel blocker, inhibits recombinant cardiac I_{Ks} by binding to the closed-state channels (Yumoto et al., 2004). The drug interaction site of I_{Ks} blockers (e.g., 293B and L-735821) is located in the pore loop and S6 domain of KCNQ1. T312 in the pore loop and I337, P339, P340, and A344 in the S6 domain are the most important molecular determinants of channel block (Seebohm et al., 2003).

Blockade of I_{Ks} usually fails to remarkably prolong cardiac APD due to the sufficient repolarization reserve carried by other K^+ channels (Lengyel et al., 2001). However, when the repolarization reserve is attenuated or depleted by inherited disorders, cardiac electrical remodeling, or drugs, blockade of I_{Ks} significantly prolongs the ventricular APD (Biliczki et al., 2002). Moreover, β-adrenergic stimulation increases I_{Ks} in epicardial and endocardial cells, but not in M cells. I_{Ks} is intrinsically small, and I_{Ks} block accentuates transmamural dispersion of repolarization (Shimizu & Antzelevitch, 1998). Similar to the I_{Kr} blocker, the I_{Ks} blocker is also proarrhythmic (Cheng & Incardona, 2009). Therefore, I_{Ks} activators, like I_{Kr} activators, may be useful in managing the cardiac arrhythmia related to delayed repolarization.

The Cl^- channel blockers mefenamic acid and 4,4'-Diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS) are the first group compounds (Table I) that enhance the recombinant I_{Ks} by reversibly speeding up channel activation in a KCNE1-subunit-dependent manner (Unsold et al., 2000). Thereafter, the novel benzodiazepine compound R-L3 is found to enhance cardiac I_{Ks} or recombinant I_{Ks}. R-L3 stereospecifically activates I_{Ks} and shortens APD in guinea-pig cardiac myocytes by slowing the rate of I_{Ks} deactivation and negatively shifting the activation conductance of I_{Ks} (Salata et al., 1998). The interaction sites of R-L3 with I_{Ks} channels are located in the S5 and S6 domains of KCNQ1 subunits (Seebohm et al., 2003).

Several natural compounds are recently demonstrated to upregulate native cardiac I_{Ks} and/or recombinant I_{Ks}. Tanshinone IIA is one of major active components from the Chinese traditional medicinal herb Danshen (*Salvia miltiorrhiza*) and directly increases recombinant I_{Ks} by accelerating the activation of the channels and negatively shifting the activation conductance (Sun et al., 2008). Ephedrine, an alkaloid isolated from the Chinese traditional medicinal herb Mahuang (*Ephedra sinica*), activates recombinant I_{Ks}, and negatively shifts the activation conductance. The binding sites of ephedrine on recombinant I_{Ks} are located in the P-loop helix F296 and Y299 of KCNQ1 (Jing et al., 2009). Ginsenoside Re, a major ingredient of *Panax ginseng*, enhances I_{Ks} via S-nitrosylation of the channel protein mediated by nitric oxide in guinea-pig cardiomyocytes (Bai et al., 2004). Moreover, various fatty acids, including docosahexaenoic acid, lauric acid,

and oleic acid, augment I_{Ks} in a KCNE1-subunit-dependent manner (Doolan et al., 2002), and phenylboronic acid is also found to activate recombinant I_{Ks} (Mruk & Kobertz, 2009).

I_{Ks} activators, like the selective I_{Kr}/hERG activators, may also offer a new approach in the treatment of delayed repolarization conditions in patients with acquired LQTs or inherited LQTs. The I_{Ks} activator benzodiazepine R-L3 displays a prominent anti-arrhythmic propensity in rescuing cellular models with acquired long QT type 2 (Nissen et al., 2009).

IV. Inward Rectifier K^+ Currents

A. The Cardiac Inward Rectifier K^+ Current I_{K1}

Cardiac I_{K1} is a strong inward rectification current and is believed to contribute only to the phase 3 rapid repolarization of action potential and maintain resting membrane potential in cardiac myocytes (Anumonwo & Lopatin, 2010). However, our recent studies have demonstrated that two components of I_{K1} are activated during cardiac action potential, one is immediately activated by action potential depolarization, and the other is activated at phase 3 repolarization. The I_{K1} activated by depolarization with comparable time course with I_{Na} may also contribute to the maintenance of cardiac excitability (Fig. 1) (Li et al., 1998b; Zhang et al., 2009). Thus, I_{K1} plays important roles in stabilizing the resting membrane potential and controlling cardiac APD and excitability (Anumonwo & Lopatin, 2010). I_{K1} density is higher in ventricular than in atrial myocytes, but there is no difference in regional cells through ventricular wall in canine and guinea-pig hearts (Schram et al., 2002) and is very low in sinoatrial and atrioventricular pacemaker cells, and therefore these cells have a more depolarized maximum diastolic potential (Schram et al., 2002).

1. Molecular Identification of I_{K1}

Cardiac I_{K1} is encoded by Kir2.1 (KCNJ2), Kir2.3 (KCNJ4), and/or Kir2.2 (KCNJ12) (Dhamoon et al., 2004; Ehrlich, 2008). Heteromeric assemblies of Kir2.1, Kir2.2, and Kir2.3 subunits underlie I_{K1} current. The unique properties of individual Kir2 isoforms, as well as their region- and species-dependent expression patterns, determine the heterogeneous profiles of I_{K1} in atrial and ventricular myocytes of the heart (Dhamoon et al., 2004).

Cardiac I_{K1} is altered under different pathophysiological conditions. I_{K1} is downregulated in ventricular myocytes from failing canine (Li et al., 2002) and human (Li et al., 2004a) hearts, but no Kir2.1 mRNA reduction is observed (Koumi et al., 1995a). In atrial myocytes from patients with chronic AF, I_{K1} is upregulated (Dobrev et al., 2002), which may be related to the

selective S-nitrosylation of Kir2.1 Cys76 residue by nitric oxide (Gomez et al., 2009).

Loss-of-function mutation of KCNJ2 causes inherited Andersen–Tawil syndrome (LQT7), which is characterized by periodic paralysis, specific T–U-wave patterns, and skeletal developmental abnormalities (Anumonwo & Lopatin, 2010; Zhang et al., 2005). On the other hand, gain-of-function mutation of KCNJ2 causes inherited SQT3 and familial AF (Anumonwo & Lopatin, 2010; Priori et al., 2005; Xia et al., 2005).

2. *Signaling Regulation of I_{K1}*

It is well recognized that the conductance of I_{K1} is highly dependent on extracellular K^+ concentration. An increase in extracellular K^+ concentration enhances the conductance of I_{K1}, while the conductance disappears with removal of extracellular K^+ (Li et al., 1998b; Zhang et al., 2009). I_{K1} is uniquely blocked by intracellular polyamines (spermine, spermindine, and putrescine) and Mg^{2+} with steep voltage dependence, which is believed to be the molecular mechanism of the inward rectification of I_{K1} (Anumonwo & Lopatin, 2010; Lopatin et al., 1994). However, the conclusion is made under symmetrical K^+ conditions. Under physiological K^+ conditions, an increase of intracellular Mg^{2+} concentration does not induce any block of I_{K1}, instead of an increase in the transient outward component of I_{K1} (Zhang et al., 2009).

Both α- and β-adrenergic stimulation suppress I_{K1} in a PKA and/or PKC-dependent way (Karle et al., 2002; Koumi et al., 1995b). PIP2 activates I_{K1} via direct electrostatic interaction between the positively charged residues in the cytoplasmic region of the channels and the negative phosphate head group of PIP2 (Xie et al., 2008). PIP2 is also an important cofactor of other regulators that modulate I_{K1} channels (Xie et al., 2007). Kir2 channels are downregulated by membrane cholesterol level (Levitan, 2009). The extracellular H^+ regulates I_{K1} in a species/tissue-dependent manner, possibly reflecting channel subunit composition (Anumonwo & Lopatin, 2010). Tyrosine kinase activation reduces the membrane density of Kir2.1 channels via promoting channel endocytosis (Tong et al., 2001).

In addition, Kir2.1 channels are posttranscriptionally suppressed by miRNA-1 in ischemia/reperfusion myocardium (Yang et al., 2007). The great reduction of miRNA-1 levels may also contribute to the upregulation of Kir2.1 subunits and the increased I_{K1} in patients with AF (Girmatsion et al., 2009).

3. *Pharmacology of I_{K1}*

Ba^{2+} is a well-known I_{K1} blocker with an IC_{50} of 20 μM (Wible et al., 1995) (Table I). Some anti-arrhythmic drugs, such as amiodarone and azimilide, show an inhibitory effect on I_{K1} (see review, Tamargo et al., 2004). Blockade of I_{K1} may cause cardiac diastolic (i.e., resting membrane

potential) depolarization, a proarrhythmic effect that offsets the membrane potential more close to the threshold potential of Na^+ channels therefore reduces cardiac excitability. I_{K1} block also slows conduction velocity due to a voltage-dependent inactivation of Na^+ channels and prolongs the QT interval (Kleber, 1994). Therefore, the drug that specifically blocks I_{K1} may not be realistic for anti-arrhthima. The compound RP58866 is initially designed to specifically block I_{K1} and is effective in treating ventricular arrhythmias induced by ischemia/reperfusion in rat, rabbit, and primate (Rees & Curtis, 1993). Later studies demonstrate that RP58866 is a non-selective K^+ channel blocker. In addition to inhibition of I_{K1}, RP58866 also suppresses I_{to1}, I_{Kr}, I_{Ks}, and I_{KACh} (Brandts et al., 2000; Yang et al., 1999).

B. The Acetylcholine-Activated K^+ Current I_{KACh}

Cardiac I_{KACh} is one of G-protein-coupled inwardly rectifying K^+ channels. It is predominantly present in sinus node, atrial myocardium, and atrioventricular node, but largely sparse in ventricles of the heart (Dobrzynski et al., 2001; Gaborit et al., 2007; Schram et al., 2002). Activation of I_{KACh} by parasympathetic signals such as acetylcholine through M_2 muscarinic receptors causes an inward rectifier K^+ current which hyperpolarizes the membrane potential, shortens cardiac APD, slows the spontaneous firing rate of pacemaker cells in sinus and atrial–ventricular nodes, and delays the atrioventricular conduction (Ehrlich, 2008; Tamargo et al., 2004). The heterogeneity of I_{KACh} expression within and between the left and right atria correlates with potentially proarrhythmic ability of vagal nerve stimulation (Arora et al., 2007). In addition, I_{KACh} plays an important role in the generation of AF (Atienza et al., 2006).

1. Molecular Identification of I_{KACh}

I_{KACh} channel in the heart is a heterotetramer constituted by two Kir3.1 (KCNJ3/GIRK1) and two Kir3.4 (KCNJ5/GIRK4) subunits (Anumonwo & Lopatin, 2010; Corey et al., 1998).

I_{KACh} is remodeled in heart disorders (Borlak & Thum, 2003; Brundel et al., 2001). I_{KACh} current is transcriptionally downregulated in chronic AF, but an agonist-independent (i.e., active in the absence of agonist) constitutively active form of I_{KACh} contributes to human chronic AF (Dobrev et al., 2005; Voigt et al., 2007). Loss-of-function mutation of Kir3.4 gene is detected in, but not associated with clear atrial disorders (Calloe et al., 2007).

2. Signaling Regulation of I_{KACh}

In addition to the acetylcholine-muscarinic receptor-G protein pathway, cardiac I_{KACh} may be activated by other G-protein-coupled receptors including A1-adenosine, α-adrenergic, etc. Adenosine activates rat atrial I_{KACh} (Bosche et al., 2003). Activation of α1-adrenoceptor reduces I_{KACh} current

in atrial myocytes (Anumonwo & Lopatin, 2010). In canine atrial cardiomyocytes, β1-adrenergic stimulation enhances I_{KACh} current via cAMP-induced activation of PKA, whereas $\alpha 1_A$-adrenergic stimulation suppresses I_{KACh} current via PLC-mediated activation of PKC (Yeh et al., 2007). PIP2 upregulates I_{KACh} in diverse manners: via direct interaction with the channels or with Gβ/γ-subunits and via the downstream PKC action (Keselman et al., 2007). Recombinant Kir3.1/Kir3.4 channels are regulated by PKA phosphorylation. Three phosphorylation sites (Ser385, Ser401, and Thr407) located within the C-terminus of Kir3.1 are responsible for PKA phosphorylation and the regulation of I_{KACh} channels (Mullner et al., 2009). Gβ/γ also affects the trafficking of I_{KACh} channels by forming Gβ/γ–Kir3.1/Kir3.4 complexes during channel biosynthesis and trafficking (Robitaille et al., 2009).

3. *Pharmacology of I_{KACh}*

The atrial-specific localization and the functionally upregulation (increased constitutive activity) of I_{KACh} during AF make it possible a promising anti-arrhythmic target devoid of ventricular side effects (Ehrlich, 2008; Kozlowski et al., 2009). Much effort is made to develop selective I_{KACh} blockers (Dobrev & Nattel, 2010) (Table I).

Tertiapin, a 21-residue peptide toxin from honey bee venom, and its derivative tertiapin-Q directly block I_{KACh} with nanomolar affinity by binding to the external end of the ion conduction pore (Jin & Lu, 1998). Tertiapin blocks I_{KACh} current in a receptor- and voltage-independent manner without affecting other cardiac ionic currents (Drici et al., 2000). It has been demonstrated that tertiapin terminates AF without affecting ventricular repolarization in canine AF model (Hashimoto et al., 2006).

The benzopyran compound NIP-151 significantly inhibits I_{KACh} and effectively terminates AF in canine model with an atrial-specific ERP-prolonging profile and lower risk of proarrhythmia compared with I_{Kr} blockers (Hashimoto et al., 2008). AVE0118, in addition to inhibiting I_{to1} and I_{Kur}, blocks I_{KACh} channels and demonstrates atrial-specific anti-arrhythmic effects in animal models (Blaauw et al., 2004; Gogelein et al., 2004). AVE1231, which has improved pharmacokinetic properties compared to AVE0118, shows similar effects on I_{KACh} current (Wirth et al., 2007). The natural flavone accacetin also inhibits I_{KACh} along with I_{Kur} and I_{to1} and prevents AF in a canine model (Li et al., 2008) (Table I).

In addition, the benzothiazepine compound JTV-519 (K201) inhibits I_{KACh} and suppresses experimental AF in isolated guinea-pig hearts (Nakaya et al., 2000). SD-3212 (levo-semotiadil fumarate), a novel benzothiazine Ca^{2+} channel antagonist, also inhibits I_{KACh} channels by depressing the function of the channel itself and/or associated guanosine-5′-triphosphate (GTP)-binding proteins (Hara & Nakaya, 1995) and shows anti-atrial arrhythmic effect (Fujiki et al., 1997).

Moreover, classical anti-arrhythmic agents with a broad channel blockade profile, such as amiodarone and the structurally related derivatives, dronedarone (SR33589) and KB130015, also block cardiac I_{KACh} with high potency by either disrupting G-protein-mediated activation or directly inhibiting interaction with the channel protein (Guillemare et al., 2000; Mubagwa et al., 2003). Blockade of I_{KACh} by flecainide contributes at least in part to the anti-AF effect in humans.

C. Cardiac ATP-Sensitive K^+ Current I_{KATP}

Cardiac I_{KATP} is carried by ATP-sensitive K^+ channels. K_{ATP} channels are closed at physiological intracellular ATP concentrations and activated by a decrease in ratio of intracellular ATP/ADP (Noma, 1983). K_{ATP} channels act as a unique metabolic sensor or a coupling between the cell metabolic status and the cellular membrane potential. I_{KATP} plays a pivotal role in maintaining cardiac homeostasis under stress, such as myocardial ischemia/reperfusion and hypoxia, and mediates the ischemia-induced electrophysiological changes and the cardioprotective effect of preconditioning (Zingman et al., 2007). Activation of I_{KATP} shortens cardiac APD by accelerating the phase 3 repolarization, reduces Ca^{2+} influx thereby preventing cardiac Ca^{2+} overload, preserves ATP levels (energy-sparing effects), and increases cell survival during myocardial ischemia (Tamargo et al., 2004; Tsuboi et al., 2004). On the other hand, activation of I_{KATP} may also be "cardiotoxic" by inducing reentrant ventricular arrhythmias (Tamargo et al., 2004; Zunkler, 2006).

1. Molecular Identification of I_{KATP}

K_{ATP} channels are composed of Kir 6.x-type subunits and sulfonylurea receptor (SUR) subunits (Fig. 2), along with additional components (Stephan et al., 2006). They are further identified by their location within the cell as being either sarcolemmal (sarcK_{ATP}) or mitochondrial (mitoK_{ATP}) (Foster et al., 2008; O'Rourke, 2004). Cardiac sarcK_{ATP} channels are a hetero-octameric complex composed of four inwardly rectifying Kir6.2 channel pore subunits (encoded by KCNJ11), which confer inhibition by ATP, and four modulatory SUR subunits SUR2A (encoded by ABCC9) (Billman, 2008; Tamargo et al., 2004). However, the subunit of mitoI_{KATP} remains to be determined conclusively (Foster et al., 2008; O'Rourke, 2004). A recent study demonstrated that the SUR2-IES is the β-subunit of mitoK_{ATP} channels (Ye et al., 2009). The SUR2A subunit (Fig. 2) has three hydrophobic transmembrane domains (TMD0, TMD1, and TMD2) that likely include five, six, and six transmembrane segments, respectively, with two hydrophilic nucleotide-binding folds (NBF1 and NBF2) following TMD1 and TMD2 (Conti et al., 2001). SUR2A contains ATPase activity harbored within NBF2 and, to a lesser degree, NBF1. NBD1/NBD2

assembly provides a molecular substrate that determines the optimal catalytic activity in SUR2A (Park et al., 2008).

Defective mutations and polymorphisms in Kir6.2 are associated with increased risk of many kinds of metabolic disorders (Reyes et al., 2009). Disruption of sarcI_{KATP} activity impairs cardiac adaptation to stress such as systolic overload (Reyes et al., 2009). Kir6.2$^{-/-}$ mice present with an aberrant regulation of cardiomyocyte membrane excitability and Ca^{2+} handling and are susceptible to ventricular arrhythmias and sudden death following sympathetic stimulation (Zingman et al., 2002).

2. *Signaling Regulation of* I_{KATP}

In addition to regulation by the intracellular ATP and ADP (Noma, 1983), I_{KATP} is regulated by PKC, protein tyrosine kinase (PTK), PIP2, nitric oxide, and many other signaling molecules. PKC activates cardiac I_{KATP} channels at near physiological levels of ATP and induces ischemic preconditioning (Light et al., 1996). Isoform-dependent activation of PKC contributes to the persistent opening of I_{KATP} channels during reoxygenation and reperfusion (Ito et al., 2001). Mitochondrial connexin 43 (Cx43) is recently found to regulate mitoK_{ATP} and protects cardiac cells from death. Genetic Cx43 deficiency, pharmacological Cx43 inhibition by carbenoxolone or by mimetic peptide, substantially reduces diazoxide-mediated stimulation of mitoK_{ATP} channels. Suppression of mitochondrial Cx43 inhibits mitoK_{ATP} channel activation by PKC (Rottlaender et al., 2010). A recent review by Akrouh et al. (2009) is recommended for the detailed information of K_{ATP} channel regulation.

3. *Pharmacology of* I_{KATP}

In addition to inhibition by intracellular ATP (Noma, 1983), K_{ATP} channels can be blocked by antidiabetic drugs including the sulfonylureas, e.g., glibenclamide, glicazide, glipizide, glimepiride, and tolbutamide, and the glinides such as repaglinide, nateglinide, and mitiglinide as well as some anti-arrhythmic drugs (e.g., flecainide) (Tamargo et al., 2004). Sulfonylurea drugs bind directly to the SUR region in the loop between TM15 and TM16 in TMD2 and S1237. These drugs have been widely used to regulate K_{ATP} channel activity (Conti et al., 2001)

Because K_{ATP} channels in pancreatic β-cells and smooth muscle regulate insulin secretion and vascular tone (Seino & Miki, 2003), I_{KATP} blockers may therefore induce hypoglycemia and coronary vasoconstriction; however, cardioselective K_{ATP} channel blockers may be beneficial for treating cardiac disorders. The cardioselective K_{ATP} channel blocker HMR 1098 (clamikalant) (Liu et al., 2001) reduces the cardiac APD shortening induced by hypoxia and prevents ventricular fibrillation induced by coronary artery occlusion in postinfarcted conscious dogs at doses that have no effect on insulin release, blood pressure, or coronary blood flow (Grover & Garlid, 2000).

Thus, specific cardiac I_{KATP} channel blockers may represent a new therapeutic approach to treat ischemia-induced ventricular arrhythmias with little or no side effects (Billman, 2008).

The K_{ATP} channel activators, e.g., pinacidil, cromakalim, rimakalim, and nicorandil, are found to open K_{ATP} channels by binding to two distinct regions of TMD2, the intracellular loop joining TM13 and TM14, and between TM15 and TM16 (residues K1249 and T1253) (Moreau et al., 2000). These compounds have ischemia preconditioning effect and are cardioprotective in experimental myocardial ischemia/reperfusion models and in patients with acute myocardial infarction (Grover & Garlid, 2000). However, they also activate vascular K_{ATP} channels (Kir6.1/SUR2B) and induce hypotension which limits their use in the treatment of myocardial ischemia.

It is believed that sarcK_{ATP} channels are responsible for ischemia preconditioning (Grover & Garlid, 2000). Indeed the mitoK_{ATP} channel activator diazoxide mimics the ischemic preconditioning, and the effect is countered by the selective mitoK_{ATP} blocker 5-hydroxydecanoate (5-HD). The selective mitoKATP opener BMS-191095 exerts cardioprotective effects without shortening APD or inducing hypotension effect (Grover et al., 2001). Therefore, cardioselective and/or mitoK_{ATP} channel blockers and activators (Table I) would be beneficial in protection of cardiac ischemia/reperfusion.

V. Cardiac Two Pore K^+ Current K_{2P}

The two pore domain K^+ (K_{2P}) channels have been discovered for more than a decade, and they support background K^+ currents and maintain membrane potential in many cell types (see reviews, Gurney & Manoury, 2009; Judge & Smith, 2009). The distinct K_{2P} channels including four subfamilies of TASK, TWIK, TREK, and THIK. K_{2P} channels comprise of four transmembrane domains and two pore-forming P loops arranged in tandem (Fig. 2) (Gurney & Manoury, 2009). K_{2P} channels are sensitive to membrane stretch, pH variation, phospholipids, fatty acids, volatile anaesthetics, and G-protein coupled receptors (Bayliss & Barrett, 2008); however, they are insensitive to conventional K^+ channel blockers such as 4-AP, TEA, Ba^{2+}, Cs^+, and glibenclamide (Gurney & Manoury, 2009; Tamargo et al., 2004).

In the heart, the resting membrane potential and cell excitability is predominantly contributed by I_{K1}. Although the mRNAs of several K_{2P} channels (e.g., TREK-1 and TASK-1) are detected, the proposed contribution of these channels to cardiac background currents and cellular physiology is still unclear. No functional current has been recorded in human cardiomyocytes (Gurney & Manoury, 2009). However, because K_{2P}

channels are sensitive to cellular or extracellular signals (pH level, membrane stretch, etc.), they likely act as cellular sensor and transducers (O′Connell et al., 2002). Effort should be made in the future to find out pharmacological tools that select for these channels in order to further understand the physiological importance and whether these channels regulate cardiac functions (Gurney & Manoury, 2009; O′Connell et al., 2002; Tamargo et al., 2004).

VI. Conclusion

The electrical properties of atria and ventricles of human heart are different in the distinct roles of cardiac physiology. Studies on cellular electrophysiology and ion channels have greatly improved our understanding of atrial and ventricular arrhythmias at cellular and molecular levels, including AF and life-threatening ventricular arrhythmias. Cardiac K^+ channels have been recognized as potential therapeutic targets. The understanding of the ion channel distribution in the atria and ventricles and the pathophysiological alteration in different ion channels induced by genetics, diseases, and/or medications has provided a basis for rational design of safer and more effective K^+ channel blockers and/or activators to prevent/treat AF and/or ventricular arrhythmias.

The improved understanding of molecular basis for cardiac I_{Kr} and I_{Ks} makes it possible for the pharmaceuticals to develop selective I_{Kr}/hERG channel activators (e.g., RPR260243, PD-118057, etc.) and/or I_{Ks} activators (e.g., RL-3, tanshinone, etc.), which may offer a new approach in the treatment of delayed repolarization conditions in patients with acquired LQTs or inherited LQTs, and congestive heart failure. In the last decade, effort has been made to develop new anti-arrhythmic agents with safer and more effective than those presently used, especially "atrial-selective drugs" that target cardiac ion channel(s) that are exclusively or predominantly expressed in the atria to avoid the proarrhythmic effect of class III anti-arrhthmic drugs. I_{Kur} is a major repolarizing current in human atria, but not in the ventricles, so that blocking of I_{Kur} is promising target for atrial-specific therapy of AF. I_{KACh} channels are predominantly present in atria, but largely sparse in ventricles of the heart. Compounds that selectively inhibit I_{Kur} (e.g., DPO-1, ISQ-1, etc.) or I_{KACh} (e.g., tertiapin, tertiapin-Q, etc.) or both I_{Kur} and I_{KACh} (e.g., AVE0118, AVE1231, acacetin, etc.) have been demonstrated to terminate or prevent experimental AF. Therefore, these compounds are thought to be promising for atrial-specific therapy of AF. Because I_{to1} channels are more significant for atrial repolarization than that in ventricles, the compounds with I_{to1} block (e.g., vernakalant, AVE0118, acacetin, etc.) would be a plus for anti-AF. These atrial-selective compounds should be further studied to demonstrate

whether they are the desired drugs that are not only effective in clinically relevant AF animal models, but also satisfied in oral bioavailability and safe in animals following repeat dosing, and, crucially, clinical efficacy and safety. The studies on cardiac selective K_{ATP} channel inhibitor (e.g., clamikalant) or mitoK_{ATP} activators (e.g., atpenin A5, P1705) (Table I) are being progressed for the protection of cardiac ischemia/reperfusion. The remained question is whether I_{K1} is a target for developing specific activators to treat certain type of arrhythmias related to delayed reploarization.

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Abbreviations

4-AP	4-aminopyrodine
AF	atrial fibrillation
APD	action potential duration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
EADs	early afterdepolarizations
ECG	electrocardiogram
EGFR	epidermal growth factor receptor
ERP	effective refractory period
hERG	human *ether-à-go-go*–related gene
I_{KACh}	acetylcholine-activated K^+ current
I_{KATP}	ATP-sensitive K^+ current
I_{Kr}	rapidly activating delayed rectifier K^+ current
I_{Ks}	slowly activating delayed rectifier K^+ current
I_{Kur}	ultra-rapidly activating delayed rectifier K^+ current
I_{to1}	transient outward K^+ current
K_2p	two pore K^+ channels
Kir	inward rectifier K^+ channels
LQTs	long QT syndrome
mitoK_{ATP}	mitochondrial ATP-sensitive K^+ channels
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

PTK	protein tyrosine kinase
$sarcK_{ATP}$	sarcolemmal ATP-sensitive K^+ channels
SQTs	short QT syndrome
SUR	sulfonylurea receptor
TdPs	Torsade de Pointes
TMD	Transmembrane domains

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Beta3-Adrenergic Receptors in Cardiac and Vascular Tissues: Emerging Concepts and Therapeutic Perspectives

Abstract

Catecholamines released by the orthosympathetic system play a major role in the short- and long-term regulation of cardiovascular function. Beta1- and beta2-adrenoreceptors (ARs) have classically been considered as mediating most of their effects on cardiac contraction. After their initial cloning and pharmacologic characterization in the late 1980s, beta3-ARs have been mostly thought of as receptors mediating metabolic effects

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(e.g., lipolysis) in adipocytes. However, definitive evidence for their expression and functional coupling in cardiovascular tissues (including in humans) has recently initiated a re-examination of their implication in the pathophysiology of cardiovascular diseases. Distinctive pharmacodynamic properties of beta3-AR, e.g., their upregulation in disease and resistance to desensitization, suggest that they may be attractive targets for therapeutic intervention. They may substitute efficient vasodilating pathways when beta1/2-ARs are inoperative. In the heart, their contractile effects, which are functionally antipathetic to those of beta1/2-AR, may protect the myocardium against adverse effects of excessive catecholamine stimulation and perhaps mediate additional ancillary effects on key aspects of electrophysiology or remodeling. Longitudinal studies in animals and patients with different stages of heart failure are now needed to identify the optimal therapeutic scheme using specific combinations of agonists or antagonists at all three beta-ARs.

I. Introduction

Among physiological regulators of the cardiovascular system, neurohormones under the control of the sympathetic nervous system play a central role, which extends in numerous cardiovascular diseases. Among these, catecholamines acting on beta-adrenoreceptors (beta-ARs) are well-established therapeutic targets, with clinically proven benefits of beta1/2-AR blockers in hypertension, ischemic, and failing cardiac diseases. However, after the classical distinction between beta1- and beta2-AR subtypes (Lands et al., 1967), was put into question in the 1980s the beta-AR family was formally enriched with a third beta-AR cloned in 1989 (Emorine et al., 1989), prompting a re-examination of the adrenergic pharmacology. In particular, the beta3-AR was formally identified in several cardiovascular cell types, and its particular pharmacologic profile supports pharmacodynamic actions that somewhat deviate from the usual beta1- and beta2-AR responses. This warrants the following review on the characteristics of the beta3-AR and its distinctive coupling in cardiovascular cells, which form the basis of a re-examination of the role of catecholamines in cardiovascular pathophysiology, as well as of the modulation of their action with subtype-specific agonists or antagonists.

II. Molecular Structure of Beta3-AR

After initial cloning of the human beta3-AR by Strosberg (Emorine et al., 1989), orthologs have been characterized in many other mammalian species (Strosberg, 1997). Contrary to other subtypes, the beta3-AR gene comprises introns with possible alternative splicing to generate different

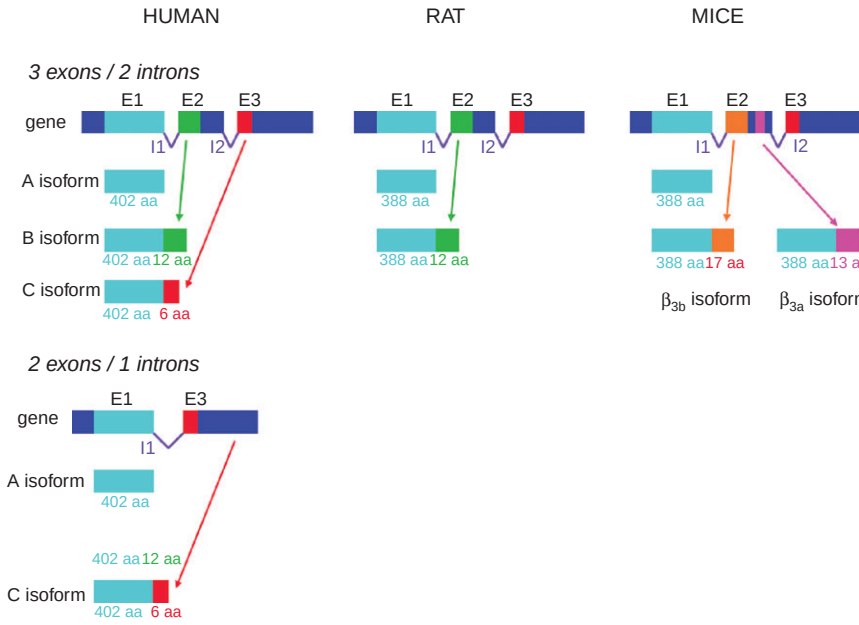

FIGURE 1 Putative structures of beta3-AR isoforms obtained after alternative splicing in human, rat, and mice. aa, amino acid; E, exon; I, intron (from Rozec & Gauthier, 2006).

isoforms. This also explains variations in length and C-terminal sequences within and between species (see Fig. 1).

The human beta3-AR gene is located on chromosome 8, with some initial controversy on the number of exons and introns. The commonly accepted structure comprises one intron and two exons, the first of which (1.7 kb in length) encodes the initial 402 amino acids of the receptor, while the second encodes the last six amino acids in the C-terminus (and 3′-UTR of the transcript) of a longer splice variant; these have been identified as the shorter "isoform A" (402 aa, from exon 1, with high homology in rodents) and longer "isoform C" (408 aa, from exon 1+2) (Granneman et al., 1992, 1993; van Spronsen et al., 1993). A similar structure with two exons and one intron was described in dogs and monkeys (Lenzen et al., 1998; Walston et al., 1997).

A structure with three exons and two introns was described in rats and mice, where exon 1 (1.4 kb) encodes the initial 388 amino acids, with some divergence for the C-terminus among these two species; a second exon encodes 12 additional C-terminal amino acids in rats, enabling two isoforms of 388 or 400 amino acids; however, in mice two different splice sites in exon 2 generate two different isoforms, with the addition of 17 amino acids for beta3-ARb, but only 13 amino acids for beta3-ARa (see Fig. 1). As expected, these isoforms also differ in their relative expression among tissues, with beta3b-AR mRNA levels relative to beta3a-AR mRNA highest in

hypothalamus, cortex, and white adipose tissue and lower in ileum smooth muscle and brown adipose tissue (Evans et al., 1999).

In all species, the resulting protein has the classical structure of seven-transmembrane (TM) domains (with three intra- and three extracellular loops), G-protein-coupled receptor, including a highly glycosylated, extracellular N-terminus, and intracellular C-terminus. Most of the homology between beta-(including beta3-) AR amino acid sequences in man and rat is concentrated in the seven-TM domains and membrane–proximal regions of the intracellular loops. Likewise, the TM domains show the highest homology (94%) between the rat and human beta3-AR (which are 79% identical), whereas the C-terminus and third intracellular loop are the least homologous.

This C-terminal divergence between beta-AR subtypes has a bearing on their pharmacologic regulation, most importantly on their sensitivity to homologous desensitization. The latter is known to involve agonist-induced phosphorylation by G-protein Receptor Kinase (GRK) of serine and threonine residues in the C-terminus, as well as consensus sequences for phosphorylation by protein kinase A (PKA). The absence of PKA phosphorylation sites as well as of several serine and threonine residues in the C-terminus of beta3-AR explains its relative resistance to agonist-induced desensitization. This was confirmed by experiments on chimeric beta2/3-AR, emphasizing the importance of sequences of the C-terminus and second and third intracellular loops for desensitization (Jockers et al., 1996; Liggett et al., 1993). Functional persistence of a response to prolonged agonist stimulation with a beta3-AR agonist was also confirmed in human myometrium (Rouget et al., 2004). Likewise, differences in the C-terminal sequences between mouse beta3a- and beta3b-AR with two additional serine residues in the beta3b-AR (Evans et al., 1999) may mediate a higher sensitivity to desensitization in the latter, although this has never been formally tested in cardiovascular tissues. This C-terminal divergence would also support differential coupling [to both Gs and Gi for beta3b-AR, but exclusively Gs for beta3a-AR, at least in Chinese hamster ovary (CHO) cells (Hutchinson et al., 2002)], raft/caveolae localization (Sato et al., 2007a) and intracellular signaling between the two isoforms, which may affect their relative roles in (patho)physiology.

III. Pharmacologic Properties of Beta3-AR

Several pharmacologic criteria help to identify a characteristic beta3-AR response, as reviewed by Gauthier et al. (Rozec & Gauthier, 2006; Rozec et al., 2006), e.g., high affinity and potency of selective agonists such as BRL37344, 5-(2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl)-1,3-benzodioxole-2,2-dicarboxylate (CL316243), and (R,S)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2

hydroethanamide hydrochloride (SR58611A); partial agonistic activity of several beta1- and/or beta2-AR antagonists, such as CGP12177A, bucindolol, and pindolol; atypically low affinity for conventional beta-AR antagonists such as propranolol and nadolol. The degree of stereoselectivity for agonists and antagonists also seems to vary between species, with higher affinities and enantiomeric ratios for the human compared with the mouse or rat beta3-AR. Notably, stereoselectivity was low for agonists such as isoproterenol and norepinephrine, but higher for antagonists such as propranolol (Popp et al., 2004).

Various agonists and antagonists for beta3-AR have been developed and described in previous reviews (Rozec & Gauthier, 2006). In general, it is important to emphasize that their affinity, selectivity, and potency widely vary between species, probably as a result of differences in beta3-AR amino acid sequences as well as levels of expression. Compared with beta1/2-AR, a general feature of beta3-AR, however, is their activation at higher concentrations of catecholamines [*Ki* around 1 μM; Strosberg (1997)]. This has supported the proposition that they may be preferentially activated in situations of high adrenergic tone, such as chronic heart failure, with sustained efficiency due to their lower propensity to desensitize (see above).

Besides endogenous catecholamines, beta3-ARs are activated with high affinity and potency by two main classes of preferential agonists, namely phenylethanolamines [comprising BRL37344 (Arch et al., 1984), SR58611A (Bianchetti & Manara, 1990), and CL316243 (Dolan et al., 1994)] and aryloxypropanolamines [including cyanopindolol and CGP12177A (Lenzen et al., 1998; Pietri-Rouxel & Strosberg, 1995; Strosberg, 1997)]. The latter is also a beta1/2-AR antagonist, as well as a weak beta3-AR agonist. Also, compounds of the first class (i.e., BRL37344 and SR58611A) share a low affinity for beta1- and beta2-AR (Dolan et al., 1994), putting their selectivity in doubt when used at high concentrations (1 μM and beyond).

Similarly, non-selective beta-AR antagonists, such as bupranolol (Galitzky et al., 1993; Langin et al., 1991), block beta3-AR with various potencies. More selective beta3-AR antagonists, such as SR59230A, exhibit quite variable potencies according to species, with efficient blockade of the rodent, but not human beta3-AR (Arch, 2002). However, it is important to note that in cells systems expressing high levels of the mouse beta3a-AR, SR59230A, exhibited partial agonist properties, whereas it antagonized the response to another beta3-AR agonist (CL316243) in cells expressing low levels of the receptor. The two agonists also exhibited reciprocal order of potencies for the activation of 3′,5′-cyclic adenosine monophosphate (cAMP) or p38-mitogen-activated protein kinase (MAPK) at the same beta3a-AR (Sato et al., 2007b). This "versatility" has been interpreted as an example of ligand-directed signaling (Ursino et al., 2009), i.e., a phenomenon where ligands favor different conformational changes in their cognate receptor driving pleiotropic downstream signaling; such ligand-directed signaling has been described at beta-ARs (Evans et al., 2010).

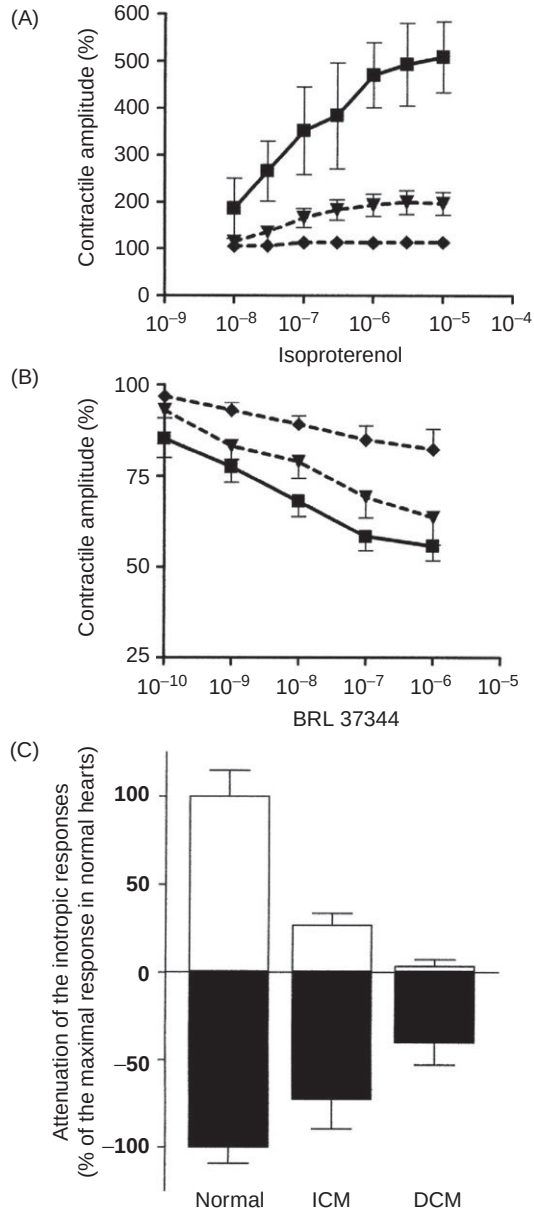

FIGURE 2 *(Continued)*

Conversely, two compounds (*S*)-*N*-[4-[2-[[3-[3-(ami-nosulphonyl) phenoxy]-2-hydroxypropyl]-amino]ethyl]phenyl]benzenesulfonamide (L-748, 328) and (*S*)-*N*-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl] amino]ethyl]phenyl]benzenesulfonamide (L-748,337) were described with good affinity and specificity for the human cloned beta3-AR, but weak activity toward the rodent receptor (Candelore et al., 1999; Rozec et al., 2005).

IV. Beta3-AR in the Cardiac Muscle

A. Differential Expression

As pointed out above, apparent discrepancies in the effect of preferential agonists are in part explained by differences in beta3-AR expression levels, which may vary between anatomical locations in the heart and between species. Expression and function have systematically been compared in a previous study by Gauthier et al. (1999) in several mammalian species. To the extent that transcripts levels accurately reflect functional receptor proteins, rodents seem to express much lower levels of cardiac beta3-AR than humans; accurate measurements of beta3-AR protein expression or density have always (and still are) hampered by the paucity of reliable reagents across species, be it specific antibodies or radiolabeled ligands. It should be reminded, also, that in most studies, human samples usually originate from diseased hearts, whereas rodent expression was mostly assessed in normal hearts. The disease state matters, because levels of beta3-AR proteins were shown to increase in human ischemic and dilated cardiomyopathic ventricles (Moniotte et al., 2001) compared to nonfailing hearts, and this was accompanied with a more prevailing beta3-AR functional influence over beta1/2-AR (see Fig. 2). A similar upregulation was observed in failing canine myocardium (Cheng et al., 2001), in human and murine sepsis (Moniotte et al., 2007) as well as in streptozotocin-induced type I diabetes in rats (Dincer et al., 2001). The latter study estimated that the ratio of

FIGURE 2 Contractile tension of human ventricular biopsy specimens in response to inotropic amines *ex vivo*. (A) Concentration-dependent positive inotropic effect of nonspecific beta-AR agonist, isoprenaline, in nondenervated nonfailing (■, $n = 4$ patients) and failing ischemic (▼, $n = 6$ patients) and dilated (◆, $n = 7$ patients) cardiomyopathic heart tissues. (B) Concentration-dependent negative inotropic effect of beta3-preferential agonist, BRL 37344, in nondenervated nonfailing (■, $n = 6$ patients) and failing ischemic (▼, $n = 9$ patients) and dilated (◆, $n = 9$ patients) cardiomyopathic heart tissues. (C) Attenuation of inotropic responses to 1 μmol/L isoprenaline (upper bars) and 1 μmol/L BRL 37344 (lower bars) in failing ischemic (ICM) and dilated (DCM) cardiomyopathic heart tissues compared with responses in nonfailing hearts (Normal). Results are normalized to response obtained with 1 μmol/L of either agonist in nonfailing hearts, expressed as 100%. Attenuation of isoprenaline responses was significantly more pronounced than that of BRL 37344, supporting a more prevailing influence of the beta3-AR pathway in pathologic (vs normal) myocardium. From Moniotte et al. (2001).

beta1- vs 2- vs -3-AR proteins in cardiac muscle varied in proportion from 62:30:8 in control rats to 40:36:23 in diabetics, and reversed to 57:33:10 after a 2-week insulin supplementation. Notably, all these diseases are associated with increased tissular and circulating catecholamines which could mediate beta3-AR upregulation *in vivo*, as previously demonstrated *in vitro* (Germack & Dickenson, 2006). Few studies have systematically examined beta3-AR expression in different anatomical locations of the heart, e.g., in atria vs ventricles. It may well be that gross variation in expression levels (or downstream signaling) explains the reported divergent functional responses between the two chambers (see below). At least, one of our study did not find differences across the different layers of the human ventricular muscle (Moniotte et al., 2001).

B. Intracellular Signaling

Contrary to the classical coupling of beta1-AR to Galpha-s/adenylyl cyclase/PKA pathway or the alternative coupling of beta2-AR to Galpha-s or alpha-i, the beta3-AR seems to preferentially couple to Galpha-i at least in the human ventricle in native conditions (Gauthier et al., 1996). This was corroborated in a mouse model with cardiac-specific (alphaMHC-driven) expression of a human transgenic beta3-AR, at least *ex vivo* (Tavernier et al., 2003), although a previous study on a similar (but not identical) transgenic model suggested coupling to a Galpha-s/PKA-mediated response (Kohout et al., 2001). This apparent discrepancy has been attributed to background strain differences, possible differences in transgene expression level (with promiscuous coupling) as well as in pharmacologic agents and protocols (*in vivo* vs *ex vivo*) used. Other studies in dog (Cheng et al., 2001), rodent (Zhang et al., 2005a, 2005b), and even eel (*Anguilla anguilla*; Imbrogno et al., 2006) myocardium have generally confirmed pertussis toxin sensitivity, indicative of Galpha-i coupling. Nevertheless, when heterologously expressed in CHO-K1 cells, the mouse beta3a- and beta3b-ARs seem to couple differentially to either exclusively G-s (for beta3a) or G-i and G-s (for beta3b), which may be explained by distinctive sequences in the C-terminal tail between the two isoforms (Hutchinson et al., 2002). Whether this applies to similar isoforms in the native mouse heart remains uncertain.

Although beta3-AR can interchangeably couple to G-s/adenylyl cyclase or G-i/MAPK in adipocytes (Soeder et al., 1999), in human (Gauthier et al., 1998), and apparently in mouse (Varghese et al., 2000), ventricles, it activates nitric oxide (NO) synthesis through still incompletely characterized mechanisms.

This was first demonstrated functionally by direct measurements of NO production and cGMP increases in response to beta3-AR agonists in human ventricular biopsies, where eNOS (but no iNOS) immunostaining was observed. Subsequent studies suggested a similar implication of eNOS in murine

ventricle, where BRL37344 activated Ser1177 phosphorylation of the enzyme in wild-type mice but not in beta3-AR knockout (KO) mice (Brixius et al., 2006). Reciprocally, BRL37344 reduced contractility in isolated adult cardiomyocytes from wild-type mice but not from $NOS3^{-/-}$ mice, further suggesting the implication of eNOS downstream beta3-AR confined in the same cell (Barouch et al., 2002). This would be consistent with our observation of beta3-AR protein enrichment together with caveolin in low-density membranes, where eNOS is known to colocalize (Belge C, Balligand JL., unpublished).

Other nitric oxide synthase (NOS) isoforms, however, may be implicated as well, at least in the diseased heart. The neuronal isoform, nNOS, was suggested to mediate beta3-AR effects in aged (Birenbaum et al., 2008) and diabetic rats (Amour et al., 2007), in which beta3-AR abundance increased, as mentioned above.

The implication of iNOS is less clear, at least in cardiomyocytes, although one study reported an upregulation of iNOS (but not eNOS or nNOS) transcripts after 5 h of Nebivolol [a beta1-AR blocker with beta3-AR agonist properties (Dessy et al., 2005)] in cardiac tissue, correlating with increased NO production (Maffei et al., 2007).

C. Physiological Effects

Consistent with earlier paradigms on the effect of cardiomyocyte NOS on cardiac (ventricular) contraction (Balligand et al., 1993, 2009; Massion & Balligand, 2003), activation of NO production downstream beta3-AR activation was shown to attenuate contraction force in ventricular preparations from several species (Cheng et al., 2001; Gauthier et al., 1999), albeit with different rank order of potencies for the different agonists used. Aside from differential affinities, as discussed above, this has been correlated with inter-species variations in beta3-AR transcripts levels, with higher expression and responses in humans and dogs than in mouse and rat ventricular tissues. Similar NO-mediated responses to beta3-AR agonists were observed in ventricular tissue from mice with cardiac-specific expression of a human beta3-AR transgene in one *ex vivo* study (Tavernier et al., 2003), which is at variance with observations in another mouse strain (Kohout et al., 2001), as discussed above. In human ventricular tissue, beta3-AR activation was associated with an increase in cGMP content, an effect attenuated by NOS inhibition (L-NAME or L-NMMA) and restored with excess L- arginine (but not D-arginine), the NOS substrate (Gauthier et al., 1998). Importantly, it was reproduced with a number of beta3-AR agonists (BRL37344, SR58611A, and CL316243) and with endogenous catecholamines, such as norepinephrine at concentrations close to pathophysiologically relevant levels, and in the presence of blockade of the alpha1- and beta1-, beta2-AR with prazosin and nadolol, respectively (Gauthier et al., 1998). More recently, the same

group observed similar responses with the third-generation beta-blocker, nebivolol, which exhibits beta3-AR agonistic properties (Rozec et al., 2009).

It is important to note that this direct effect on contractility does not translate into negative inotropism upon infusion of beta3-AR agonists *in vivo* in dogs, due to peripheral vasodilatation (see vascular effects, discussed in the next section) and reflex orthosympathic activation, which translates into tachycardia (Berlan et al., 1994; Shen et al., 1994; Tavernier et al., 1992) and positive inotropic responses (Donckier et al., 2001; Shen et al., 1996). Similar reflex tachycardic responses blocked by beta1/2-AR antagonists have been observed in humans (Wheeldon et al., 1993, 1994a).

Notably, in contrast with ventricular tissue, beta3-AR agonists fail to decrease contractility (Cohen et al., 1999; Kaumann & Molenaar, 1996) and even evoke positive inotropic effects in isolated atrial myocytes from several species, including human (Skeberdis et al., 2008). This is probably related to differential coupling to downstream signaling in atrial compared with ventricular cardiomyocytes, as is known, e.g., for NOS signaling between the two cell types (Kirstein et al., 1995).

This is particularly true for the effects of beta3-AR activation on elements of excitation–contraction (EC)coupling. NO regulates L-type calcium channel activity either through cGMP/PKG modulation of phosphorylation state of the channel alpha1 subunit, cGMP-dependent regulation of cAMP/PKA activity through phosphodiesterases (Massion & Balligand, 2003; van der Heyden et al., 2006), or direct, cGMP-independent S-nitrosylation of the channel (Burger et al., 2009). Consistent with decreased contractility, beta3-AR agonists inhibited L-type calcium channel currents in ventricular myocytes from failing rats, an effect partly reversible upon NOS inhibition with L-NAME (Zhang et al., 2005b). The NOS isoform involved may be eNOS, as suggested from the abrogation of beta3-AR effects on calcium transients and shortening in $eNOS^{-/-}$ cardiomyocytes (Barouch et al., 2002), but could be nNOS as well, given previous evidence for the regulation of calcium transient and EC coupling by this isoform (Zhang et al., 2008). Notably, some or all of these NO-mediated effects on EC coupling (and more broadly, on cardiomyocyte biology) may be lost or even reversed upon NOS "uncoupling," a state when the NOS enzyme shifts from NO to superoxide production, with increased oxidant stress. Notably, such "uncoupling" was shown to be promoted in response to hemodynamic stress in mice with beta3-AR genetic deletion (Moens et al., 2009) suggesting a protective role of the beta3-AR against oxidative stress.

V. Vascular Beta3-ARs

Stimulation of the vascular beta-AR evokes a vasodilation. In the last three decades, the use of selective agonists and antagonists had amended earlier believes of a relaxation mediated only through the stimulation of the

beta2-AR subtype. Anyway, the contribution of beta1-AR remains marginal and varies depending on the vascular beds, the species studied and even the precontracting stimulus. As in cardiac muscle, the implication of a third beta-AR has arisen from the use, in *in vivo* and *in vitro* studies, of dual beta1- and beta2-AR antagonists and selective beta3-AR agonists. These studies have provided undisputable evidence for functional expression of beta3-AR in some vessels in addition to beta1- and beta2-AR subtypes.

A. *In Vivo* Effects of Beta3-AR Stimulation

In anesthetized mice, beta3-AR stimulation with CL316243 results in a robust and sustained reduction in blood pressure. Interestingly, in mice deficient in both beta1- and beta2-AR, infusion of CL316243 induced an exaggerated hypotensive response, which might suggest a compensatory upregulation of the beta3-AR or of the signaling machinery distal to beta-AR (Rohrer et al., 1999).

CL316243 infusion evoked hypotension and peripheral vasodilation in anesthetized rats (Shen et al., 1994, 1996), whereas BRL37244 induced a more profound effect. After beta1- and beta2-AR blockade, there were almost no differences between BRL37344 and CL 316243, suggesting that BRL37344 is a less specific but more potent agonist than CL316243 in the rat vasculature (Shen et al., 1994, 1996).

In aorta of spontaneously hypertensive rats (SHR), in spite of an upregulation of beta3-AR, beta3-AR-mediated relaxation was not modified (Mallem et al., 2004). Notably, nerve-activated beta1- and beta3-AR-mediated vasodilation was not present in SHR, which might suggest that beta3-AR could participate in modulating alpha1-AR-mediated vasoconstriction (Berg et al., 2010). CL316243 induced a marked increase in islet blood flow without changes in whole pancreatic blood flow. This increase is totally prevented by beta-nonspecific blockade with bupranolol, but not with nadolol, a beta1/beta2-AR antagonist (Atef et al., 1996). BRL26830A did not induce any significant modification in blood pressure but markedly increased blood flow in brown adipose tissue (Takahashi et al., 1992). Other selective beta3-AR agonists (BRL35135, CL316243, and SR58611A) increase antral gastric mucosal blood flow in rats anesthetized with halothane (Kuratani et al., 1994).

As mentioned in previous sections, in conscious dogs, BRL37344 exerts hypotensive effects, which persist in sinoaortic-denervated animals (i.e., animals deprived of baroreceptor pathways) (Tavernier et al., 1992). Berlan et al. had extended this observation to CL316243 and reported that both beta3-specific agonists evoke an increase in cutaneous blood flow (Berlan et al., 1994). Similar hypotensive response and a peripheral vasodilation have been observed both in control conscious dog infused with SR58611A and in a dog model of perinephretic hypertension (Donckier et al., 2001).

In conscious dogs instrumented with aortic and left atrial catheters and ascending aortic flow probes, BRL37344 reduced mean arterial pressure and increased total peripheral conductance which were resistant to blockade by propranolol (Shen et al., 1994). Beta3-AR stimulation is reported to be more profound in dogs than in other species (Shen et al., 1996). However, as expected from its pharmacologic properties (see previous sections), *in vivo* activation of beta3-AR requires higher doses of catecholamine than those necessary for beta1- and/or beta2-AR stimulation in anesthetized animals (Pelat et al., 2003).

In comparison to the hypotensive effects of CL316243 and BRL37344 observed in dogs, the same drugs induced only marginal reduction in blood pressure in primate (Shen et al., 1996). Very few studies have specifically investigated the vascular effects of beta3-AR stimulation in human. A weak role of beta3-AR has been demonstrated in the control of lipolysis and nutritive blood flow in subcutaneous abdominal adipose tissue from human submitted to a sustained infusion of CGP12177 (selective beta3-AR agonist having beta1- and beta2 antagonist properties) (Barbe et al., 1996). A study aimed to investigate the cardiac effects of the beta3-AR agonist BRL35135, a prodrug for BRL37344, proposed that most effects were attributable to beta2-AR stimulation (Wheeldon et al., 1994b). There was no modification in systolic or diastolic pressure in patients treated with L-796,568, a selective beta3 agonist, during 28 days (Larsen et al., 2002).

B. Vascular Effects of Beta3 Stimulation in *In Vitro* Studies in Animal Models

Pindolol, a nonspecific beta-AR antagonist with partial beta3-AR agonistic activity, induces a vasodilation of rat thoracic aorta that is resistant to propranolol (Clark & Bertholet, 1983). This study was the first to suggest the presence of an atypical beta-AR. Similarly, non specific stimulation of rat isolated common carotid (MacDonald et al., 1999; Oriowo, 1994) and thoracic aorta (Trochu et al., 1999) with isoprenaline induced a relaxation that was resistant to beta1- and beta2-AR blockade. CGP12177 and BRL37344 concentration-dependently relaxed ring segments of the rat aorta and carotid artery precontracted with noradrenaline (Oriowo, 1995). Rat thoracic aorta relaxation to SR58611A was antagonized by SR59230A, a beta3-AR blocker, but was left unaltered by pretreatment with beta1/beta2 blockade with nadolol (Trochu et al., 1999). In phenylephrine-constricted rings, but not PGF2alpha-constricted rings of rat aorta, relaxations to isoprenaline showed a propranolol-resistant component (Brahmadevara et al., 2003). In ET1-precontracted rings, the beta3-AR agonist, SR58611A, and nebivolol (a beta1 blocker with beta3 agonistic properties) induced a concentration-dependent relaxation, which was

unaffected by nadolol (a beta1/beta2-AR antagonist) but was significantly reduced by L-748,337 (a beta3-AR antagonist) (Rozec et al., 2006). These studies are disputed by two reports putting into doubt the existence of functional beta3-AR in rat conductive vessels. Indeed, although nonconventional partial agonists and selective beta3-AR agonists cause relaxation of the rat aorta, it was reported that the observed vascular atypical beta-AR does not appear to rely on the stimulation of beta3-AR but could correspond to a putative beta4-AR (Brawley et al., 2000) or alpha1-AR antagonist properties (Brahmadevara et al., 2004).

According to Kozlowska and colleagues (Kozlowska et al., 2003), beta2-AR and atypical beta-AR relax the rat main mesenteric artery, which seems to lack the beta3 subtype. Similarly, in rat mesenteric microarteries, Briones and colleagues provided some evidence against the implication of beta2- and beta3-AR and in favor of a relaxation evoked by beta-AR stimulation that is mainly due to the activation of beta1-AR subtypes (Briones et al., 2005). Nevertheless, mouse mesenteric microarteries preconstricted with PGF2alpha showed a dose-dependent relaxation to nebivolol, which is virtually absent in mesenteric microarteries from mice genetically deficient of beta3-AR (Dessy et al., 2005).

In rat isolated perfused lung preparations, isoprenaline and the three beta3-AR agonists SR59104A, SR59119A, and SR58611A caused concentration-dependent relaxations of hypoxia-induced pulmonary vasoconstriction (Dumas et al., 1998). Propranolol and SR59230A, a beta3 antagonist, inhibited the relaxant effects of isoprenaline. SR59230A but not propranolol inhibited those of SR59104A. Surprisingly, propranolol and SR59230A failed to oppose SR59119A- and SR58611A-induced relaxant effects. These results suggested the existence of an atypical beta-AR in the rat pulmonary vessels (Dumas et al., 1998), but could be reinterpreted in the light of "ligand-mediated signaling" with SR59230A, as discussed in Section III. Conversely, Pourageaud and colleagues had suggested that the beta2-AR subtype mediates the relaxation to beta-AR stimulation of rat intralobar pulmonary artery precontracted with PGF2alpha. In agreement, experiments carried out in pulmonary arteries from normoxic and hypoxic mice demonstrated that isoproterenol and procaterol (selective beta2-AR agonist) elicited relaxation, while cyanopindolol and CL316243 (beta3-AR agonists) were ineffective (Leblais et al., 2008). Together, these results argued against a major implication of beta3-AR in pulmonary vessels, at least in rodents. However, in dogs, CL316243 and BRL37344 induced a dose-dependent relaxation of isolated canine pulmonary arterial rings under isometric conditions *in vitro*, after preconstruction with norepinephrine (Tagaya et al., 1999).

In aortic and carotid arteries and in portal veins, the vasodilating effect of isoproterenol was reduced in mice lacking either beta1- or beta2-AR. However, in these vessels, the vasodilating effect was only abolished in

double KO mice lacking both beta1- and beta2-AR (Chruscinski et al., 1999). Accordingly, isoprenaline but not BRL37344 significantly reduced noradrenaline-induced contractions of the rat portal vein (Yousif & Oriowo, 2002). These results are in contradiction with *in vivo* experiments showing an enhanced hypotensive effect of beta3-specific agonist in the same mice (Rohrer et al., 1999), but can be explained by differential distribution of the beta3-AR between vascular beds.

C. Effects of Beta3 Stimulation in Human Isolated Vessels

Early work from Shafiei et al. had suggested that human internal mammary arteries relax to the stimulation of an atypical beta-AR together with beta1- and beta2-AR isotypes, as they observed that BRL37344 produced a concentration-dependent relaxation in phenylephrine-precontracted rings (Shafiei et al., 2000). Molecular and biochemical approaches have since confirmed this observation in the same vessel (Rozec et al., 2005). Beta3-AR transcripts and protein have been detected in extracts from human coronary microarteries (see Fig. 3) (Dessy et al., 2004). In the same vascular bed, BRL37344 evokes a sustained dilation of microvessels preconstricted with endothelin-1 (Dessy et al., 2004). Interestingly, the endogenous catecholamine, norepinephrine, also evoked a relaxation that was resistant to beta1/beta2 blockade but fully abrogated by bupranolol providing physiological relevance to these receptors (Dessy et al., 2004). Functional expression of beta3-AR has also been demonstrated in human placental artery obtained from women with uncomplicated or pre-eclamptic pregnancies, where SR59119A evokes a relaxation (Rouget et al., 2006). Conversely, human umbilical arteries showed only a weak vasodilatory response to BRL37344 (Dennedy et al., 2002).

The vasorelaxation evoked by BRL37344 in corpus cavernosum has also suggested a role for beta3-AR in erection (Cirino et al., 2003).

D. Cellular Location and Signaling in Vascular Tissue

Many studies concur to propose an endothelial localization of the beta3-AR subtype to mediate the relaxation through the activation of an NO-dependent pathway. Indeed, endothelium removal or NOS inhibition strongly inhibited the relaxation of human coronary microarteries to BRL47344 and nebivolol (Dessy et al., 2004, 2005). Similar results were obtained in rat thoracic aorta, indicating that beta3-ARs were mainly located on endothelial cells (Trochu et al., 1999) where they mediate a relaxation through the release of NO leading to subsequent increases in cyclic GMP levels. In rat carotid arteries, L-NAME attenuates vasodilating responses to both isoprenaline and the beta3-AR agonist BRL37344, suggesting a role

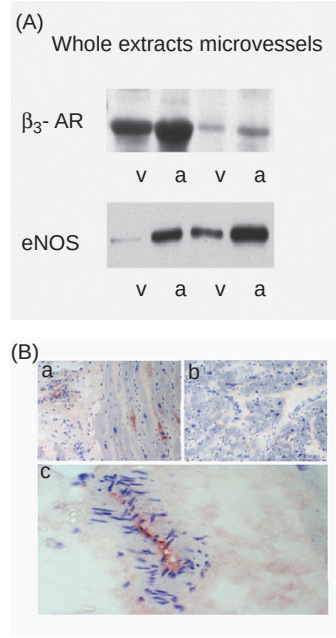

FIGURE 3 Endothelium-restricted expression of beta3-AR in coronary microarteries. (A) Immunoblots for beta3-AR (top lane) and eNOS (bottom lane) from protein homogenates of human coronary microarteries isolated from left ventricle (v) or right atria (a) and from protein homogenates prepared from whole left ventricular (v) and right atrial (a) pieces. (B) Immunostaining for beta3-AR in human right atrial appendages; a, lower magnification; b, negative control obtained in the absence of specific antibodies; c, longitudinal section of microartery at higher magnification. Modified from Dessy et al. (2004).

for endothelial release of NO (MacDonald et al., 1999). However, in these vessels, the relaxant effect of BRL37344 was attenuated by L-NAME to a lesser extent than that of isoprenaline, which suggest that there may be a differential contribution of endothelium to classical beta1/2- and beta3-AR-mediated effects, with endothelium contributing less to beta3-AR-mediated relaxation, at least through NO release (MacDonald et al., 1999). Endothelial cell localization was confirmed by molecular and immunohistochemical approaches in rat aorta (Rautureau et al., 2002). More importantly, beta3-AR transcripts have been detected in endothelial cells by laser capture from human coronary microarteries (Moniotte et al., 2001). The presence of beta3-AR in the endothelial layer of the human coronary microarteries was further demonstrated by immunohistological localization (see Fig. 3) (Dessy et al., 2004). Rozec and colleagues had also provided molecular and biochemical evidence of an endothelial localization of the beta3-AR in human mammary arteries (Rozec et al., 2005). However, these results are

still debated, as BRL37344 was shown to evoke a relaxation of endothelium-denuded human internal mammary arteries suggesting the existence of an endothelium-independent pathway in this vascular bed (Shafiei et al., 2000).

Also, reverse transcription-polymerase chain reaction showed that beta3-ARs were expressed in rat portal vein myocytes together with beta2- and beta3-AR subtypes (Viard et al., 2000).

In human coronary microarteries, we have observed the persistence of an endothelium-dependent relaxation in the presence of NOS and COX inhibitors. BRL37344-mediated hyperpolarization of smooth muscle cell membrane together with the inhibitory effect of the combination of apamin and charybdotoxin have confirmed the hypothesis that the beta3-AR-evoked relaxation is partly mediated through the release of endothelium-derived hyperpolarizing factor (EDHF) (Dessy et al., 2004) (Fig. 4). In agreement, the inhibitory effect of SR59104A, another selective beta3 agonist, on the hypoxic increase in perfusion pressure in rat is partially blocked by L-NAME or apamin and charybdotoxin (Dumas et al., 1999). Iberiotoxin, glibenclamide, tolbutamide, or 4-aminopyridine, partially but significantly inhibited the SR58611A-mediated vasorelaxation of rat aorta, suggesting that other K^+ channels (i.e., B_{KCa}, K_{ATP}, and K_v, among others), are involved in the signaling cascade of beta3-AR in conductive vessels (Rautureau et al., 2002). In canine pulmonary artery, relaxation to BRL37344 and CL316243 resisted to endothelial denudation. Moreover, intracellular cAMP levels were increased by CL316243 in a concentration-dependent fashion, an effect that was not altered by ICI118551. Altogether, this suggests that beta3-AR may exist in canine pulmonary artery smooth muscle, the stimulation of which causes vasodilation through a cAMP-dependent pathway (Tagaya et al., 1999).

E. Beta3-Mediated Response to Nebivolol, an Atypical Beta1 Blocker with Additive Beta3 Agonistic Properties

Nebivolol combines a potent beta1-AR antagonistic activity with vasodilating properties, thereby providing cardioprotection, as recently demonstrated in the SENIORS trial in heart failure, together with a modulation of peripheral vasoconstriction. We provided evidence that nebivolol dilates human coronary resistance microarteries through an agonistic effect on endothelial beta3-AR, relaxes mesenteric resistance arteries, and promotes neoangiogenesis (see Fig. 5). Both effects are mediated through the release of NO as they were reduced by NOS inhibition or NOS genetic deficiency, respectively (Dessy et al., 2004). Similar demonstration of beta3-AR-dependent vasodilatory properties of nebivolol was obtained in animal models. Indeed, relaxation of rat aorta rings to nebivolol was unaffected by the selective 5-HT(1A) antagonist, NAN-190, the 5-HT(1/2) antagonist methysergide, beta2 blockade with

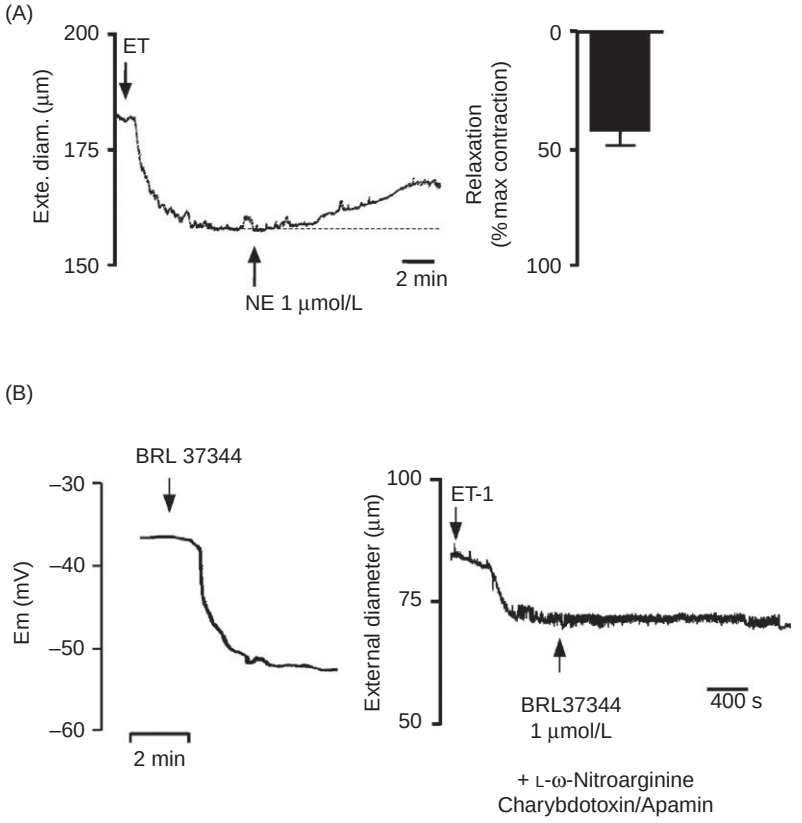

FIGURE 4 (A) *Left panel*, Norepinephrine evokes a beta3-mediated relaxation of coronary microarteries. Representative tracing showing the relaxation to norepinephrine (NE) (1 µmol/L) of a human coronary microarteriole constricted with ET-1 in the presence of an alpha1/alpha2 blocker (phentolamine) and a beta1/2 blocker (nadolol). *Right panel*, quantification of the norepinephrine relaxation. (B) Beta3 agonist stimulation hyperpolarizes coronary microvessels; involvement of Ca^{2+}-activated K^+ channels. *Left panel*, typical recording showing the BRL37344 evoked hyperpolarization of smooth muscle cell membrane from isolated human coronary arteries. *Right panel*, representative tracing of the contraction of a human coronary microarteriole with ET-1 after incubation with the NOS inhibitor L-ω-nitroarginine and K^+ channel inhibitors charybdotoxin and apamin (100 µmol/L each). Under these conditions, no residual relaxation is observed in response to the beta3 agonist BRL37344. Modified from Dessy et al. (2004).

butoxamine but antagonized by the beta3 blocker *S*-(-)-cyanopindolol (de Groot et al., 2003). Similarly, in ET1-precontracted rings, nebivolol induced a concentration-dependent relaxation, which was unaffected by nadolol (a beta1/2-AR antagonist) but was significantly reduced by L-748,337 (a beta3-AR antagonist) (Rozec et al., 2006). Results in cellular models have substantiated the demonstration and allowed the dissection of the

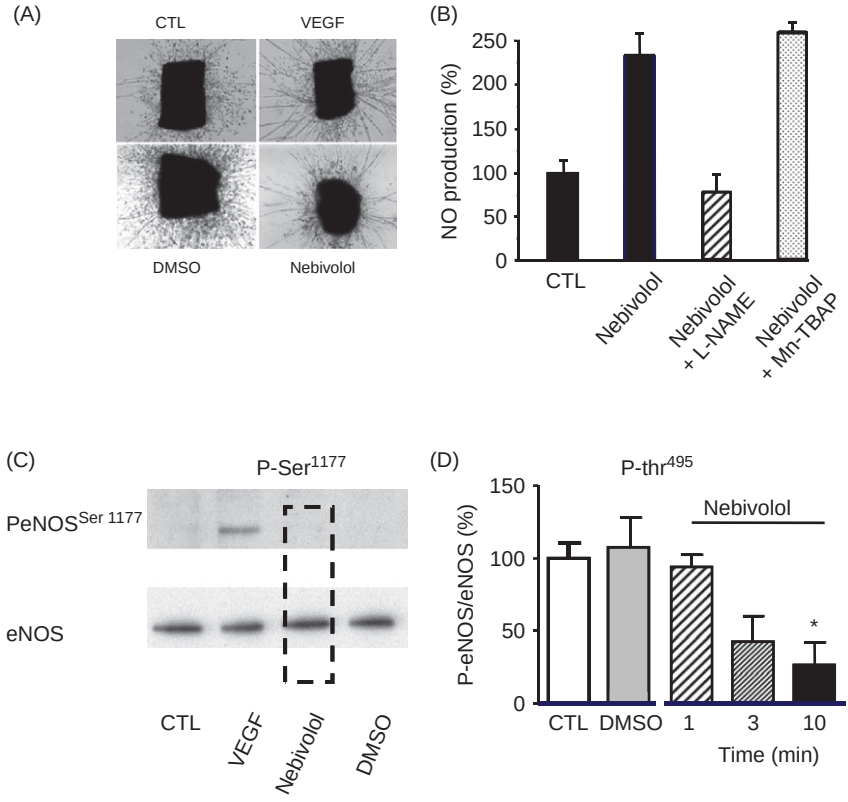

FIGURE 5 (A) Nebivolol-evoked angiogenesis is dependent on the presence of eNOS and beta3-AR. Photomicrographs showing the angiogenic response of explanted aortic rings from C57BL/6J mice by day 12 cultured in autologous serum in the absence of drug or in the presence of VEGF (40 ng/mL), solvent (0.03% DMSO), or 3 μmol/L nebivolol. (B) Mean amplitudes of EPR signals obtained from $[Fe(II)NO(DETC)_2]$, mean amplitude of the third component of the EPR signal in extracts of BAECs, either untreated (open column) or after incubation with 1 μmol/L nebivolol in the absence (filled column, $n = 3$) or the presence (hatched column, $n = 3$) of 1 mmol/L L-NAME or 50 μmol/L MnTBAP (dotted column). (C) Phosphorylation (P) of eNOS on Ser^{1177} by VEGF or nebivolol in BAECs stimulated with 40 ng/mL VEGF or 1 μmol/L nebivolol for 10 min. (D) eNOS-Thr^{495} dephosphorylation by nebivolol in BAECs stimulated with 1 μmol/L nebivolol for 1, 3, or 10 min. Modified from Dessy et al. (2005).

NOS pathway (see Fig. 5). Nebivolol-evoked NO release has been quantified both by Electron Paramagnetic Resonance (EPR) and by specific amperometric electrode. NO release measured in endothelial cells results from the increased NOS activity that relies both on cystosolic calcium and on modification of the NOS phosphorylation pattern (Dessy et al., 2005). In rat aorta, nebivolol-induced relaxation has been shown to result from both inhibition of alpha1-AR and activation of beta3-AR (de Groot et al., 2003;

Rozec et al., 2006). Surprisingly, as noted in previous sections, the beta3-AR vasodilation of nebivolol in the mouse heart vasculature has been attributed to a chronic upregulation of iNOS (after 5 h) (Maffei et al., 2007), which may act in addition to the constitutive NOS implicated in acute responses. These effects are of therapeutic importance for the treatment of pathological conditions associated with an elevation of sympathetic tone, such as heart failure and hypertension.

VI. Clinical Perspectives

Initially, beta3-ARs have been considered mainly as receptors mediating metabolic effects (e.g., lipolysis) in adipocytes, although their low expression and lipolytic activity in (at least normal) human white fat tissue have dampened enthusiasm for their exploitation as therapeutic targets of obesity (Tavernier et al., 1996). However, after the discovery of their expression and functional role in human cardiovascular tissues, as described in previous sections, they raised renewed interest in the cardiovascular field. Indeed, beta-ARs play a major role in the short- and long-term regulation of cardiac function, including in disease states. Although acute stimulation of beta1-AR increases all aspects of cardiomyocyte contractility and mediates positive inotropic effects on heart function, their prolonged activation results in adverse remodeling of the myocardium, in part due to toxic effects on cardiomyocytes (and other cells composing cardiac muscle). This was exemplified by the cardiac phenotype of mice with cardiac-specific overexpression of beta1-AR (Engelhardt et al., 1999). Similarly, chronic overactivation of the orthosympathetic system in heart failure, albeit initially adaptive, is generally considered to be deleterious on the long term (Lohse et al., 2003). To a large extent, this view is supported by clinical evidence of the beneficial effect of beta-adrenergic blockade in failing patients.

How do cardiovascular beta3-ARs fit in this picture? As detailed above, their activation with preferential agonists (in the presence of combined beta1/2-AR blockade) produced effects functionally antipathetic to those classically ascribed to the other two isotypes, i.e., a decrease in developed cardiac muscle tension. Again, contrary to beta1- (and perhaps beta2-) AR, their expression increases in the failing myocardium (possibly under chronic exposure to raised catecholamines levels), and their functional coupling is better preserved, due to lower desensitization. Overall, this makes beta3-AR very attractive candidate targets for an efficient pharmacologic modulation in the diseased heart.

A major unanswered question, however, is whether they should be blocked with antagonists or activated with specific agonists to preserve or improve cardiac function on the long term. In the absence of prospective, controlled trials with “bona fide” beta3-AR agonists or antagonists, we can

only resort to indirect evidence from animal studies and a limited number of human trials using specific beta blockers with ancillary beta3-AR agonist activity. In dogs with pacing-induced heart failure, acute administration of the beta3-AR antagonist L-748,337 resulted in increase in the slope of the left ventricular (LV) pressure–volume relation and decrease in LV relaxation constant, indicative of improved LV function (Morimoto et al., 2004). This would agree with the negative inotropic effect mediated by myocardial beta3-AR stimulation *ex vivo*. However, whether such benefit would be maintained on the long term is questionable, as positive inotropic interventions on a failing heart usually result in adverse outcomes.

Reciprocally, antagonizing the adverse long-term effects of beta1-AR activation has received ample validation with the use of beta1-AR blockers in clinical trials, and beta3-AR agonists may functionally reproduce such effect. They have never been used in chronic heart failure models, though, raising an important caveat. The only experimental evidence in support of such a putative benefit comes from mouse models with either systemic deletion or cardiac-specific overexpression of beta3-AR. Beta3-AR KO mice submitted to transaortic constriction faired worse than WT littermate, with excessive oxidant stress and exacerbated LV remodeling (Moens et al., 2009). However, this was a systemic KO, and some of the adverse phenotype may originate from indirect effects of blood pressure or compensatory overexpression of beta1-AR (not evaluated in the study). Mice with cardiac-specific (alphaMHC-driven) overexpression of beta3-AR exhibit a phenotype that is at the opposite of beta3-AR KO, i.e., under catecholaminergic stress, they are protected from hypertrophic remodeling (Belge et al., unpublished).

Therefore, even though stimulation of the cardiac beta3-AR pathway may decrease cardiac contraction (as reflected by short-term positive inotropic effect of its blockade), long-term stimulation may result in myocardial protection by antagonizing adverse effects of beta1-AR overstimulation. The beta3-AR pathway may be viewed as an "endogenous beta1-AR blocker," operating as a "safety brake" in circumstances of adrenergic overdrive. The effects of beta3-AR agonists on vasodilation (including resistance coronary arteries) and NO release in the myocardium would promote additional benefits, according to previously described effects of cardiac NO on ortho-/parasympathetic balance (Massion et al., 2004), LV relaxation, coronary perfusion, and possibly, even angiogenesis (Dessy et al., 2005), also reviewed in (Balligand et al., 2009) (see also Fig. 5). Similar benefits may operate in hypertension through persistent beta3-AR-mediated vasorelaxation despite beta1/2-AR receptors desensitization. Finally, it has been suggested that NO downstream beta3-AR promotes the activity of the cardiac Na^+/K^+-ATPase, which may counteract Na^+ overload in the failing cardiac muscle and perhaps contribute to anti-arrhythmic as well as antiremodeling effects, thereby preserving LV function (Rasmussen et al., 2009).

In humans, some indirect evidence pro- or against beta3-AR activation can be found in clinical trials with beta1-AR blockers combining beta3-AR agonism. One of these, bucindolol (a nonspecific beta1/2-AR blocker also combining alpha1-AR antagonism), did not confer survival benefit in a non-selected patient population with advanced heart failure (NYHA III-IV) (Beta-Blocker Evaluation of Survival Trial Investigators, 2001) but did so in a selected patient cohort bearing the beta1-AR Arg-389 polymorphism associated with enhanced agonist-promoted activity, and on which bucindolol selectively exerts inverse agonism (Liggett et al., 2006). At least, this would suggest that combined beta3-AR agonism of the drug, if real, does not abrogate the benefit of efficient beta1-AR blockade. Whether the previously described Trp to Arg mutation at position 64 in the beta3-AR (Walston et al., 1995) similarly affects its coupling in cardiac or vascular tissue is still uncertain. Another such beta-blocker is nebivolol, combining beta1-AR-specific antagonism with beta3-AR agonist activity (see Fig. 6). In an early small trial on a limited number of patients with ischemic cardiomyopathy, nebivolol (but not the specific beta1-AR blocker, atenolol) improved diastolic reserve (Stoleru et al., 1993); such distinctive property may explain some of the clinical benefit in elderly patients, as included in the SENIORS trial (Flather et al., 2005), because of the high prevalence of heart failure with normal LV ejection fraction (and altered diastolic reserve) in this population.

VII. Conclusion

Definitive molecular and functional evidence for the expression of beta3-AR in human cardiac and vascular tissue now broadens our perspectives for therapeutic modulation of beta-adrenergic signaling in cardiovascular diseases. Although a complete understanding of beta3-AR function has been (and perhaps still is) hampered by the difficulty in finding specific pharmacologic and molecular (e.g., antibodies) reagents, inter-species variability in expression and affinity, and low expression levels compared with beta1/2-AR isotypes, convergent evidence points toward a functional antagonism of the beta1/2-AR effects on cardiac contraction and a vasodilatation (concordant with beta1/2-AR effects) in most vascular beds. Although these effects may appear subtle in most healthy animal (e.g., rodent) models, one should bear in mind that the expression of beta3-AR increases with cardiac disease, thereby reinforcing their influence relative to the other isotypes. Although beneficial effects can reasonably be expected from such upregulated beta3-AR response in the vasculature, the consequences on cardiac function are less predictable. They probably vary according to the stage of the cardiac disease, with possible protection against catecholamine-induced tissue remodeling at early stages (without significant degradation of LV contractility), but, possibly, a prohibitive deterioration of inotropy in

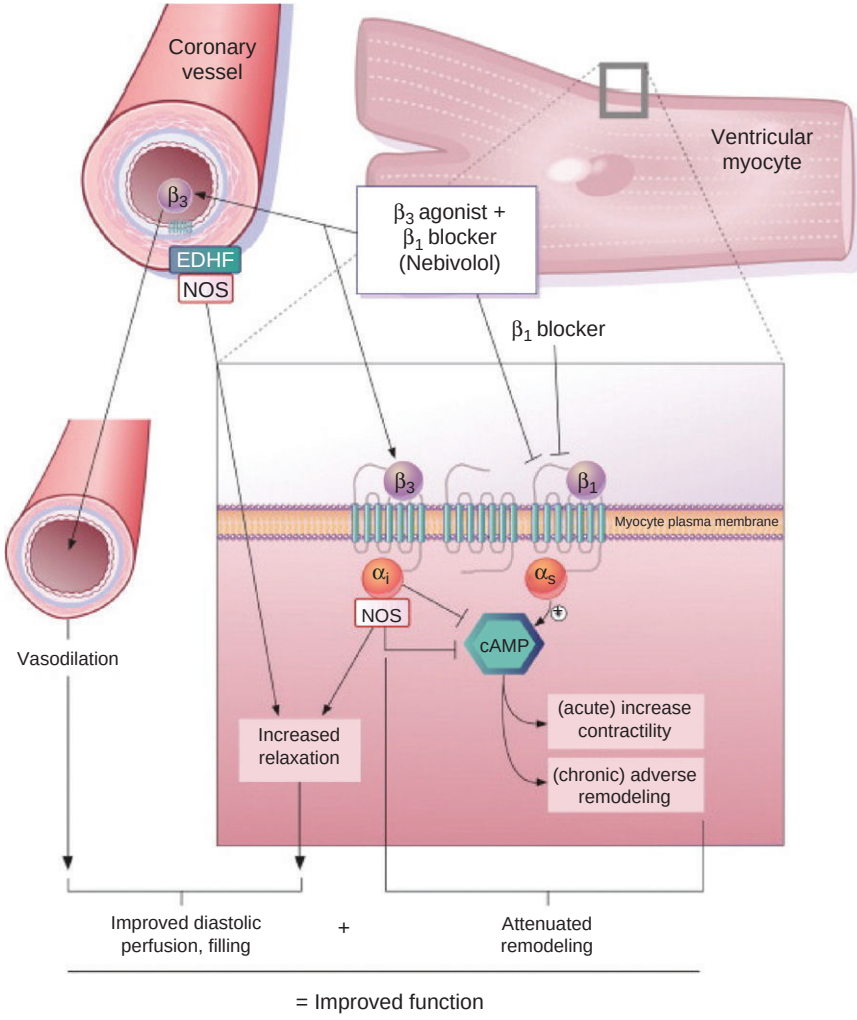

FIGURE 6 Putative beneficial effects of combined activation of beta3-AR and blockade of beta1-AR on cardiac function Nebivolol combines the properties of a beta1-AR antagonist (beta1 blocker) and a beta3-AR agonist. In human myocardium, beta3-ARs are expressed both on endothelial cells of the coronary microvasculature and on cardiac myocytes. (*Left*) Activation of beta3-AR on coronary endothelium produces the activation of endothelial nitric oxide synthase (NOS) and the release of nitric oxide (NO) and of an endothelium-derived hyperpolarizing factor (EDHF), both of which contribute to increased vasodilation and coronary perfusion. In addition, NO may paracrinally promote cardiomyocyte relaxation and left ventricular (LV) diastolic filling, thereby enhancing diastolic reserve. (*Right*) In ventricular cardiomyocytes, nebivolol combines the effects of beta1-AR blockade, which prevents the deleterious effects of chronic adrenergic stimulation on myocardial remodeling, and activation of beta3-AR; the latter, through G-alpha-i coupling, (e)NOS activation, and NO production may oppose the acute effects of adrenergic stimulation of contractility. Hypothetically, agonism on beta3-AR may contribute to additional protection against adverse remodeling under chronic adrenergic stress through sustained functional antagonism of the beta1-AR/cyclic adenosine monophosphate (cAMP) pathway or other (yet undetermined) pathways. The combined effects on remodeling, ventricular perfusion, and filling ultimately would result in improved LV function. From Balligand (2009).

advanced heart failure. Important aspects of the beta3-AR effects on human cardiac tissue, such as their influence on cardiac electrophysiology or metabolism, remain underexplored and should be resolved for a safer prediction. Longitudinal studies in animal models or even in patients using some of the new specific beta3-AR agonists currently under development for urologic or neurologic diseases are now needed to clarify this important issue.

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Modulation of Vascular Sarco/Endoplasmic Reticulum Calcium ATPase in Cardiovascular Pathophysiology

Abstract

Endothelial dysfunction associated with decreased nitric oxide (NO) bioactivity is a major feature of vascular diseases such as atherosclerosis or diabetes. Sodium nitroprusside (SNP)-induced relaxation is entirely dependent on cyclic guanosine monophosphate (cGMP) and preserved in atherosclerosis, suggesting that smooth muscle response to NO donor is intact. However, NO gas activates both cGMP-dependent and -independent

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signal pathways in vascular smooth muscle cells, and oxidative stress associated with vascular diseases selectively impairs cGMP-independent relaxation to NO. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which regulates intracellular Ca^{2+} levels by pumping Ca^{2+} into store, is a major cGMP-independent target for NO. Physiological levels of reactive nitrogen species (RNS) S-glutathiolate SERCA at Cys674 to increase its activity, and the augmentation of RNS in vascular diseases irreversibly oxidizes Cys674 or nitrates tyrosine residues at Tyr296-Tyr297, which are associated with loss of function. S-glutathiolation of various proteins by NO can explain redox-sensitive cGMP-independent actions, and oxidative inactivation of target proteins for NO can be associated with the pathogenesis of cardiovascular diseases. Oxidative inactivation of SERCA is also implicated with dysregulation of smooth muscle migration, promotion of platelet aggregation, and impairment of cardiac function, which can be implicated with restenosis, pathological angiogenesis, thrombosis, as well as heart failure. Analysis of posttranslational oxidative modifications of SERCA and the preservation of SERCA function can be novel strategies against cardiovascular diseases associated with oxidative stress.

I. Introduction

The impairment of endothelium-dependent relaxation is widely regarded as a major feature of vascular pathophysiology. In various animal models of vascular diseases such as hyperlipidemia, hyperglycemia, hypertension, hyperhomocysteinemia, or in the case of toxins associated with smoking, acetylcholine (ACh)- or bradykinin (BK)-induced relaxation is impaired (Cohen, 1995; Harrison, 1993). In human studies, methacholine-induced relaxation and flow-mediated relaxation in brachial arteries are impaired in vascular diseases such as coronary artery disease, peripheral artery disease, and stroke. Secession of smoking, diet modification, or treatment with drugs for vascular diseases such as statin, ARB/ACEI, and Ca^{2+} antagonists improve endothelium-dependent relaxation. Because endothelial dysfunction is associated with vascular diseases, the assessment of endothelial function has spread to the many fields of cardiovascular research. For example, many vascular laboratories in clinics assess endothelial function for the early marker of atherosclerosis and for evaluating the beneficial effects of drugs (Ganz & Vita, 2003; Vita, 2005).

More than 20 years ago, nitric oxide (NO) was identified as the major endothelium-derived relaxation factor especially in various vessels (Cohen, 1999; Vahnoutte, 2009). NO, which is released from endothelial NO synthase (eNOS), not only relaxes the arteries, but has pleiotropic effects, such as antiaggregatory, antiatherosclerotic effects; promotion of angiogenesis; suppression of vascular spasm and restenosis. Since the discovery of

NO, many studies have focused on the pathological mechanisms for vascular dysfunction related with "NO bioactivity."

It is believed that endothelium is injured at a very early stage and smooth muscle functioning is preserved until the later stages of disease, because endothelium-independent relaxation induced by sodium nitroprusside (SNP) or nitroglycerine is preserved (Schmidt et al., 1993). These findings indicate that smooth muscle relaxation due to "NO" is intact and that the underlying mechanisms for the impairment of relaxation are related to decreased NO release from eNOS or scavenging and destruction of NO during diffusion. Reactive oxygen/nitrogen species (ROS/RNS) are overproduced in various vascular diseases, which can impair eNOS function and/or scavenge NO. The elevation of ROS/RNS is clearly associated with decreased NO bioactivity (Cai & Harrison, 2000). When I joined Dr. R. A. Cohen's laboratory in the late 1990s, the topic of preservation of smooth muscle response to NO came into the limelight again for the following two reasons. First, there were differences between NO and SNP with regard to the signaling mechanisms for smooth muscle relaxation (Adachi et al., 2001; Plane et al., 2000; Weisbrod et al., 1998). Second, smooth muscle response to NO, but not to SNP, was also impaired in atherosclerosis and diabetes, and endothelial dysfunction was simultaneously observed (Yaghoubi et al., 2000). A short-term incubation of aorta with antioxidant had no effects (Adachi & Cohen, 2000; Miller et al., 1998). Even at the highest concentration of NO gas, smooth muscle response was decreased in atherosclerosis, suggesting that NO scavenging with ROS/RNS was not the primary reason for the reduced response to NO in smooth muscles (Weisbrod et al., 1997, Fig. 1). In this chapter, I will discuss the signaling mechanism of NO in diseased smooth muscle, with the implication for other cardiovascular diseases.

II. Mechanism of cGMP-Independent Relaxation to NO

A. cGMP-Independent Relaxation to NO and ACh in Normal Arteries

In 1998, Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad received the Noble Prize for the discovery of NO as a signaling molecule. NO can diffuse through the plasma membrane, bind heme iron on soluble guanylyl cyclase (sGC), and activate the enzyme. sGC produces cyclic guanosine monophosphate (cGMP) and activates protein kinase G (PKG) in intracellular spaces to induce vasodilation. It takes part in gas signaling and therefore research was undertaken in many fields including physiology, pathology, pharmacology, neuroscience, immunology, and cardiovascular/pulmonary medicine (Vanhoutte, 2009). Researchers have found that most of the actions involving NO do not depend on the cGMP/PKG pathway,

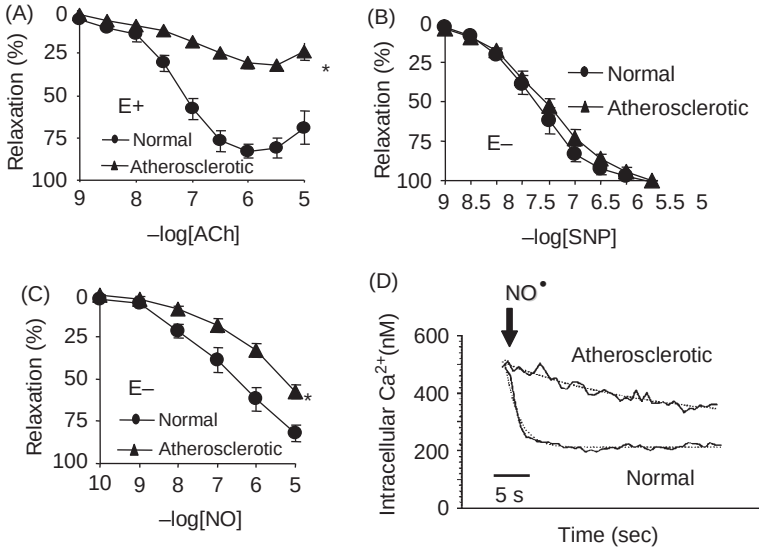

FIGURE 1 Impairment of ACh- and NO- but not SNP-induced relaxation in atherosclerosis. (A) ACh-, (B) SNP-, and (C) NO-induced relaxation in normal and atherosclerotic rabbit aorta. Both endothelium-dependent relaxation and NO-induced relaxation are impaired in atherosclerosis. (D) The measurement of intracellular calcium ($[Ca^{2+}]_i$) in cultured smooth muscle cells from normal and atherosclerotic aorta from rabbits. NO sharply dropped $[Ca^{2+}]_i$ in cells from normal aorta. The response to NO in atherosclerotic smooth muscle cells was much slower than normal, suggesting abnormal smooth muscle response to NO (Adachi et al., 2001, Permission from AHA).

although cGMP is an important target of NO for its biological function. NO may bind other heme proteins such as hemoglobin (Allen et al., 2009), cytochrome oxidase (Taylor & Moncada, 2010), or NO synthase to modulate their functions. NO may produce *S*-nitrosothiol in extra- or intracellular spaces. *S*-nitrosothiol has a much longer biological half-life than NO gas and can modulate the protein functions related with metabolism, intracellular calcium uptake, transcription, cell shape, as well as phosphorylation signaling. Nitroxyl anion (NO^-) is a poor reactant with thiols since the negative charges reject each other. Nitrosonium (NO^+) or N_2O_3 would be the better and more important choice for formation of *S*-nitrothiols. The thiol-dependent effects of NO are potentially mediated by secondary produced RNS such as $^{\bullet}NO_2$, NO^-, and peroxynitrite ($ONOO^-$) (Lima et al., 2010).

Thus, cGMP-independent effects of NO and the involvement of cGMP in NO-induced vasodilation were assessed using sGC inhibitors such as methylene blue or 1H-[1,2,4]-oxadiazole-[4,3-*a*]quinoxalin-1-one (ODQ). ACh-dependent vasodilation in some vessel types (e.g., rabbit thoracic aorta) is almost completely abolished by ODQ (up to 90%). However,

ODQ decreases ACh-induced relaxation by about 50% only and almost eliminates a rise in vascular cGMP levels in rabbit carotid arteries, suggesting that a substantial portion of endothelial-dependent relaxation is induced by a cGMP-independent mechanism in some vessels. ODQ also inhibits NO-induced relaxation in endothelium-denuded arteries in rabbits by about 50%. On the other hand, SNP-induced relaxation was almost abolished with ODQ, suggesting that it is entirely a cGMP-dependent mechanism (Fig. 2A–B). From these observations, we hypothesized the diverse signaling mechanisms for NO-induced relaxation in a cGMP-dependent and -independent manner (Adachi et al., 2001; Plane et al., 1998, Weisbrod et al., 1998).

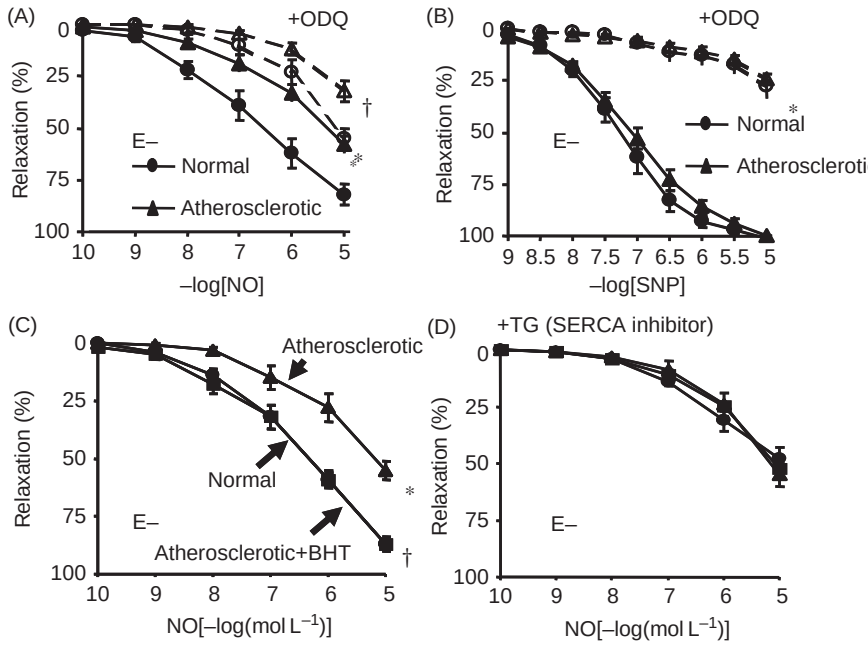

FIGURE 2 Contribution of guanylyl cyclase (sGC) and SERCA in NO-induced relaxation in normal and atherosclerotic aorta. (A) An sGC inhibitor, ODQ, inhibited NO-induced relaxation in both normal and atherosclerotic aorta. After the inhibition of sGC with ODQ, NO-induced relaxation is still less in atherosclerotic aorta. (B) SNP-induced relaxation was almost entirely eliminated with ODQ in both normal and atherosclerotic aorta. These data suggest that cGMP-independent relaxation to NO was impaired in atherosclerosis. (C–D) NO-induced relaxation was impaired in atherosclerosis, which is restored with the treatment of antioxidant BHT for 3 weeks. TG, a SERCA inhibitor, decreased NO-induced relaxation in both the normal and BHT group, but not in the atherosclerosis group. After treatment with TG, NO-induced relaxation was identical among three groups, suggesting that SERCA-mediated relaxation to NO was selectively impaired in atherosclerosis and restored with an antioxidant (Adachi et al., 2001, 2002, Permission from AHA).

Vasodilation can be elicited by two distinct mechanisms: by lowering intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and by decreasing Ca^{2+} sensitivity of contractile proteins. To understand the mechanism of cGMP-independent relaxation induced by NO, we measured $[Ca^{2+}]_i$ with Fura-2 in vascular smooth muscle cells cultured from rabbit aorta. NO gas sharply dropped $[Ca^{2+}]_i$. Surprisingly, pretreatment of cells with ODQ had no effects on a drop in $[Ca^{2+}]_i$ by NO, suggesting that the modulation of $[Ca^{2+}]_i$ by NO gas in smooth muscle cells is mostly independent of cGMP/PKG. From this observation, we concluded that iron channels or transporters regulate $[Ca^{2+}]_i$ for the cGMP-independent targets of NO.

B. cGMP-Independent Relaxation in Diseased Arteries

We further analyzed cGMP-independent relaxation in atherosclerotic aorta from rabbits fed a high-cholesterol diet. Unlike SNP-induced relaxation, NO-induced relaxation decreased in atherosclerotic aorta. Following treatment with ODQ, the residual relaxation induced by NO gas was still less in atherosclerotic aorta. SNP-induced relaxation, which is mostly dependent on cGMP/PKG, was preserved in atherosclerosis (Fig. 2A–B). Moreover, 8-bromo-cGMP, a cell-permeable analogue of cGMP, elicited relaxation in atherosclerotic aorta as in normal aorta (Adachi & Cohen, 2000; Adachi et al., 2001). Although the expression or function of sGC per molecule may be altered (Schmidt et al., 1993), cGMP-dependent relaxation to NO is largely preserved in atherosclerosis. Moreover, a drop in $[Ca^{2+}]_i$ with NO gas was markedly slower in cultured smooth muscle cells from atherosclerotic aorta (Fig. 1D). These data indicate that cGMP-dependent relaxation was preserved and cGMP-independent relaxation to NO, which was caused by the modulation of $[Ca^{2+}]_i$, was mainly impaired in smooth muscle from diseased arteries. Similar data were obtained with high glucose levels (Cohen & Tong, 2010).

C. Potassium Channels

Endothelium-dependent relaxation is associated with hyperpolarization of underlying smooth muscle. It is now recognized that the endothelium-derived hyperpolarization factors (EDHFs) activate Ca^{2+}-activated potassium (BKCa) channels to induce hyperpolarization, especially in small arterioles (Félétou & Vanhoutte, 2006). NO activates BKCa channels in a cGMP-dependent (Archer et al., 1994) or -independent manner (Bolotina et al., 1994). Hyperpolarization of plasma membrane in smooth muscles inhibits Ca^{2+} influx from an L-type channel; thus, a drop in $[Ca^{2+}]_i$ by NO is potentially induced by the activation of BKCa (Cohen et al., 1997).

Najibi assessed the effects of charybdotoxin, a BKCa inhibitor, on NO-induced relaxation in atherosclerosis. Charybdotoxin-sensitive relaxation with ACh and NO was augmented in carotid arteries from rabbits fed a

high-cholesterol diet (Najibi & Cohen, 1995, Najibi et al., 1994). BKCa was reported to be impaired in diabetes (Li et al., 2004). EDHF-mediated relaxation, which is mediated by the activation of BKCa, is preserved in the early or middle stage of atherosclerosis, when NO bioactivity is decreased (Shimokawa & Morikawa, 2005). Charybdotoxin is not utilized as a specific antagonist for BKCa. However, these data indicate that the decreased BKCa (or other potassium channels)-mediated pathway may not be the primary cause for the reduced smooth muscle response in atherosclerotic arteries.

D. Sarco/Endoplasmic Reticulum Ca^{2+} ATPase

Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) refills Ca^{2+} into the sarco/endoplasmic reticulum (SR) store, which plays a critical role in regulating $[Ca^{2+}]_i$. In each cycle, it hydrolyzes ATP to ADP and uses high energy to transport two Ca^{2+} ions from the cytoplasm to the lumen of SR. SERCA proteins consist of a large cytoplasmic head with a phosphorylation domain including the site of acylphosphate formation (Asp 351), a β-pleated transduction domain, a nucleotide-binding domain, and a hinge domain. They also consist of 10–11 transmembrane helixes. There are several isoforms encoded by four SERCA genes. SERCA1 is expressed in the fast-twitch skeletal muscle, SERCA2a is expressed in cardiac and slow-twitch skeletal muscle whereas SERCA2b is expressed in vascular smooth muscle and brain, and SERCA3 is expressed in various nonmuscle cells.

Vascular smooth muscle cells have dual pathways for Ca^{2+} influx: one is an L-type channel and the other is a store-operated Ca^{2+} channel (STOC). Even in the presence of nifedipine, an L-type channel blocker, phenylephrine (PE) causes similar contraction in rabbit aorta, suggesting the significant role of STOC in vascular tone. STOC opens when the Ca^{2+} store in SR decreases. SERCA, as the SR Ca^{2+} pump, refills cytoplasmic Ca^{2+} in the SR Ca^{2+} store, and decreases Ca^{2+} influx through STOC, which consequently decreases $[Ca^{2+}]_i$. Cohen and Bolotina found that inhibition of SERCA eliminated a drop in $[Ca^{2+}]_i$ by NO in vascular smooth muscle cells and platelets. They used three kinds of SERCA inhibitors—cyclopiazonic acid (CPA), thapsigargin (TG), and 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ)—and obtained similar results. Blockade of plasma membrane Ca^{2+} ATPase or Na^+/Ca^{2+} exchange had no effects on a drop in $[Ca^{2+}]_i$ by NO (Cohen et al., 1999; Trepakova et al., 1999). From these data, we hypothesized that SERCA is the major target of NO in vascular smooth muscle cells.

On the basis of these findings, I assessed the involvement of SERCA in smooth muscle relaxation to NO using aortic rings from rabbits. CPA decreased NO-induced relaxation to 50% and mostly blocked ODQ-resistant relaxation to NO. TG had similar effects as CPA, suggesting

that SERCA plays a critical role in cGMP-independent relaxation to NO. CPA had negligible effects on SNP-induced relaxation, which is mostly a cGMP-dependent mechanism. cGMP/PKG was proposed to modulate $[Ca^{2+}]_i$, and the cGMP/PKG pathway was reported to modulate SERCA through phosphorylation of its adaptor protein phospholamban or through SR Ca^{2+} releasing channels (Cornwell et al., 1991; Komalavilas & Lincoln, 1996). However, this pathway may not be prominent or may be spared in SNP-induced aortic relaxation. cGMP-dependent relaxation can be potentially due to reduced Ca^{2+} sensitivity. In fact, myosin light chain phosphatases or Rho/Rho kinase were reported to be the major targets of the cGMP/PKG pathway activated with NO (Shimokawa & Takeshita, 2005; Surks et al., 1999).

Because SERCA was found to be involved in cGMP-independent relaxation to NO, I used these pharmacological blockers of SERCA for assessing relaxation in atherosclerotic aorta. After treatment of aortic rings with ODQ, NO-induced relaxation was mostly eliminated in atherosclerotic aorta and addition of CPA to ODQ mostly negated the response to NO in both normal and atherosclerotic aorta. Following treatment of aortic rings with TG, NO-induced relaxations were almost similar in normal and atherosclerotic aorta (Fig. 2C–D), and the residual relaxation was also eliminated by adding ODQ. From these observations, it can be concluded that SERCA plays an essential role in cGMP-independent relaxation and the cGMP-independent SERCA-mediated relaxation to NO is impaired in atherosclerosis. Basal SERCA activity, which was assessed with $^{45}Ca^{2+}$ uptake into aortic SR, decreased by about 50% in atherosclerosis without there being a change in protein expression (Adachi et al., 2001). From these data, it can be concluded that NO activates dual pathways to relax arteries: cGMP-dependent modulation of Ca^{2+} sensitivity and cGMP-independent modulation of SERCA activity to decrease $[Ca^{2+}]_i$ (Fig. 3).

At this stage, we had many unsolved questions. First, SERCA blockers clearly eliminate cGMP-independent relaxation; however, we had no evidence about whether NO directly activates SERCA. Second, other Ca^{2+} regulators, ryanodine receptors, Na^+/K^+ ATPase, Na^+/Ca^{2+} exchanger, L-type channels, and BKCa can be influenced by inhibition of SERCA, because SERCA plays a critical role in the regulation of $[Ca^{2+}]_i$. Other molecules may mediate the cGMP-independent response to NO, and SERCA blockers can influence them. Third, we did not know how the activation of SERCA by NO was impaired in atherosclerosis. If NO could directly activate SERCA, there should be molecular changes on SERCA to interfere a cGMP-independent signal in atherosclerosis. Because oxidative stress is an extremely important factor in atherosclerosis (Cai & Harrison, 2000), we characterized redox changes in vascular smooth muscle that resulted from atherosclerosis.

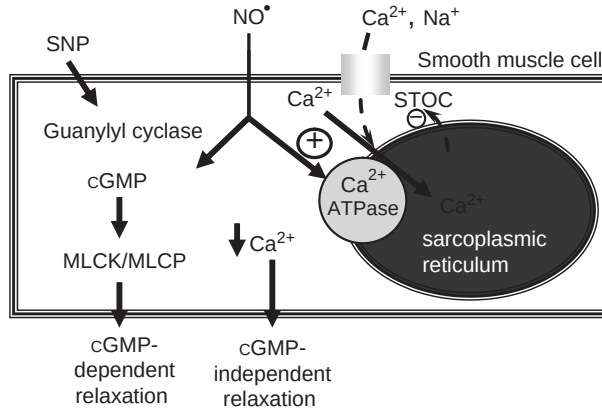

FIGURE 3 NO relaxes smooth muscle cells by cGMP-dependent and -independent mechanisms. NO activates sGC, increases cGMP, and activates protein kinase G (PKG). cGMP/PKG predominantly regulates contractile proteins by modulating myosine light chain kinases/phosphatases (MLCKs/MLCPs) or Rho/Rho kinases and relaxes smooth muscles. Unlike SNP, NO directly activates SERCA and refills Ca^{2+} into store. Subsequently store operated Ca^{2+} channels (STOC) are inhibited, which decreases intracellular calcium and relaxes arteries. This mechanism is predominantly cGMP independent and is impaired in vascular diseases (Cohen, 2007).

III. Oxidative Stress, NO Bioactivity, and Vascular Physiology/ Pathophysiology

A. Role of Superoxide and Peroxynitrite

In 1986, it was found that superoxide anion inactivated endothelium-derived relaxing factor (EDRF) and later NO was identified as EDRF (Rubanyi & Vanhoutte, 1986). Atherosclerosis and other vascular diseases elevate the levels of ROS/RNS by augmenting superoxide production from NADPH oxidase, xanthine oxidase, NO synthase, and the mitochondrial electron transport chain. NO reacts very rapidly with superoxide anion to form $ONOO^-$, which rapidly breaks down to nitrate, a poor vasodilator. This reaction is the major reason for decreased NO bioactivity by superoxide. $ONOO^-$ also induces eNOS uncoupling by either oxidation of tetrahydrobiopterin (Landmesser et al., 2003) or eNOS monomerization (Zou et al., 2002), which further increases vascular superoxide levels. $ONOO^-$ is a very strong oxidant, which oxidizes metals, DNA, lipids, tyrosine, thiols, ascorbate, and other cell components (Beckman & Koppenol, 1996; Szabó et al., 2007). We hypothesized that intracellular oxidative stress might be an important factor in reducing smooth muscle response to NO by changing the intracellular redox state.

B. Glutathione

Glutathione (GSH) is the most abundant intracellular low-molecular-weight thiol that detoxicates drugs, toxins, and oxidants. To assess intravascular redox, I measured the GSH levels in aortic homogenate using the Tietz method. The vascular GSH levels in atherosclerotic aorta were about 50% of that in normal aorta. We also employed a cell-permeable thiol oxidant—diamide—to decrease intravascular GSH levels. A preincubation of aorta with diamide (1 mM) decreased vascular GSH levels at 40% of control. Diamide also decreased ACh- and NO-induced relaxation and had little effect on SNP-induced relaxation. Diamide eliminated ODQ-resistant relaxation to NO (Fig. 4); thus, the effects of atherosclerosis and diamide were very similar.

At that time, we thought that the decreased vascular GSH levels could be responsible for reduced NO-induced relaxation and we employed a GSH ester to supply intracellular GSH. Although the GSH ester normalized vascular GSH levels in atherosclerotic aorta, it had little effect on ACh- and NO-induced relaxation. We concluded that "the other factors, perhaps related to the long-term decrease in GSH levels, are responsible for reduced NO bioactivity in hypercholesterolemia"

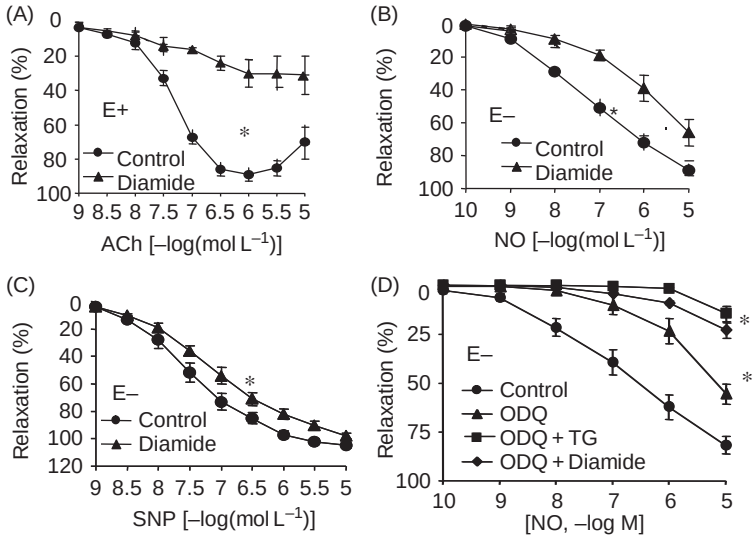

FIGURE 4 The effects of diamide in NO-induced relaxation. (A–C) A thiol oxidant diamide (1 mM) decreased ACh- and NO-induced relaxation, but had little effect on SNP-induced relaxation. The effect of diamide is similar to that in atherosclerosis, as shown in Fig. 1A–C. (D) After inhibition of sGC by ODQ, the residual relaxation to NO was almost eliminated with TG or diamide. These data suggest that cGMP-independent relaxation to NO is mediated by SERCA and impaired with thiol oxidation (Adachi et al., 2000, Permission from Nature Pub).

(Adachi & Cohen, 2000). Although a decrease in vascular GSH levels may not be responsible for the reduced response to NO, we observed that the effects of thiol oxidation and atherosclerosis on NO-induced relaxation are similar.

C. Antioxidants

Short-term (30–60 min) incubation with antioxidants had little effect on the reduced smooth muscle response to NO in atherosclerosis. Since the supply of antioxidants does not immediately improve NO bioactivity in vascular tissue, it is obvious that the beneficial effects of antioxidants is not dependent on the rapid scavenging action of ROS/RNS. To test the mechanism of antioxidants for reserved NO bioactivity in atherosclerotic rabbits, butylated hydroxytoluene (BHT) was added to a high-cholesterol diet during the last 3 weeks of the 13-week diet. BHT did not change plasma cholesterol levels; however, decreased aortic cholesterol levels indicated inhibition of atherosclerosis. BHT normalized ACh- and NO-induced relaxation in atherosclerotic aorta. SERCA-mediated component of NO-induced relaxation and vascular SERCA activity were also restored with BHT without changing the protein expression of SERCA (Adachi et al., 2002, Fig. 2C). Antioxidants improved SERCA activity in 3 weeks; thus, they may be effective in protecting the SERCA from oxidative modifications. With this hypothesis, we started to analyze the posttranslational oxidative modifications of SERCA protein.

IV. Posttranslational Oxidative Modifications of SERCA

A. Oxidative Modifications on Proteins

A multitude of ROS/RNS, including $O_2^{\bullet -}$, H_2O_2, HOCl, $HO^{\bullet}$, $NO^{\bullet}$, $^{\bullet}NO_2$, NO^-, and $ONOO^-$, are generated *in vivo*. ROS/RNS preferentially react with similar protein constituents such as transition metals, methionine, tryptophan, tyrosine, and cysteine. Under physiological conditions, ROS/RNS may act as potential signaling molecules, mostly producing reversible protein modifications such as methionine sulfoxide, S-nitroso derivative, sulfenic acid, and S-glutathiolated proteins (Finkel, 2000). As the disease progresses, more irreversible types of oxidative modifications are generated, such as nitrotyrosine, chlorotyrosine (Podrez et al., 2000), aldehydes (Endo et al., 2009), and cysteine sulfinic/sulfonic acid (Park et al., 2009). These posttranslational oxidative modifications can alter protein function without changing protein expression (Adachi et al., 2005) (Fig. 5).

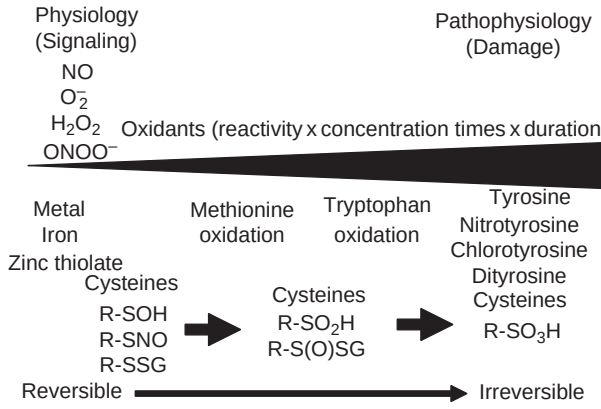

FIGURE 5 Reactive oxygen and nitrogen species (ROS/RNS) and posttranslational oxidative modifications. Under physiological conditions or mild elevation of ROS/RNS, reversible oxidative modifications of metals or thiols regulate protein function and may subserve cell signaling mechanisms. In cardiovascular diseases, the elevated ROS/RNS levels produce irreversible oxidation of proteins and alter enzyme function permanently. Nitrotyrosine or S-sulfonylation cause irreversible oxidation and are produced on SERCA in atherosclerosis (Adachi, 2005).

B. Nitrotyrosine

As the disease progresses, nitrotyrosine is generated on the tyrosine residues in the specific proteins, which represents a marker of oxidative stress associated with protein dysfunction. The precise reaction for the generation of nitrotyrosine *in vivo* is not clear yet. It may be generated by the chemical oxidation of tyrosine with $ONOO^-$ or may be enzymatically generated by peroxidases with nitrite and hydrogen peroxide. Polyclonal and monoclonal antinitrotyrosine antibodies, raised using tyrosine-nitrated keyhole limpet hemocyanin (KLH) peptides from plants, were widely used for the detection of nitrated proteins using immunohistochemistry and Western blotting (Beckman & Koppenol, 1996). We employed them for the detection of nitrotyrosine formations in SERCA of atherosclerotic aorta with or without BHT. The generation of nitrotyrosine was observed in the whole layer of aorta in atherosclerosis using antinitrotyrosine antibodies for immunohistochemistry. Treatment with BHT for 3 weeks decreased the stain of nitrotyrosine especially in medial smooth muscle region. The data suggest that elevated ROS/RNS generation in smooth muscle with protein nitration is associated with decreased ACh- and NO-induced relaxations. Tyrosine-nitrated SERCA2 (NY-SERCA2) was reported in aging skeletal muscle, which was associated with decreased SERCA function (Viner et al., 1999a). We measured NY-SERCA2 in atherosclerosis in collaboration with Schöneich's laboratory. NY-SERCA2 was increased about 20 times in atherosclerosis (1.6 ± 0.7 vs

0.08 ± 0.06) (Adachi et al., 2002). The Schöneich group also identified that Tyr296-Tyr297 were primary sites for the generation of NY-SERCA2 proteins. Using corresponding peptides for SERCA with nitrotyrosine modifications, we raised an anti-NY-SERCA2 antibody. Using this antibody for immunohistochemical studies, NY-SERCA2 were detected in atherosclerotic humans and rabbits, in skeletal muscle of aged rats, and in the atrium of patients with heart failure (Xu et al., 2006). Many tyrosine-nitrated proteins were thus identified and our method may be potentially useful for detecting modifications on specific proteins in various diseases.

NY-SERCA2 was closely associated with dysfunction of SERCA activity and decreased NO bioactivity in atherosclerosis. However, it is not clear whether the generation of nitrotyrosine is the cause for reduced SERCA function. High concentrations of $ONOO^-$ (>100 μM) decrease SERCA activity with tyrosine nitration. However, exogenous $ONOO^-$ generates nitrotyrosine in tyrosine 122 (Sharov et al., 2002). Tyr296-Tyr297 are located in the luminal site of SR. The localization of oxidative stress may be different in disease states and with exogenous $ONOO^-$.

The generation of nitrotyrosine on proteins is potentially a good marker for enhanced RNS formation in diseases. It is controversial whether nitrotyrosine formation is reversible. The modification is at least resistant to millimolar concentrations of dithiothreitol (DTT). Even if it can be reduced *in vivo*, aminotyrosine may be a secondary product. So far, nitrotyrosine is regarded as a marker of diseases and may work as an intracellular signal.

C. S-Glutathiolation

The augmented oxidative stress in diseases may produce irreversible modifications that change protein functions permanently. However, low oxidative stress plays a role in cell signaling, which can reversibly modify proteins. The thiol modifications on proteins are complex and potentially responsible for redox-sensitive signaling. Many thiols on intracellular proteins exist in the reduced form, and are relatively difficult to be oxidized in the basal state, because of their protonation state and an overall reducing environment. Some cysteine residues are present in intra- and intermolecular disulfides, which stabilize protein structure and folding. Because of the local electrostatic microenvironment, which is affected by neighboring amino acids, some thiols exhibit an unusually low pK_a, which results in a higher reactivity toward ROS/RNS. These thiols can be easily modified with high physiological concentration of ROS/RNS through oxidation (formation of sulfenic acid, RSOH; sulfinic acid, RSO_2H), thiolation (S-glutathiolation, RSSG; formation of thiosulfinate, RS(O)SG; formation of *S*-cysteine, *S*-homocysteine, and other mixed disulfides), and S-nitrosation (RSNO). S-glutathiolation can be a representative modification in redox-sensitive signaling (Klatt & Lamas, 2000). S-nitrosation or sulfenic acid formation

may not be stable for a long period in intracellular space, because ascorbate or other intracellular antioxidants can reverse them. On the other hand, S-glutathiolation is stable in the presence of millimolar levels of ascorbate or GSH (Konorev et al., 2000). S-glutathiolation can be chemically and enzymatically reversed by DTT or a thioredoxin/glutaredoxin (TRX/GRX) system with a reducing capacity of NADPH (Adachi et al., 2004a; Yamamoto et al., 2003). S-glutathiolation is also reversible by dihydrolipoic acid. NADPH is mainly generated through the pentose phosphate pathway, and glucose 6-phosphate dehydrogenase may be important for the regulation of S-glutathiolated proteins (Ayene et al., 2008; Leopold & Loscalzo, 2005).

It is still unclear how S-glutathiolation can be induced in intracellular spaces with ROS/RNS. High concentrations of oxidized GSH (GSSG) were used to generate S-glutathiolated proteins *in vitro*, because it was believed that thiol–disulfide exchange (reaction 1) is the major mechanism for S-glutathiolation.

$$RSH + GSSG \rightarrow RSSG + GSH \quad (1)$$

Usually, more than 50% of GSSG is required for this reaction; however, such high concentrations of GSSG are unlikely to exist in cells even in disease states.

ROS/RNS can directly attack reactive thiols on proteins and generate thiyl radicals that can be an intermediate for S-glutathiolated proteins (reactions 2–4).

$$RS^- \rightarrow RS^\bullet + e^- \quad (2)$$

$$RS^\bullet + GSH \rightarrow RSSG^{\bullet -} + H^+ \quad (3)$$

$$RSSG^{\bullet -} + O_2 \rightarrow RSSG + O_2^{\bullet -} \quad (4)$$

Alternatively, S-glutathiolation might proceed using sulfenic acid (RSOH), *S*-nitrosothiols, or *S*-nitrothiols (reactions 5–7).

$$RSOH + GSH \rightarrow RSSG + H_2O \quad (5)$$

$$RSH + NO_2^+ \rightarrow RSNO_2 + H^+ \quad (6)$$

$$RSNO_2 + GSH \rightarrow RSSG + NO_2^- + H^+ \quad (7)$$

These are chemical explanations and the role of enzymes in S-glutathiolation in cells is not completely understood yet. (Adachi et al., 2005)

S-glutathiolation has been known to regulate the various protein/enzyme activities *in vitro* (Klatt & Lamas, 2000). For example, S-glutathiolation of Cys93 on the beta-chain of hemoglobin increases oxygen affinity and prevents

aggregation against the exposure to low oxygen tension in sickle cell disease (Garel et al., 1986). S-glutathiolation of Cys34 on actin regulates cell polymerization (Wang et al., 2001). S-glutathiolation regulates various metabolic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mohr et al., 1999), creatine kinase, branched chain amino acid transferase (Coles et al., 2009), and matrix metalloproteinases (Okamoto et al., 2001). Especially, mitochondrial enzyme is susceptible to oxidative modifications and S-glutathiolation can be induced in mitochondrial proteins such as complex I (Tayler et al., 2003), complex II (Chen et al., 2007), and isocitrate dehydrogenase (Shin et al., 2009). S-glutathiolation can regulate the phosphorylation signal to modulate hypertrophy, migration, metabolism, inflammation, and apoptosis. Many of proteins which modulate intracellular signal such as protein kinase A, protein kinase C, mutagen-activated protein kinase/extracellular-signal-regulated kinase kinase kinase, calcium/calmodulin kinase (Anselmo & Cobb, 2004; Kambe et al., 2010), phosphatases (protein tyrosine phosphatase 1B, protein phosphatase 2A, PTEN), inhibitory kappa beta kinase beta (Reynaert et al., 2006), and caspase-3 (Pan & Berk, 2007) are S-glutathiolated to modulate their activities. Moreover, S-glutathiolation of the Ras family, small GTPases, may be part of various signal cascades involving angiotensin II (Adachi et al., 2004a), oxidized low-density lipoprotein (LDL) (Clavreul et al., 2006a), insulin (Clavreul et al., 2006b), α-adrenergic receptor (Kuster et al., 2005), as well as mechanical stretch (Pimentel et al., 2006). S-glutathiolation of the transcription factors (c-Jun of AP-1 or p50 of NF-κβ, HIF-1) within their DNA binding sites inhibits their DNA binding affinity (Klatt & Lamas, 2000). Sirtuin-1, an NADH-dependent histone deacetylase, is inhibited by S-glutathiolation (Zee et al., 2010). S-glutathiolation also affects various antioxidant enzymes, such as SOD1 (Choi et al., 2005), TRX (Heo & Campbell, 2005; Wilcox et al., 2009), GRX, protein disulfide isomerase (Townsend et al., 2009b), peroxiredoxin, and GSH-S-transferases (Townsend et al., 2009a), which may potentially generate networks for positive/negative feedback in a thiol-mediated redox signal. Ion channels, such as the ryanodine receptor (Aracena et al., 2003) and chloride channels (Wang et al., 2005), are also S-glutathiolated, which regulate ion transport activity. We hypothesized that S-glutathiolation with ROS/RNS might regulate SERCA activity.

D. Thiol Oxidations on SERCA

SERCA was reported to be S-glutathiolated with $ONOO^-$ including Cys674 and S-nitrosated with DEA/NO including Cys364. Interestingly, target Cys sites of S-glutathiolation and S-nitrosation were different (Viner et al., 1999b, 2000). To investigate the functional relevance of thiol (s) modifications of SERCA, we prepared the purified SERCA reconstituted in artificial liposome. Up to 100 μM of $ONOO^-$ had no effect on SERCA

activity in the absence of GSH; however, 10–100 μM of $ONOO^-$ increased Ca^{2+}-uptake activity of SERCA in the presence of GSH. The activation of SERCA was reversed with DTT, suggesting that S-glutathiolation is responsible for the enzymatic change. Using GSH–sepharose and mass spectrometry, we identified Cys674 as the primary site for S-glutathiolation of SERCA2. To test the functional relevance for S-glutathiolation of SERCA, we used cell lysate with the expression of wild-type SERCA and SERCA-mutated Cys674 to serine (C674S SERCA). Even in the presence of GSH, $ONOO^-$ failed to S-glutathiolate and activate C674S SERCA. From these data we concluded that SERCA was activated with S-glutathiolation at Cys674.

When higher concentrations of $ONOO^-$ (100–1000 μM) were applied to SERCA reconstituted in liposome or aortic homogenate, SERCA activity decreased, which was irreversible with DTT. Using antinitrotyrosine antibody and mass spectrometry, we detected NY-SERCA2 formations and irreversible Cys oxidation (sulfinylation/sulfonylation) at Cys674. From these studies, we found the dual effects of $ONOO^-$ on SERCA activity: low concentrations activate SERCA via S-glutathiolation at Cys674 and high concentrations inhibit SERCA activity with irreversible oxidation including 3-nitrotyrosine and Cys-sulfonylation (Adachi et al., 2004b, Favero et al., 1998, Grover and Samson, 1989).

V. Physiological and Pathophysiological Roles of Thiol Modifications on SERCA in NO-Induced Relaxation

A. Physiological S-Glutathiolation of SERCA by NO

Activation of SERCA by S-glutathiolation at Cys674 indicates that modifications occur in NO-induced relaxation. We employed a biotin–GSH ester (Bio-GEE) for detecting intracellular S-glutathiolation (Ying et al., 2007b). After aortic rings from rabbits were pretreated with Bio-GEE, they were constricted with PE and relaxed with NO gas. Rings were freeze-clamped at each point and S-glutathiolated SERCA was identified using Western blotting. PE had no effect and after exposure to NO gas for 1 min, S-glutathiolated SERCA markedly increased. After 30 min when aortic tone was reversed, S-glutathiolated SERCA was reversed. Although we were not able to identify the enzyme that reversed S-glutathiolated SERCA, other studies indicated TRX/GRX/NADPH system could effectively reverse S-glutathiolated proteins (Adachi et al., 2004a; Han, 2007; Kuster et al., 2005; Pimentel et al., 2007; Tsou et al., 2009). I also tested the involvement of S-glutathiolated SERCA in endothelium-dependent relaxation. ACh-induced relaxation in carotid arteries from rabbits was associated with S-glutathiolation of SERCA. In arteries exposed to NO or ACh,

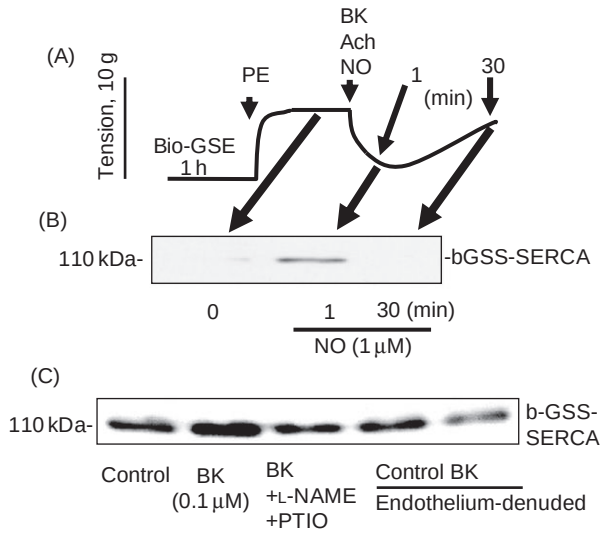

FIGURE 6 Increased S-glutathiolated SERCA by either endothelium-derived or exogenous NO. (A) Tracing of endothelium-denuded aortic rings contracted by PE and relaxed with exogenous NO gas solution. (B) S-Glutathiolated SERCA observed at contraction with PE, plus NO (1 min, 30 min). NO relaxes arteries and concomitantly increases S-glutathiolated SERCA. After 30 min, relaxation was reversed and S-glutathiolated SERCA was erased at the same time. (C) BK S-glutathiolates SERCA in porcine carotid arteries. L-NAME + PTIO (eNOS inhibitor and NO scavenger) or the denudation of endothelium prevented S-glutathiolated SERCA with BK, suggesting that endothelium-derived NO caused S-glutathiolated SERCA. b-GSS-SERCA: biotin-labeled S-glutathiolated SERCA (Adachi et al., 2004, Permission from Nature Pub).

SERCA activity increased in the absence of DTT to preserve S-glutathiolation. BK-induced relaxation in porcine carotid arteries was also accompanied with S-glutathiolation of SERCA, which is prevented by L-NAME/PTIO or denuded endothelium (Fig. 6). These data suggest that either exogenous or endogenous NO activated SERCA with S-glutathiolation, which occurred simultaneously with activation of sGC (Adachi et al., 2004b).

B. Involvement of Intracellular $ONOO^-$ in Physiological Regulation of Vascular Tone

Many researchers consider $ONOO^-$ to be a toxic RNS. Our *in vitro* data suggest that low concentrations of $ONOO^-$ can be physiologically generated in intracellular space and can work as a second messenger for NO (Pagano et al., 1999). A superoxide scavenger, Tiron, or an RNS

scavenger, uric acid, decreased ACh- and NO-induced relaxation in arteries from normal rabbits. Moreover, overexpression of SOD in smooth muscle cells prevented S-glutathiolation by NO. In SOD transgenic mice, aortic relaxation to ACh and NO was rather decreased. In *in vitro* studies with SERCA-reconstituted liposome, NO gas (20 μM) did not cause activation and S-glutathiolation of SERCA; however, similar or lower concentrations of $ONOO^-$ activated SERCA by S-glutathiolation. Moreover, in HEK293 cells expressing SERCA, NO S-glutathiolated and activated wild-type SERCA, but not C674S SERCA (Ying et al., 2007a). From these observations, we hypothesized that a certain amount of superoxide was generated in intracellular space and NO reacted with it to generate $ONOO^-$. Low concentrations of intracellular $ONOO^-$ activate SERCA by S-glutathiolation at Cys674, which can account for the regulation of vascular tone. There is no method for measuring the low levels of $ONOO^-$ in intracellular space and there is no direct evidence for $ONOO^-$ as the second messenger. Because NO is a weak oxidant for oxidizing thiol(s), the reaction with superoxide to produce RNS is required for S-glutathiolation of SERCA (Adachi et al., 2004b). Hydrogen peroxide is identified as EDHF (Shimokawa & Morikawa, 2005), which may modulate vascular tone via thiol modifications. It is not clear whether the enzyme(s) catalyze S-glutathiolation of proteins by NO.

C. Pathophysiological Thiol Oxidation of SERCA

As discussed in Chapter 5, cGMP-independent, SERCA-mediated relaxation to NO was selectively impaired in atherosclerotic aorta. Based on this finding, we assessed S-glutathiolation of SERCA by NO in atherosclerotic aorta. NO failed to S-glutathiolate and activate SERCA in atherosclerosis (Fig. 7). We hypothesized that oxidative stress in atherosclerosis modified Cys674 to interfere with S-glutathiolation. Redox state of Cys674 was detected with biotin iodoacetamide labeling, because nearly 90% of iodoacetamide preferentially binds the highly reactive Cys (Bishop et al., 1998). Biotin iodoacetamide labeling markedly decreased in atherosclerotic aorta, suggesting the oxidation of Cys674. High concentrations of $ONOO^-$ irreversibly oxidized Cys674 to sulfonylation *in vitro,* which might be produced in atherosclerosis. To assess the thiol modifications of SERCA further, we employed mass spectrometry for the detection of S-glutathiolated sites of SERCA by BK in porcine carotid arteries and sulfonylated sites of SERCA in atherosclerosis. BK S-glutathiolated multiple Cys on SERCA and sulfonylation of these Cys including Cys674 was detected in atherosclerosis (Fig. 8). Mass spectrometry did not produce quantitative data, and we did not know how much Cys674 was sulfonylated. On the basis of the data obtained using iodoacetamide labeling for the loss of reduced Cys674 in atherosclerosis, we concluded that the

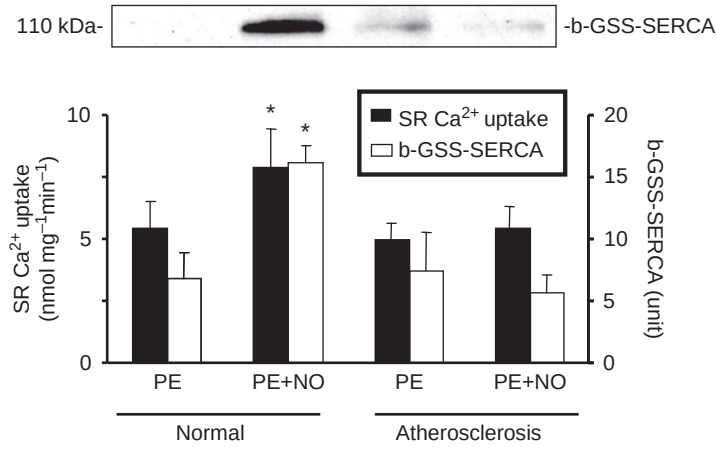

FIGURE 7 Atherosclerosis prevented S-glutathiolation and activation of SERCA by NO. NO increased S-glutathiolation of SERCA and TG-sensitive SERCA activity in normal aorta but failed to do so in atherosclerotic aorta. b-GSS-SERCA: biotin-labeled S-glutathiolated SERCA (Adachi et al., 2004, Permission from Nature Pub).

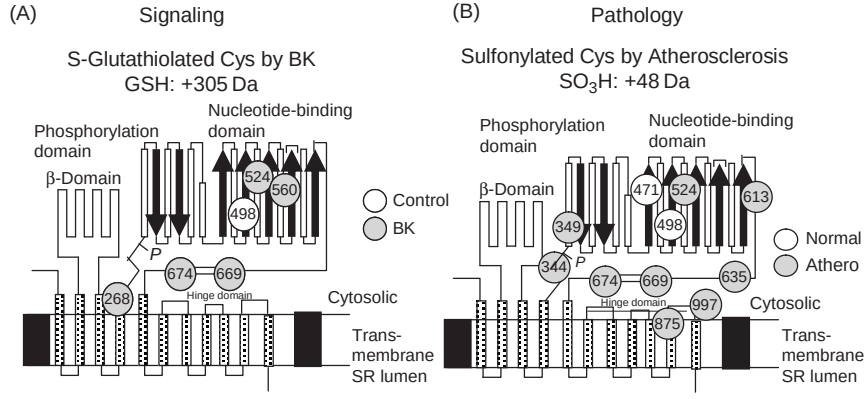

FIGURE 8 Summary of MALDI–TOF mass spectrometry analysis of Cys residues modified physiologically by NO and by atherosclerosis. (A) MALDI–TOF mass spectrometry analysis of GSS–Cys residues on SERCA purified from porcine carotid artery exposed to BK. NO released from endothelium with BK increased the number of GSS–Cys including Cys674, which is responsible for its activation. (B) Sulfonylated Cys (RSO_3H) detected on SERCA derived from rabbit aorta by MALDI–TOF mass spectrometry Atherosclerosis increased the number of RSO_3H including Cys674, suggesting the irreversible oxidation of reactive thiols in diseases, which can interfere with physiological S-glutathiolation to activate SERCA by NO (Adachi et al., 2004, Permission from Nature Pub).

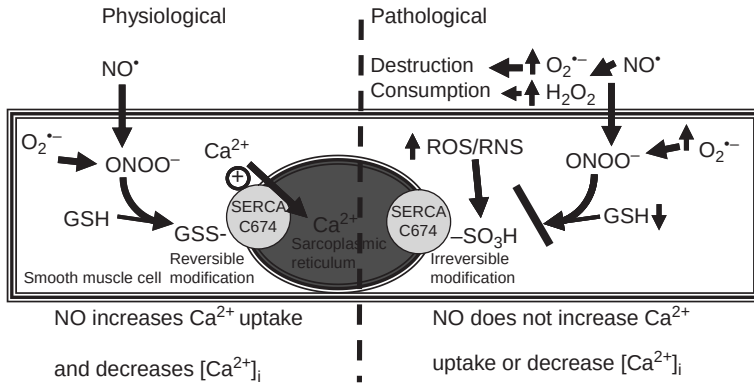

FIGURE 9 The proposed mechanism of cGMP-independent vasodilatation to NO and the influence of disease. In intact cells and arteries, NO forms *S*-glutathione adducts with SERCA. Physiological levels of NO likely react with superoxide anion ($O_2^{\bullet-}$) to form intracellular RNS, possibly $ONOO^-$, which S-glutathiolates Cys674 and increases SERCA activity to decrease $[Ca^{2+}]_i$ (*left*). In pathological states like atherosclerosis or diabetes, increased levels of extracellular superoxide anion scavenge and destroy NO. NO is also consumed by (myelo)peroxidases in the presence of hydrogen peroxide. Augmented intracellular ROS/RNS irreversibly oxidize thiols, such as by sulfonylation. This prevents thiols that are critical for physiological function (left), like Cys674, from reversibly reacting with GSH, thus preventing NO-induced relaxation in pathological states (*right*) (Adachi et al., 2004, Permission from Nature Pub).

irreversible oxidation of Cys674 in atherosclerosis prevented physiological S-glutathiolation and activation of SERCA (Adachi et al., 2004b; Cohen & Adachi, 2006) (Fig. 9).

This can explain many of our earlier doubts: (i) The activation of SERCA by S-glutathiolation plays a critical role in cGMP-independent relaxation. (ii) Atherosclerosis selectively decreases cGMP-independent relaxation to NO, because of irreversible oxidation at Cys674 that interferes with S-glutathiolation. (iii) A short period of incubation with antioxidants has no effects, because sulfonylation is irreversible. However, treatment with an antioxidant for 3 weeks prevented irreversible oxidation in proteins. The half-life of SERCA is about 13 days and BHT treatment for 3 weeks may prevent irreversible oxidation for proteins, until SERCA is *de novo* synthesized. Oxidized SERCA is susceptible to rapid degradation in atherosclerosis (Ying et al., 2008).

VI. Other Diseases Related with the Inactivation of SERCA

In this chapter, I will review the recent advances in the studies investigating the oxidation of SERCA in cardiovascular diseases.

A. Migration of Smooth Muscle Cells in High Glucose

S-glutathiolation of SERCA causes migration of smooth muscle cells, which is associated with restenosis or atherosclerosis. Migration of smooth muscle cells requires the elevation of $[Ca^{2+}]_i$ and NO inhibits migration by decreasing $[Ca^{2+}]_i$. Ying observed the migration distance in cultured smooth muscle cells or HEK293 cells expressing wild-type SERCA or C674S SERCA. S-nitrosopenicillamin (SNAP), which activates the cGMP-independent pathway, decreased $[Ca^{2+}]_i$ in cells expressing wild-type SERCA but not C674S SERCA. Strikingly, SNAP inhibited migration in cells expressing wild-type SERCA but not C674S SERCA. Inducible NO synthase (iNOS) were reported to inhibit restenosis in the case of balloon injury in rat carotid arteries (Yan et al., 1996). Induction of iNOS with interleukin-1β (IL-1β) also decreased migration of smooth muscle cells expressing wild-type SERCA but not C674S SERCA. These data indicate the potential role of iNOS for suppression of smooth muscle cell migration via S-glutathiolation of SERCA at Cys674.

High glucose induces oxidative stress in diabetes (Brownlee, 2001); oxidative thiol modifications of SERCA in atherosclerosis are also observed in smooth muscle exposed to high glucose. Aorta exposed to high glucose decreases ACh- and NO-induced relaxation. Using mass spectrometry, we observed that high glucose oxidized Cys674 on SERCA, which could account for reduced NO bioactivity in diabetes (Cohen & Tong, 2010).

Tong et al. cultured smooth muscle cells in high glucose and observed cell migration. To assess the irreversible thiol oxidation of SERCA, anti-sulfonyl SERCA antibodies were raised with peptides corresponding to Cys674-sulfonylated SERCA. Sulfonyl SERCA increased in smooth muscle cells exposed to high glucose. NO inhibited migration of smooth muscle cells in normal glucose, but failed to inhibit migration in smooth muscle cells exposed to high glucose. Thus, irreversible oxidation of Cys674 on SERCA in high glucose decreased the inhibitory effects of NO on smooth muscle migration. Indeed, diabetes augmented atherosclerosis and increased the incidences of restenosis in coronary angioplasty, and the irreversible oxidation of SERCA at Cys674 may be implicated (Tong et al., 2008). High glucose in diabetes may elevate ROS/RNS from mitochondria (Brownlee, 2001) or NADPH oxidases (Jay et al., 2006). NOX4 may be responsible for the oxidation of SERCA in high glucose (Tong & Schröder, 2009); however, the source of ROS/RNS for oxidation of SERCA in various vascular diseases is not clear.

B. Angiogenesis, Apoptosis, and ER Stress

NO from eNOS is essential for angiogenesis (Aicher et al., 2003). Unlike in the case of migration of smooth muscle cells, NO may promote migration

of endothelial cells for angiogenesis. One potential mechanism may be the activation of matrix metalloproteinase by S-glutathiolation, which is essential for angiogenesis (Okamoto et al., 2001). Further studies are required for the role of S-glutathiolation of proteins by NO in angiogenesis.

SERCA can regulate apoptosis and endoplasmic reticulum (ER) stress (Misquitta et al., 1999; Townsend et al., 2009b; Vangheluwe et al., 2005), and SERCA inhibitors induce apoptosis and ER stress. Apoptosis of smooth muscle cells markedly promotes atherosclerosis (Clarke et al., 2006). Interestingly, antiapoptotic protein bcl-2 interacts with SERCA in SR membrane (Dremina et al., 2004). TG also induces ER stress, which can contribute to vascular diseases in diabetes and atherosclerosis (Thorp et al., 2009). Because SERCA activity is impaired in the case of oxidative stress, ER stress may be induced in vascular diseases. The relationship between oxidative inactivation of SERCA and ER stress in cardiovascular diseases is not known yet.

C. Aggregation of Platelets

Aggregation of platelets is important for the pathogenesis of acute coronary syndrome, peripheral artery disease, or stroke. NO activates SERCA and decreases Ca^{2+} influx to inhibit platelet aggregation, which is at least partially mediated by a cGMP-independent process (Trepakova et al., 1999). Oxidative stress related with vascular diseases can promote aggregation of platelets by modulation of PI3 kinase and NO release from eNOS in platelets (Freedman, 2008). Oxidative stress in vascular diseases can impair SERCA in platelets, which increases $[Ca^{2+}]_i$ and promotes thrombosis.

NY-SERCA2 and an increase in $[Ca^{2+}]_i$ were detected in platelets from patients with diabetes (Randoriamboavonjy et al., 2007). The exposure of platelets to $ONOO^-$ also increases NY-SERCA. Interestingly, a treatment of peroxisome proliferator-activated receptor-gamma agonist, rosiglitazone, for patients with diabetes increased SERCA2 expression and decreased NY-SERCA. Oxidation of SERCA2 in platelets can be implicated with atherothrombosis in diabetes.

D. Cardiac Function and Heart Failure

SERCA plays a critical role in refilling Ca^{2+} into SR, which has an impact on cardiac $[Ca^{2+}]_i$ recycling, thereby modulating cardiac function; thus, reduced SERCA function leads to heart failure (Wehrens & Marks, 2004). SERCA is the primary candidate for gene therapy to treat heart failure. In rodents and sheep, gene transfer of SERCA markedly improves cardiac function (Byrne et al., 2008; Monte & Hajjar, 2005). However, the regulation of SERCA by ROS/RNS in heart is relatively poorly understood yet.

The heart expresses three types of NO synthase and various types of NADPH oxidases. Xanthine oxidoreductase activity in the heart and the mitochondrial electron transport chain are the potential sources of oxidative stress (Zimmet & Hare, 2006). Also, multiple sources for ROS/RNS may be implicated with pathogenesis of heart failure. Moreover, many proteins involved in excitation–contraction coupling have redox-sensitive thiols, including ryanodine receptors (Aracena et al., 2003), L-type Ca^{2+} channels (Campbell et al., 1996), Na^+–K^+ ATPase (Figtree et al., 2009), Na^+/Ca^{2+} exchanger (Kuster et al., 2010), actin (Wang et al., 2001), as well as SERCA. These thiols on multiple proteins may be susceptible to S-nitrosation, S-glutathiolation, as well as irreversible oxidation. Thus, physiological/pathophysiological release of ROS/RNS may regulate these ion channels, and subcellular localization of RNS may have a different role. eNOS is localized in plasma membrane especially on calveolae to modulate Ca^{2+} influx through L-type channels. Interestingly, neuronal NO synthase (nNOS) is present in SR to modulate Ca^{2+} recycling and S-glutathiolation of SERCA by nNOS may physiologically regulate cardiac function. Xanthine oxidoreductase may be colocalized with nNOS to modulate RNS in SR (Zimmet & Hare, 2006). Exogenous nitroxyl has positive inotropic and lusitropic effects, and the molecular mechanism involves activation of SERCA by S-glutathiolation at Cys674 (Lancel et al., 2009). NY-SERCA2 in myocytes increases in dilated cardiomyopathy in humans (Lokuta et al., 2005). Thus, physiological regulation of SERCA by S-glutathiolation and the impairment of SERCA function with irreversible oxidation may be important in the regulation of cardiac function. Ischemia/reperfusion induces S-thiolation in heart, which plays an important role in signaling for adaptation (Eaton, 2006). In diabetes model of rats, transcoronary gene transfer of SERCA increases coronary blood flow and reduces the size of myocyte (Sakata et al., 2007). Preservation of SERCA in both coronary arteries and cardiac myocytes may be a novel strategy for heart failure related with cardiac hypertrophy.

VII. Conclusion

Either exogenous or endothelial NO elicits arterial relaxation in both a cGMP-dependent and -independent manner, and cGMP-independent relaxation to NO is the primary target for vascular diseases such as atherosclerosis and diabetes. RNS derived from endogenous NO (possibly $ONOO^-$) S-glutathiolates SERCA at Cys674, resulting in the activation of SERCA, refilling Ca^{2+} into the SR store, inhibiting STOC, and decreasing $[Ca^{2+}]_i$, which is the primary mechanism for cGMP-independent relaxation. ROS/RNS, which are augmented in vascular diseases, nitrates tyrosine at Tyr296-Tyr297 and oxidizes multiple thiols on SERCA. Especially,

irreversible oxidation of Cys674 on SERCA (including sulfonylation) prevents physiological S-glutathiolation of SERCA. Pathological oxidation of SERCA may impair the physiological effects of NO including vasodilation, the suppression of migration, the inhibition of platelet aggregation, and the augmentation of cardiac function in heart. The mutation of Cys674 to serine mimicks the state of disease. This novel signaling pathway for NO and its impairment in disease states can be implicated with other cardiovascular diseases including restenosis, angiogenesis, atherothrombosis, and heart failure.

Further studies are required to resolve important questions. NO S-glutathiolates multiple proteins, which also play a role in cell signaling. For example, S-glutathiolation of Ras at Cys118 causes downstream signaling including mitogen-activated kinase and Akt (Adachi et al., 2004a; Clavreul et al., 2006a; Pimentel et al., 2006). The irreversible oxidation of these key proteins may be implicated in the pathogenesis of cardiovascular diseases. The role and regulation of other S-glutathiolated proteins in intracellular space have not been largely clarified.

The precise molecular mechanisms for the regulation of S-glutathiolation and dethiolation are still unclear. Although $ONOO^-$ chemically produces S-glutathiolation, the enzymatic regulation of S-glutathiolation is poorly understood. We cannot detect dynamic regulation of S-glutathiolation in the body using current techniques. Many kinds of posttranslational modifications can be induced in diseases and other posttranslational oxidative modifications related with augmented ROS/RNS levels may reveal the pathogenesis in various cardiovascular diseases.

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Abbreviations

ACh	acetylcholine
ARB/ACEI	angiotensin II type I receptor blocker/angiotensin-converting enzyme inhibitor

BHT	butylated hydroxytoluene
Bio-GEE	biotin–GSH ester
BK	bradykinin
BKCa channels	Ca^{2+}-activated potassium channels
C674S SERCA	SERCA mutated Cys674 to serine
$[Ca^{2+}]_i$	intracellular Ca^{2+} levels
cGMP	cyclic guanosine monophosphate
CPA	cyclopiazonic acid
DTT	dithiothreitol
EDHF	endothelium-derived hyperpolarization factor
EDRF	endothelium-derived relaxing factor
eNOS	endothelial NO synthase
ER	endoplasmic reticulum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRX	glutaredoxin
GSH	glutathione
GSSG	oxidized glutathione
IL-1β	interleukin-1β
iNOS	inducible NO synthase
LDL	low-density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal NO synthase
NO	nitric oxide
NY-SERCA2	tyrosine-nitrated SERCA 2
ODQ	1H-[1,2,4]-oxadiazole-[4,3-*a*]quinoxalin-1-one
ONOO	peroxynitrite
PE	phenyrephrine
PKG	protein kinase G
PTEN	phosphatase and tensin homologue
PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSNO	S-nitrosation
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase
sGC	guanylyl cyclase
SNAP	S-nitrosopenicillamin
SNP	sodium nitroprusside
SOD	superoxide dismutase
STOC	store-operated Ca^{2+} channel
TG	thapsigargin
TRX	thioredoxin

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Advances in the Renin Angiotensin System: Focus on Angiotensin-Converting Enzyme 2 and Angiotensin-(1–7)

Abstract

The contribution of the renin angiotensin system to physiology and pathology is undergoing a rapid reconsideration of its mechanisms from emerging new concepts implicating angiotensin-converting enzyme 2 and angiotensin-(1–7) as new elements negatively influencing the vasoconstrictor, trophic, and pro-inflammatory actions of angiotensin II. This component of the system acts to oppose the vasoconstrictor and proliferative

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effects on angiotensin II through signaling mechanisms mediated by the mas receptor. In addition, a reduced expression of the vasodepressor axis composed by angiotensin-converting enzyme 2 and angiotensin-(1–7) may contribute to the expression of essential hypertension, the remodeling of heart and renal function associated with this disease, and even the physiology of pregnancy and the development of eclampsia.

I. Introduction

Research on the regulatory actions of the renin angiotensin system continues to provide a wealth of information on how cells maintain their internal homeostatic environment, regulate metabolic processes, and adapt and contribute to disease. Not that long ago, the active product of the system—angiotensin II (Ang II)—was considered the sole hormone product of an endocrine system involved in regulating blood volume and vascular tone. The demonstration of renin and angiotensinogen in multiple tissues led to a revision of concepts and the suggestion for independent regulation of tissue and circulating renin angiotensin systems, with the former acting to modulate cell growth, cell differentiation, and intercellular communication. Today, a broader and complex system is revealed by advanced genetic and molecular tools, as well as the outcome of clinical studies using medications selective for one or more of the enzymes and proteins contributing to the generation of angiotensin peptides. The further discovery that the renin angiotensin system contains both a pressor and depressor arm in exerting regulatory functions on vascular tone and cellular signaling paved the way for the generation of alternate hypothesis as to how an unbalance of their

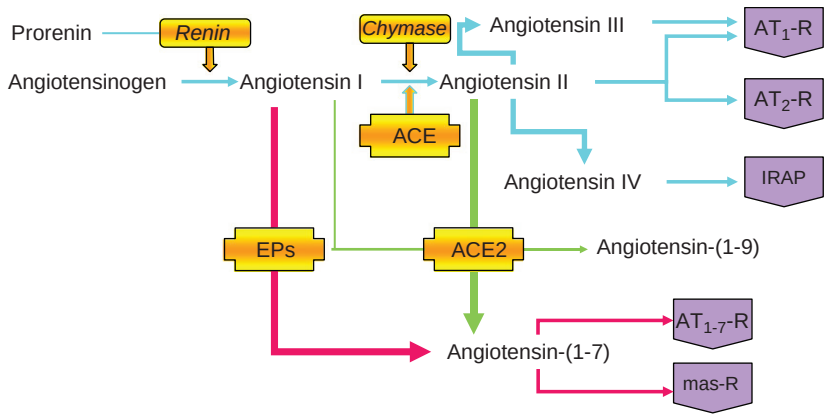

FIGURE 1 Schematic diagram of the key biochemical steps involved in the formation of angiotensin peptides.

function could contribute to cardiovascular disease. In this chapter, we elaborate on these discoveries. The general tenet of the chapter focuses on providing evidence that the axis composed by angiotensin-converting enzyme 2 (ACE2), the heptapeptide angiotensin-(1–7) [Ang-(1–7)], and the mas receptor (mas-R) function to oppose the activity of the pressor and proliferative axis composed by angiotensin-converting enzyme (ACE), Ang II, and its actions at the type 1 Ang II receptor (AT_1) (Fig. 1).

II. The Paradigm Shift

The period from approximately 1970 to 1980 may be viewed as the beginning of a shift in the concept of how the renin angiotensin system is involved in cardiovascular pathology. Renewed enthusiasm for its study resulted from the introduction of ACE inhibitors as the response to the administration of teprotide (Ondetti et al., 1977), a synthetic nonapeptide mimicking the substance contained in the venom of the *Bothrops Jararaca*, had a dramatic effect in reducing the blood pressure of hypertensive subjects (Gavras et al., 1978). Accelerated by the encouraging data regarding the antihypertensive actions of ACE inhibition, increased funding from the National Heart, Lung, and Blood Institute of the National Institutes of Health and pharmaceutical companies catapulted many laboratories to undertake newer approaches to study the physiology of Ang II, isolate its receptor, and undertake the eventual synthesis and introduction of first systemic (Brunner et al., 1976; Ondetti et al., 1977; Streeten et al., 1975a, 1975b) and then orally active Ang II receptor antagonists (Timmermans et al., 1993, 1995; Wong et al., 1990). In truth, the important work in the design, experimental, and clinical characterization of losartan, the first prototypical Ang II receptor blocker (ARB) built on the work that my mentor Irvine H. Page and his colleagues Merlin F. Bumpus, Robert Kharallaih, Robert Smeby, and Mahesh Khosla from the Research Division of the Cleveland Clinic Foundation (Cleveland, OH, USA) had done in the characterization of the biological effect on amino acid substitutions in the conformational structure of the Ang II molecule. Their meticulous efforts along these lines documented the critical importance of the eighth amino acid residue, the C-terminal phenylalanine in the natural peptide, for binding and expression of the pressor and secretagogue actions of Ang II in adrenal tissue (Bumpus, 1977; Bumpus & Khosla, 1975; Bumpus et al., 1973, 1984; Khairallah et al., 1970, 1978; Khosla et al., 1972, 1973, 1974, 1976, 1977; Masaki et al., 1977; Smeby et al., 1962; Yamamoto et al., 1972).

The progress made throughout these exciting periods of discovery, nevertheless, continued to posit Ang II as the sole biologically relevant product of the biochemical cascade initiated by the proteolytic action of

renin on hepatic angiotensinogen and the formation of Ang II through the cleavage of Ang I by ACE. Identification of alternate enzymes and Ang I metabolites, though demonstrated in several biological systems, was considered to have no major relevance in terms of biological function or their role in cardiovascular pathology. Although this is not the place for a discussion of these other components, it is worth mentioning the demonstration that tonin could cleave Ang I (Boucher et al., 1977; Cemassieux et al., 1976; Garcia et al., 1976, 1979; Schiffrin et al., 1981), that angiotensin-(1–9) could be produced from Ang I (Campbell et al., 1991, 1995; Donoghue et al., 2000), and that a synthetic tetradecapeptide substrate, angiotensin-(1–14) [Ang-(1–14)], was capable of forming Ang II *in vitro* (Skeggs, 1993; Skeggs et al., 1958).

III. The ACE2/Ang-(1–7)/Mas Axis

In 1998, the publication of the first description of the biological activity of the N-terminal-derived heptapeptide Ang-(1–7) would over time decisively alter the prevailing view. The initial demonstration that Ang-(1–7) stimulated the release of vasopressin from rat hypothalamic explants with a potency comparable to Ang II (Schiavone et al., 1988) suggested that more complex processes participated in the biochemical mechanisms accounting for angiotensin peptide formation and function. The heptapeptide Ang-(1–7) [Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-] constituted a truncated form of Ang II since it lacked phenylalanine in the eighth position. This truncation had important consequences in terms of accepting a biological activity for this peptide because structural work on previous amino acid substitutions of the Ang II molecule indicated the criticality of maintaining the C-terminus intact for both pressor and adrenal catecholamine release (Bumpus, 1977; Khosla et al., 1973, 1974). This interpretation was in keeping with Greene et al. (1982) previous conclusion that the absence of a vascular effect of Ang-(1–7) suggested that Ang-(1–7) was an inactive fragment of Ang II.

A. Hemodynamic and Neuro-Humoral Actions of Ang-(1–7)

Vasodilator effects of Ang-(1–7) were first observed in Sprague Dawley rats made areflexic by spinal cord transection (Benter et al., 1993). In this condition, the dose-dependent reduction in arterial pressure mediated by intravenous Ang-(1–7) infusions was abolished by administration of either the cyclooxygenase inhibitor, indomethacin, or the nonselective Ang II receptor antagonist [Sar^1-Thr^8]Ang II (Benter et al., 1993). A more prolonged vasodepressor effect of Ang-(1–7) was then demonstrated in spontaneously hypertensive rats (SHRs), in which the antihypertensive action of Ang-(1–7) at day 7 of its chronic administration was preceded by reduction

in plasma vasopressin concentration and increases in urinary prostaglandin E_2 and 6-keto prostaglandin F1 *alpha* (6-keto-PGF_1 α) excretion (Benter et al., 1995). The possibility that Ang-(1–7) depressor actions were in part modulated by the state of the baroreceptor reflexes was further confirmed in experiments in which the heptapeptide blunted the pressor actions of systemically injected Ang II or phenylephrine (Benter et al., 1995). These earlier observations were both confirmed and extended by additional studies showing the existence of immunoreactive Ang-(1–7) within brainstem pathways involved in the central control of blood pressure (Block et al., 1988) and evidence that the heptapeptide modulated the baroreceptor reflexes at postsynaptic nerve endings in the vagal-solitarii nuclei (Campagnole-Santos et al., 1990) of the medulla oblongata (Campagnole-Santos et al., 1989, 1990). Other studies showed that the facilitatory effects of Ang-(1–7) on central baroreceptor mechanisms may involve an action on substance P pathways and the blunting of the tonic action of the heptapeptide in the brain of older rats (Sakima et al., 2005) and the (mRen-2)27 transgenic hypertensive rat (Diz & Ferrario, 1996; Diz & Pirro, 1992; Diz et al., 1997, 2008). The latter observation gained support from studies showing that immunosuppression of brain Ang-(1–7) was associated with increased blood pressure in this model of transgenic hypertension (Moriguchi et al., 1995). Over the following decades, research would demonstrate that Ang-(1–7) contributes to the cardiorenal control of blood pressure via actions that within the heart, the kidney, and the blood vessels opposed the activity of Ang II (Ferrario, 2003, 2010; Ferrario et al., 1997, 2005).

B. Ang-(1–7) and the Heart

Ang-(1–7) suppresses the hypertrophic and profibrotic effects of Ang II in the heart (Ferreira et al., 2007b, 2010; Giani et al., 2010; Gomes et al., 2010; Grobe et al., 2006, 2007b; He et al., 2004; Iwata et al., 2005; Li et al., 2009; Mendes et al., 2005; Mercure et al., 2008; Neves et al., 1997; Santiago et al., 2010; Santos et al., 2004, 2006; Wang et al., 2005) and reduces cardiac arrhythmogenic activity (De Mello, 2004, 2009a, 2009b; De Mello et al., 2007) possibly through the release of endogenous prostaglandins (Ferreira et al., 2001).

Following the original observation of Santos et al. (1990) of the presence of increased coronary sinus levels of Ang-(1–7) after acute myocardial infarction, studies confirmed a local contribution of this hormone in the adaptation of the heart to ischemia and the progression of heart failure. Myocardial ischemia due to coronary artery ligation increases the content of Ang-(1–7) in myocytes surrounding the infarcted zone of the left ventricle (Averill et al., 2003) while administration of Ang-(1–7) attenuates the progression of cardiac fibrosis induced by chronic administration of isoproterenol in Wistar rats (Ferreira et al., 2007b) and in transgenic rats

expressing an Ang-(1–7)-producing fusion protein driven by the alpha-myosin heavy chain promoter (Ferreira et al., 2010). Additional studies in the isolated heart have confirmed a cardioprotective effect of Ang-(1–7) (Ferreira et al., 2001, 2002, 2010). While the bulk of studies indicate that the counter-regulatory effect of Ang-(1–7) on cardiac function is mediated through Ang-(1–7) expression (Ferrario, 2002, 2005; Ferrario et al., 2005a; Ferreira et al., 2001, 2002, 2007a, 2007b, 2010; Grobe et al., 2006, 2007b), a study by Wang et al. (2005) suggested that circulating Ang-(1–7) accounted for the attenuation of cardiac remodeling and fibrosis induced by aortic coarctation. Since this interpretation was based on assessing the cardioprotective actions of chronically infused Ang-(1–7) on the content of cardiac Ang II, their data do not negate the possibility of a local action of Ang-(1–7) through myocyte uptake or increased autocrine expression (Wang et al., 2005).

Emerging evidence also demonstrates an action of Ang-(1–7) in regulating cardiac chronotropic activity and a consequent antiarrhythmic function. De Mello (2004) first reported that Ang-(1–7) activates the sodium pump, hyperpolarizes the heart cell, and reestablishes the impulse conduction during ischemia/reperfusion in the isolated hamster's heart. Additional studies showed that at higher concentrations [10^{-7} M], the heptapeptide induced early afterdepolarizations probably through an action on AT_1 receptors (De Mello et al., 2007). This interpretation is in keeping with previous studies showing that Ang-(1–7) binds to AT_1 receptors when administered at supraphysiological concentrations (Clark et al., 2001).

Consistent with an action of Ang-(1–7) on attenuation of cardiac remodeling are studies demonstrating that the heptapeptide has beneficial effects on the progression of heart failure. Loot et al. (2002) showed that an 8-week administration of Ang-(1–7) to Sprague Dawley rats developing heart failure due to coronary artery ligation was associated with a 40% reduction in the elevated left ventricular (LV) end-diastolic pressure, increase in the rate of ventricular pressure incline (dp/dt) as well as restoration of endothelial function. Similar conclusions have been reported in other studies including those in which a nonpeptide analog of Ang-(1–7) (AVE-0991) was used both *in vitro* and *in vivo* (Ferreira et al., 2007a; Grobe et al., 2007b; Li et al., 2009; McMurray & Davie, 2002).

C. Ang-(1–7) Vascular Actions

Weak vasodilator actions of Ang-(1–7) given systemically contrasts with the more pronounced effects of the peptide when administered to isolated blood vessels (Almeida et al., 2000; Brosnihan et al., 1996, 1998; Fernandes et al., 2001; Gorelik et al., 1998; Kozlovski et al., 2007; Machado et al., 2002; Meng & Busija, 1993; Neves et al., 2004; Oliveira et al., 1999; Osei et al., 1993; Sampaio et al., 2007; Soares de et al., 2004;

Tirapelli et al., 2006), a finding that underscores a paracrine rather than a systemic effect of this angiotensin peptide. There is agreement that Ang-(1–7) vasodilator responses are in part related to release of bradykinin and endothelium-derived nitric oxide as well as a direct effect of Ang-(1–7) on the mas-R (Almeida et al., 2000; Brosnihan et al., 1996, 1998; Davie & McMurray, 1999; Fernandes et al., 2001; Gorelik et al., 1998; Heitsch et al., 2001; Kozlovski et al., 2007; Lemos et al., 2005; Li et al., 1997a; Li et al., 1997b: Marangoni et al., 2006; Oliveira et al., 1999, 2002, 2003; Sampaio et al., 2007; Soares de et al., 2004; Tirapelli et al., 2006). In the preconstricted mesenteric bed of SHR, Ognibene et al. (2009) reported that Ang II induces a type 2 Ang II (AT_2) receptor-mediated vasodilator effect via activation of Ang-(1–7) and bradykinin receptors. In addition, there is evidence that vasodilator prostaglandins may contribute as well in the effects of Ang-(1–7) on vascular tone (Oliveira et al., 1999, 2003).

Consistent with direct actions of Ang-(1–7) on vascular function are reports that Ang-(1–7) opposes the proatherogenic effects of Ang II (Dantas & Sandberg, 2005; Fraga-Silva et al., 2008; Heringer-Walther et al., 2009; Igase et al., 2005, 2008; Langeveld et al., 2005, 2008; Nie et al., 2009; Nishimura et al., 1999; Strawn et al., 1999, 2000, 2004) and acts as an anti-inflammatory factor in part via the inhibition of oxidative stress and the generation of radical oxygen species (Esteban et al., 2009; Gava et al., 2009; Heitsch et al., 2001; Polizio et al., 2007; Rajendran et al., 2005, 2007; Santos et al., 2010). Additional actions of Ang-(1–7) in the regulation of insulin resistance and the evolution of diabetes underscore a role for this peptide in antagonizing the actions of Ang II (Benter et al., 2007; 2008; Dharmani et al., 2007; Ebermann et al., 2008; Oliveira et al., 2002; Rastelli et al., 2007; Santos et al., 2010; Yousif et al., 2007).

D. Renal Actions of Ang-(1–7)

The functional importance of Ang II action on the kidneys in the long-term regulation of blood pressure has been shown through its critical involvement in maintaining body volume and electrolyte homeostasis. However, pathological activation of Ang II not only modifies renal hemodynamics and water and sodium balance but also imposes significant alteration in cell growth and proliferation resulting in hypertension and progression of renal disease. Ang-(1–7) counteracts the effects of Ang II on nephron function by mechanisms that are often not associated with changes in renal hemodynamics (Ferrario & Varagic, 2010).

As shown in the systemic circulation, the renal production of Ang-(1–7) from Ang I is influenced by prolyl oligopeptidase, neprilysin, and thimet oligopeptidase (Ferrario & Varagic, 2010). Activities of all three enzymes are documented in the kidney (Berti et al., 2009; Ebihara et al., 2003; Edwards et al., 1999; Kato et al., 1980; Li et al., 1996; Pendergrass et al.,

2008; Schulz et al., 1991; Shaltout et al., 2007). Velez et al. (2009) recently showed that Ang-(1–7) production from Ang I in fresh isolated glomeruli depends heavily on neprilysin activity. Interestingly, renal cortical neprilysin activity and protein expression were higher in female congenic (mRen-2)27 hypertensive rats when compared to their male counterparts (Pendergrass et al., 2008). Since both Ang-(1–7) and Ang II can be metabolized into Ang-(1–4) by neprilysin (Ferrario et al., 2002a), the observed lower renal Ang II but not Ang-(1–7) may be accounted for by higher neprilysin activity and, at least in part, explain the gender difference in blood pressure and proteinuria in this hypertensive strain.

The alternative pathway of Ang-(1–7) generation in the kidney includes degradation of Ang II by ACE2 which is highly expressed in the kidney (Chappell, 2010; Shaltout et al., 2009). Several reports showed that ACE and ACE2 colocalize on the apical surface of the proximal tubules while ACE2 is expressed in podocytes and parietal epithelial cells of the Bowman's capsule within the glomerulus (Li et al., 2005; Wysocki et al., 2006; Ye et al., 2006). Similarly, in renal arterioles, ACE2 expression was demonstrated in vascular smooth muscle cells while expression of ACE was confined in the vascular endothelium (Soler et al., 2009). While these reports clearly support the concept of the functionally active Ang-(1–7) machinery, they also underline the necessity for further studies aiming to functionally dissect the specific localization of the participating enzyme and corresponding substrates in structurally diverse nephron segments.

Ang-(1–7) exerts its renal action by acting on the G-protein-coupled receptor mas (Santos et al., 2003). The mas-R expression in afferent arterioles, glomerular mesangial cells, and apical surface of the tubular epithelium (Alenina et al., 2008; Su et al., 2006; Zhang et al., 2010) are consistent with a critical role of Ang-(1–7) in the regulation of renal hemodynamics, and tubuloglomerular structure and function. In isolated blood vessels including the renal afferent arterioles, Ang-(1–7) induced robust vasodilation (Ren et al., 2002) supporting the concept that Ang-(1–7) acts in an autocrine/paracrine fashion rather than exerting classical hormonal effects. Additionally, Ang II-induced renal vasoconstriction in isolated kidney was abolished by mas-R-mediated Ang-(1–7) effects in both Wistar Kyoto (WKY) and SHR (Dharmani et al., 2007). Chronic increases in circulating Ang-(1–7) in transgenic rats expressing an Ang-(1–7)-producing fusion protein did not affect blood pressure but profoundly decreased vascular resistance in many tissues including the kidneys (Botelho-Santos et al., 2007). Similarly, the increases in regional blood flow, including the renal vascular bed, in response to Ang-(1–7) infusion was partially abolished by the specific mas-R antagonist [D-Ala^7]-Ang-(1–7) (A-779) (Sampaio et al., 2003).

After the first report that Ang-(1–7) increased glomerular filtration rate and water and sodium excretion in isolated kidney (DelliPizzi et al., 1994), studies from our laboratory showed that the heptapeptide facilitated diuresis and natriuresis by inhibiting tubular Na^+/K^+-ATPase at doses that did not alter renal blood flow (Handa et al., 1996). At the time when the mas-R-mediated effects of Ang (1–7) were not yet known, Heller et al. (2000) reported that the increases in water and sodium excretion produced by intrarenal Ang-(1–7) infusion in dogs were mediated through a non-AT_1/AT_2 receptor. Subsequent studies revealed that Ang-(1–7) abolished the stimulatory effect of Ang II on the Na^+/K^+-ATPase activity in proximal tubule through the mas-R (Lara et al., 2002). These effects of Ang-(1–7) in the kidneys are linked to the release of cyclooxygenase products coupled with downregulation of AT_1 receptors (Clark et al., 2001; 2003), activation of phospholipase A (Andreatta-van et al., 1993), and increased phosphatidylcholine (Gironacci et al., 2002). There is some discrepancy regarding the receptors involved in mediating the actions of Ang-(1–7) in the kidney. In a distal tubule epithelial-derived cell line from Madin–Darby canine kidney (MDCK), Ang-(1–7) inhibitory action on Na^+/K^+-ATPase involved the activation of a protein kinase C signaling pathway via AT_1 receptors (Lara et al., 2005). By contrast, inhibitory effects of both Ang II and Ang-(1–7) on Na^+/K^+-ATPase of inner cortex from pig kidney were completely reversed by AT_2 but not by either AT_1 or mas-R antagonists (De Souza et al., 2004). The same group has also reported an antidiuretic action of Ang-(1–7) in water-loaded rats which is abrogated by blockade of AT_1 receptors or genetic deletion of the mas-R (Baracho et al., 1998; Santos et al., 1996, 2003; Simoes e Silva et al., 1998). Consistently, A-779, a selective mas-R antagonist, blocked the antidiuretic effects of Ang-(1–7) mimetic AVE 0991 in water-loaded mice (Pinheiro et al., 2004). Although the foregoing studies demonstrate a modulatory role of Ang-(1–7) in the regulation of both proximal and distal tubular function, continuing research efforts are warranted to further clarify the role of Ang-(1–7) signaling mechanisms in water and electrolyte transport along the different parts of the nephron. In addition, little regard has been taken in evaluating whether species differences influenced the nature of the receptors involved in Ang-(1–7) renal function. As suggested by Ferrario & Varagic (2010), the interaction between the angiotensins and other autocoid and their receptors in terms of allosteric regulation or heterodimerization cannot be ruled out and should be considered when specific effects of different components of the renin angiotensin system are evaluated.

As in other tissues, Ang-(1–7) exerts antigrowth activity in proximal tubular cells inhibiting Ang II-induced phosphorylation of mitogen-activated protein kinases (MAPK) [p38, extracellular signal-related kinase, (ERK1/2), and *c*-Jun N-terminal kinase (JNK)] and Ang II stimulation of transforming growth factor β_1 (TGF-β_1) (Su et al., 2006). Consistently, in

genetically modified mice lacking the mas-R, tubulointerstitial and glomerular fibrosis as well as renal dysfunction were associated with upregulation of AT_1 receptors and TGF-β1 mRNA. Interestingly, a MAPK phosphatase may mediate Ang-(1–7) antiproliferative actions in vascular smooth muscle cells and cardiac fibroblasts (Tallant et al., 2006 and Tallant and Gallagher, unpublished observations).

E. Renoprotective Effects of Ang-(1–7)

A large body of experimental and clinical evidence supports the concept that the Ang-(1–7) axis counterbalances the renal effects of Ang II. Similar to the effects in the heart and aorta (Igase et al., 2005, 2008; Ishiyama et al., 2004), ACE2 gene expression in kidney arterioles increased in response to blockade of AT_1 receptors (Soler et al., 2009). In accord with these studies, a differential regulation of ACE and ACE2 by Ang II has been observed in hypertensive renal disease (Koka et al., 2008). Upregulation of renal ACE mRNA accompanied by activation of ERK1/2 and p38 MAPK was associated with downregulation of renal ACE2 mRNA in hypertensive patients (Koka et al., 2008). Moreover, inhibition of endogenous ACE2 accelerated hypertension and decreased renal hemodynamics in two-kidney one-clip (2K1C) hypertensive rats by further increasing kidney content of Ang II while averting the protective increase in Ang-(1–7) (Burgelova et al., 2009). A protective role of Ang-(1–7) axis has also been suggested in other forms of nonhypertensive renal disease such as in renal failure (Simoes e Silva et al., 2006; Velkoska et al., 2010), nonhypertensive glomerulopathy (Lely et al., 2004), chemically induced glomerulonephritis (Zhang et al., 2010), renal fibrosis resulting from mas-R genetic deletion (Pinheiro et al., 2009), and diabetic nephropathy (Benter et al., 2007, 2008; Konoshita et al., 2006; Mizuiri et al., 2008; Oliveira et al., 2002; Reich et al., 2008; Soler et al., 2008; Tikellis et al., 2003, 2008; Wang et al., 2008a, 2009; Zhang et al., 2008; Wysocki et al., 2008, 2010).

The existence of Ang-(1–7) as a functional nephron component is buttressed by prior observations of reduced urinary content of Ang-(1–7) in untreated hypertensive subjects (Ferrario et al., 1998) or hypertensive rats (Yamada et al., 1999), and a significant increase in urinary excretion rates of Ang-(1–7) in response to chronic administration of captopril (Luque et al., 1996) or the dual peptidase inhibitor omapatrilat (Ferrario et al., 2002a, 2002b). These prior studies are reinforced by the observation of a strong positive correlation between proteinuria and urinary ACE2 expression in diabetic patients (Wang et al., 2008a).

F. Ang-(1–7) and the Mas Receptor

Prior studies (Brosnihan, 1998; Brosnihan et al., 1996, 1998; Diz & Pirro, 1992; Iyer et al., 1998; Jaiswal et al., 1992) showing that Ang-(1–7)

actions were not abrogated by blockade of either AT_1 or AT_2 receptors culminated with the identification of the mas-R as the protein responsible for its physiological effects (Santos et al., 2003). Engagement of the mas-R as a site for Ang-(1–7) binding and actions has been confirmed to mediate attenuation of [^{3}H]leucine incorporation in neonatal cardiac myocytes while transfection of cultured myocytes with an antisense oligonucleotide to the mas-R blocked the Ang-(1–7)-mediated inhibition of serum-stimulated MAPK activation (Tallant et al., 2005). Findings involving the application of A-779 to evaluate Ang-(1–7) actions appear to confirm the involvement of the mas-R (Santos et al., 1994). Using this antagonist, studies have reported that A-779 prevents the inhibitory actions of Ang-(1–7) on nerve stimulation-mediated release of norepinephrine and neuropeptide Y (Byku et al., 2010), myocardial contractility (Castro-Chaves et al., 2009), and stimulation of cardiac Akt phosphorylation and Ang II-stimulated ERK1/2 and Rho kinase phosphorylation (Giani et al., 2008). The use of mice with genetic deletion of the mas-R has affirmed conclusions derived from pharmacological blockade of Ang-(1–7) (Santos et al., 2003, 2006). Further evidence for a role of the mas-R in mediating Ang-(1–7) actions has been derived from the observation of hypertension in mas-deficient mice backcrossed to the inbred mouse strain FVB/N genetic background (Alenina et al., 2008), the disappearance of Ang-(1–7) reduction in coronary perfusion pressure in mas knockout mice (Castro et al., 2005), and the suppression of Ang-(1–7)-mediated release of nitric oxide from cardiomyocytes isolated from mas-deficient mice (Dias-Peixoto et al., 2008) or Chinese hamster ovary (CHO)-mas cells (Sampaio et al., 2007). Transfection of COS or Human Embryonic Kidney 293 (HEK293) cells with an assortment of mas-related gene (Mrg) receptors shows that of the six studied receptors, MrgD and MRG initiated significant arachidonic acid release after stimulation with Ang-(1–7), a finding that implicates other Mrg's in mediating the actions of Ang-(1–7) (Gembardt et al., 2008).

IV. Angiotensin-Converting Enzyme 2 as a Component of the Ang-(1–7)/Mas Axis

The pace of research on the counter lever role of Ang-(1–7) on Ang II was expanded with the identification of an ACE homologue, ACE2, that cleaves Ang II into Ang-(1–7) (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 cloned from a human lymphoma (Tipnis et al., 2000) and the heart of a heart failure patient (Donoghue et al., 2000) was found to be a close homologue of the ACE cDNA library. Despite a close similarity between ACE and ACE2, the two enzymes function in a completely different way. ACE functions as a dipeptidyl carboxypeptidase, whereas ACE2 functions

as a mono carboxypeptidase. In addition, ACE2 activity is not inhibitable by the administration of ACE inhibitors.

A. ACE2 Enzyme Kinetic and Protein Structure

Shortly, after the discovery of ACE2, Vickers et al. (2002) showed that ACE2 cleaved Ang II into Ang-(1–7) with high affinity (catalytic efficiency of ACE2 for Ang II, $k_{cat}/K_m = 1.9 \times 10^6\ M^{-1}\ s^{-1}$). The ACE2 catalytic efficiency is 400-fold higher for Ang II as compared to Ang I. In addition, ACE2 competes with ACE for Ang I, forming Ang-(1–9) with a lower turnover number ($k_{cat} = 0.035\ s^{-1}$). ACE2 also cleaves apelin-13 and dynorphin A 1-13 substrate with high catalytic efficiency (K_m value is <10 μM, and k_{cat}/K_m value is $>1 \times 10^6\ M^{-1}\ s^{-1}$). In addition, ACE2 cleaves des-Arg^9-bradykinin peptides, β-casomorphin, and neurotensin 1-8 with substantial catalytic efficiency ($k_{cat}/K_m \geq 1 \times 10^5\ M^{-1}\ s^{-1}$) (Vickers et al., 2002). The optimum proteolytic activity of ACE2 is pH 6.5 and this enzyme is almost inactive at pH 5.0. However, ACE2 maintains substantial catalytic activity under basic condition (pH 7.0–9.0). ACE2 proteolytic activity is greatly enhanced by high chloride or fluoride ions while changes in bromide ion concentrations have no effect. Vickers et al. (2002) also noted that ACE2 catalytic activity is optimal in the presence of 1.0 M NaCl and is stable for more than 6 h at room temperature in assay buffer. Additional studies by Rice et al. (2004) confirmed the high catalytic efficiency of ACE2 for Ang II ($k_{cat}/K_m = 2.2 \times 10^6\ M^{-1}\ s^{-1}$).

Detailed information about ACE2 protein crystal structure and the schematic view of active site was reported by Guy et al. (2003), Natesh et al. (2003), and Towler et al. (2004). Unlike ACE which has two catalytic sites, ACE2 has one catalytic domain. ACE2 shares 42% sequence identity in the catalytic regions and 61% sequence similarity with ACE (Tipnis et al., 2000). In contrast to ACE, ACE2 contains a single His-Glu-Xaa-Xaa-His (HEXXH) zinc active-site motif. The gene encoding human ACE has been localized to the chromosome 17q23 and is encoded by a 21 kb gene that consists of 26 exons and 25 introns (Rigat et al., 1990). The human ACE2 gene, on the other hand, is located on the X chromosome (Tipnis et al., 2000). Analysis of the DNA sequence of mouse ACE2 shows 83% identity with human ACE2 and the gene is also mapped to chromosome X 70.5 cM (Komatsu et al., 2002).

B. Tissue Distribution of ACE2

ACE2 is widely distributed in many tissues with higher levels in the heart, kidney, and testis (Batlle et al., 2006, 2008; Ferrario & Varagic, 2010; Ferrario et al., 2005a, 2005b; Gembardt et al., 2005; Hamming et al., 2004, 2008; Ohtsuki et al., 2010; Oudit et al., 2006). Our studies

showed that ACE2-dependent Ang-(1–7) formation from Ang II was significantly higher from the heart of hypertensive rats as compared to normal rat heart (Trask et al., 2007). ACE2 is a cell surface protein distributed evenly to detergent-soluble regions of the plasma membrane in CHO cells. However, in polarized MDCK cells under steady-state conditions, ACE2 is localized predominantly to the apical surface (92%) where it is proteolytically cleaved within its ectodomain to release a soluble form (Warner et al., 2005). ACE2 protein was also detected in membrane preparation from whole testis and Leydig cells from adult rats (Douglas et al., 2004). In this study, they also reported the cloning and sequencing of ACE2 from a human testis and noted that the cDNA sequence was identical to that reported for ACE2 from cardiac tissue.

C. Role of ACE2 in Cardiovascular Function

Crackower et al. (2002) demonstration that deletion of the ACE2 gene in mice resulted in major defects in cardiac contractility and upregulation of hypoxia-inducible genes awakened the interest of investigators to consider ACE2 and Ang-(1–7) as components of the renin angiotensin system involved in cardiovascular function. Studies in transgenic Ren-2 hypertensive rats from our group revealed an ACE2-dependent cardiac generation of Ang-(1–7) from Ang II as well as increased collagen deposition following chronic ACE2 inhibition (Trask et al., 2007, 2010). Other studies involving genetic knockout mice models also showed the *in vivo* importance of ACE2 in cardiac hypertrophy and fibrosis (Gurley & Coffman, 2008; Huentelman et al., 2005; Yamamoto et al., 2006). A recent study from our laboratory showed that a 28-day intravenous infusion of the specific ACE2 inhibitor, MLN-4760, resulted in increased cardiac Ang II levels, worsening of cardiac hypertrophy, and augmented content of cardiac interstitial collagen fraction in (mRen-2)27 transgenic rats (Trask et al., 2010).

Der Sarkissian et al. (2008) showed that cardiac overexpression of ACE2 exerts protective influence on the heart during myocardial infarction by preserving cardiac function, LV wall motion, and contractility, and by attenuating LV wall thinning. Earlier, a study from Huentelman et al. (2005) found that ACE2 overexpression induced by intracardiac injection of a lentiviral vector encoding the mouse ACE2 attenuated the development of Ang II-induced cardiac hypertrophy and fibrosis. Additional studies agree with the conclusion that ACE2 counteracts cardiac remodeling induced by myocardial injury, renal hypertension, and heart failure, as well as retarding the progression of atherosclerosis and vascular injury (Burgelova et al., 2009; Burrell et al., 2005; Crackower et al., 2002; Diez-Freire et al., 2006; Dong et al., 2008; Epelman et al., 2008; Ferrario, 2005; Ferrario et al., 2005a; Goulter et al., 2004; Grobe et al., 2007a, 2007b; Hamming et al., 2008; Heeneman et al., 2007; Hu et al., 2007; Igase et al., 2005, 2008; Ji

et al., 2008; Kaiqiang et al., 2009; Kassiri et al., 2009; Lovren et al., 2008; Ohtsuki et al., 2010; Pan et al., 2007; Sluimer et al., 2008; Trask et al., 2010; Varagic et al., 2010; Velkoska et al., 2010; Yamamoto et al., 2006; Zulli et al., 2006). These findings demonstrate that ACE2 plays a central role in balancing the pressor and proliferative activity of the ACE/Ang II/AT_1 receptor axis.

The role of ACE2 in the development of human heart failure is not fully understood. Ohtsuki et al. (2010) found that the upregulation of the ACE2 gene in the myocardium of the LV of patients with severe heart failure correlated with the degree of LV dilatation. These data are in keeping with the observation that the hypertensive effects of infused Ang II in mice can be prevented by coinfusion of human recombinant ACE2 (hrACE2) enzyme in association with increased formation of Ang-(1–7) from Ang II (Wysocki et al., 2010). Since blunting effect of hrACE2 on blood pressure was unaffected by the specific Ang-(1–7) receptor blocker A-779, the mechanism of hrACE2 action may result from the combination of increased systemic ACE2 activity and reduction in plasma Ang II. Further validation of the results obtained in these experiments (Wysocki et al., 2010) may lead to the design of therapeutic approaches geared to increasing ACE2 activity in states of Ang II overactivity. Since the loss of ACE2 facilitates adverse postmyocardial infarction ventricular remodeling by potentiation of Ang II effects on AT_1 receptors, supplementation of ACE2 may be a potential therapy for ischemic heart disease (Kassiri et al., 2009). The studies reported by Wysocki et al. (2010) and Kassiri et al. (2009) provide strong evidence for a direct role of ACE2 in modulation of adverse cardiac remodeling and tissue expression of Ang-(1–7).

Several studies suggest that Ang II peptide play a significant role in regulation of ACE2 expression, but molecular mechanisms of ACE2 regulation in cardiovascular system are poorly understood. Signaling pathways studies show that the downregulation of ACE2 by Ang II or endothelin-1 was blocked by mitogen-activated protein extracellular kinase (MEK) inhibitors to reduce ERK activities, suggesting that both agonists downregulate ACE2 through activation of the MAPK (ERK1 and/or ERK2) pathways (Koka et al., 2008). The influence that Ang II exerts on the expression and activity of ACE2 requires further study since a recent report in human cardiac fibroblast showed that both Ang II and Ang-(1–7) upregulate ACE2 expression via AT_1 and mas-R-mediated actions, respectively (Lin et al., 2010). This finding is not consistent with observations of increased ACE2 mRNA and activity induced by AT_1 receptor blockade *in vivo* (Agata et al., 2006; Ferrario et al., 2005a, 2005b; Igase et al., 2005, 2008; Ishiyama et al., 2004; Jessup et al., 2006) and *in vitro* (Gallagher & Tallant, 2004; Gallagher et al., 2006, 2008a, 2008b), and the demonstration that Ang-(1–7) activates a MAPK phosphatase to counteract the inhibitory effect of Ang II on ACE2 (Gallagher et al., 2008a).

The clinical translation for a role of ACE2 in human disease is buttressed by the report of Calo et al. (2010) who showed increased ACE2 expression and Ang-(1–7) levels in mononuclear cells obtained from Bartter's/Gitelman's patients. In addition, ACE2-mediated Ang II degrading activity is significantly increased in prehypertensive patients (Keidar et al., 2007). Completion of the Human Genome Project has opened a new window to investigate genetic means for the treatment of hypertension through overexpression of ACE2 gene (Ferreira & Raizada, 2008; Katovich et al., 2005). Polymorphisms of the ACE2 gene have been associated with essential hypertension (Fan et al., 2007; Huang et al., 2006; Liu et al., 2005; Mo et al., 2010; Niu et al., 2007; Yi et al., 2006), LV hypertrophy, coronary artery disease, and myocardial infarction (Lieb et al., 2006; Palmer et al., 2008; Sotoodehnia et al., 2009; Wang et al., 2008b; Yan et al., 2008; Yang et al., 2006).

D. Role of ACE2 in Renal Axis

ACE2, expressed at high levels in the kidney, has been demonstrated as renoprotective in mouse model of chronic kidney disease (Dilauro et al., 2010). In 5/6 nephrectomized mice, administration of an ACE2 inhibitor worsened proteinuria in association with increased renal content of Ang II. While AT_1 receptor blockade reversed proteinuria, a similar effect could not be obtained by Ang-(1–7) administration (Dilauro et al., 2010). These data suggest that some of the effects induced by ACE2 inhibition may not be expressed to increased renal expression of Ang-(1–7). Dilauro et al.'s (2010) interpretation of these findings agree with the report that administration of hrACE2 blunts the hemodynamic effects of acute increases in Ang II by a mechanism that is independent of circulating Ang-(1–7) (Wysocki et al., 2010). Likewise, the role of ACE2 in the development of diabetic nephropathy remains unclear. A recent study on a streptozotocin-induced diabetic ACE2-knockout mice (ACE2-KO) model shows more severe progression of albuminuria, as well as a severe time-dependent increase in glomerular/tubulointerstitial damage in diabetic ACE2-KO mice compared to wild-type mice (C57BL/6) (Shiota et al., 2010). The renal-protective effect of ACE2 might involve more than just suppressing Ang II-mediated AT_1 receptor signaling because Senador et al. (2010) found that short-term AT_1 blockade treatment in C57BL/6 mice (fed with a 60% fructose diet for 8 weeks in combination with losartan treatment with 30 mg/kg / day on week 9) reversed the pressor effect in a high fructose diet group, demonstrating that there are prominent interactions between a dietary regimen that produces glucose intolerance and an antihypertensive drug that antagonizes Ang II signaling. These findings suggest that the mechanism of change may be via renal Ang II rather than the ACE2/Ang-(1–7) pathway because the fructose losartan combination resulted in lowered renal Ang II content without changes in Ang-(1–7) level (Senador et al., 2010).

Protection against oxidative stress and cell damage through the ACE2/Ang-(1–7) pathway has also been reported within the nucleus in the kidneys. Studies showed that the ACE2 inhibitor (MLN-4760) reduced the exacerbation of Ang II-dependent formation of reactive oxygen species in renal nuclei isolated from older sheep (Gwathmey et al., 2010). These changes were associated with abolition of Ang-(1–7) formation from Ang II (Gwathmey et al., 2010). A protective role of hrACE2 to slow the progression of kidney injury has also been reported in diabetic Akita mice [Ins2 (WT/C96Y)] (Oudit et al., 2010). Treatment with hrACE2 increases the Ang-(1–7) levels, lowers Ang II levels, and reduces NADPH oxidase activity in diabetic Akita mice [Ins2(WT/C96Y)] as compared to control C57BL/6J mice [(WT/WT)] (Oudit et al., 2010). Furthermore, an *in vitro* study also reported that hrACE2 attenuated both high glucose and Ang II-induced oxidative stress and NADPH oxidase activity which suggest that the protective effect of hrACE2 is due to reduction in Ang II and increases in Ang-(1–7) signaling. The changes in renin angiotensin system components appear to be tissue specific and possibly related to enhancement of fatty acid synthesis, epididymal adipose tissue mass, and hypertension. Coelho et al. (2010) found that the tissue level Aogen, ACE, AT_1, and AT_2 genes as well as protein expression were unaltered in sucrose-fed rats while Ang II, Ang-(1–7), and ACE activity increased in their kidneys.

Although the expression of ACE2 in the diabetic kidney has been well studied (Mizuiri et al., 2008; Moon et al., 2008; Tikellis et al., 2003, 2008; Wysocki et al., 2006), little is known about the ACE2 expression in non-diabetic renal disease. Recently, a study conducted in acute kidney injury induced by subtotal nephrectomy showed a marked reduction in cortical and medullary ACE2 activity compared with control rats (Velkoska et al., 2010). ACE inhibition with ramipril produced beneficial effects in association with increased renal ACE2 activity (Velkoska et al., 2010). Renal biopsies obtained from both control and renal diseased patients showed that ACE2 is present in human kidney (Lely et al., 2004). In control kidneys, the immunohistochemical staining for ACE2 was localized in tubular and glomerular epithelium. However, in diseased patients (all primary and secondary renal diseases, and renal transplants) neo-expression of ACE2 was found to be restricted to the glomerular and peritubular capillary endothelium (Lely et al., 2004).

V. The Ang-(1–7)/ACE2/Mas-R Axis in the Regulation of Pregnancy

Normal pregnancy is a physiological condition characterized by decreased total peripheral resistance, decreased or normal blood pressure (August et al., 1990), and an increased renin angiotensin system activity

(Nasjletti & Masson, 1972; Oelkers, 1996). Within the first few weeks of pregnancy, a rise in plasma Ang II levels is accompanied by increases in angiotensinogen, plasma renin activity (PRA), urinary and plasma aldosterone, and downregulation of AT_1 receptors (Baker et al., 1990). Pregnant women possess decreased vascular responsiveness to Ang II (Gilbert et al., 2008; Rosenfeld, 2001; Shah, 2005) which is associated with downregulation of the AT_1 receptor (Baker et al., 1990, 1992). New data suggest that increased estrogen and Ang-(1–7) levels compensate for the increased renin angiotensin system in pregnancy (Li et al., 1997). When nulliparous third trimester normal pregnant patients were compared to nonpregnant subjects, plasma Ang-(1–7) levels were significantly elevated in normal pregnant women as compared to nonpregnant women (Merrill et al., 2002). According to Merrill et al. (2002), plasma Ang-(1–7) and Ang II levels were 34 and 50% higher, respectively, in normal pregnant women as compared to nonpregnant subjects. Nogueira et al. (2007) confirmed the increase in plasma Ang I and Ang-(1–7) levels in a small sample of women with pregnancy and further reported a blunting of the Ang-(1–7) increase in pregnant women with gestational diabetes. Involvement of Ang-(1–7) in the evolution of normal pregnancy has been demonstrated by Valdes et al. (2001), who described a progressive increase in Ang-(1–7) urinary excretion throughout the gestational period to levels that were 10-fold higher than those reported during the menstrual cycle. In agreement with these observations, 24 h urinary excretion of Ang I, Ang II, and Ang-(1–7) was increased by 93, 44, and 60%, respectively, as compared to virgin animals in the diestrus phase of the estrus cycle of pregnant rats (Neves et al., 2003). The increase in urinary Ang-(1–7) levels may reflect local synthesis of the heptapeptide as kidney Ang-(1–7) and ACE2 immunostaining were enhanced in the inner cortex/outer medulla proximal and distal tubules throughout pregnancy in Sprague Dawley rats (Joyner et al., 2007). These results suggested that Ang-(1–7) through its associated enzyme ACE2 may function in blood pressure and/or hydro mineral balance during pregnancy. Additional studies involving blockade of Ang-(1–7) concluded that the diuresis seen during late gestation in normal pregnancy can be mechanistically regulated by Ang-(1–7) through increased water intake, decreased plasma arginine vasopressin (AVP), and downregulation of kidney aquaporin 1 (Joyner et al., 2008). Recent studies have shown wide distribution and generally colocalization of Ang-(1–7) and ACE2 throughout the human and rat uteroplacental unit during gestation (Joyner et al., 2008; Valdes et al., 2006). In the pregnant human uterus, Ang-(1–7) and ACE2 were found in the invading trophoblasts and in trophoblasts cells lining the uterine spiral arteries (Anton et al., 2009). While the uterine concentration of Ang I and Ang-(1–7) and ACE2 mRNA did not change with pregnancy, the concentration of Ang II was lower in the human uterus during pregnancy as compared to nonpregnant subjects (Anton et al., 2009). The relative gene expression of AT_1 receptor, AT_2 receptor, and mas-R

was decreased in the uterus during normal pregnancy as compared to the uterus of nonpregnant subjects (Anton et al., 2009).

Temporal–spatial studies in the rat uterus during early and late gestation suggest that Ang-(1–7) and ACE2 may play an important role in implantation (Neves et al., 2008). During early pregnancy, Ang-(1–7) and ACE2 immunostaining was present in the implantation and interimplantation sites (decidua, luminal, and glandular epithelium, embryo, and ectoplacental cone) (Neves et al., 2003), whereas during late gestation, Ang-(1–7) and ACE2 were found on epithelial cells of the yolk sac and amnion.

The main sites of human placental Ang-(1–7) expression were in the syncytiotrophoblasts, cytotrophoblasts, blood vessel endothelium, and vascular smooth muscle of the primary and secondary villi (Valdes et al., 2006). Ang-(1–7) was also expressed in the maternal stroma in extravillous cytotrophoblasts, intravascular cytotrophoblasts, and decidual cells. ACE2 was expressed mainly in the syncytiotrophoblasts, cytotrophoblasts, villous blood vessel endothelium, and vascular smooth muscle cells of the primary villi while in the maternal stroma, ACE2 was expressed in the invading and intravascular trophoblast and the decidual cells (Valdes et al., 2006). The coincident location of Ang-(1–7) and ACE2 suggests an autocrine function of Ang-(1–7). Additionally, Ang-(1–7) may integrate with other vasodilators including nitric oxide, bradykinin, and prostaglandins to produce an autocrine/paracrine role in endometrial receptivity; trophoblast invasion; regulation of fetal, placental, and uterine perfusion; and the prevention of platelet aggregation in the intervillous space. Ang-(1–7) in the placenta may regulate vascular growth since Ang-(1–7) was shown to have antiangiogenic effects on human umbilical vein endothelial cells through a unique AT_{1-7} receptor that was sensitive to losartan (Anton et al., 2007).

The regulatory role of the Ang-(1–7)/ACE2/mas-R axis in blood pressure regulation in the pregnant state is further strengthened by reports of reduced plasma Ang-(1–7) levels in preeclamptic women (Velloso et al., 2007) and nulliparous preeclamptic third trimester patients matched for parity, race, and gestational age as compared to third trimester normal pregnant patients (Merrill et al., 2002). Additionally, Merrill et al. (2002) observed that there was a negative correlation between plasma Ang-(1–7) and both systolic and diastolic blood pressures, suggesting a potential contribution of reduced Ang-(1–7) on elevated blood pressure in preeclampsia.

VI. Conclusion

As reviewed elsewhere (Ferrario, 2010; Ferrario & Varagic, 2010), even from this limited overview of the intricate internal mechanisms regulating the pathways determining the production of angiotensin peptides, it is obvious that the renin angiotensin system is embodied with a great capacity

to use alternate mechanisms in bypassing blockade of primary pathways. In unraveling the complexity of the biochemical physiology of the system, it is also apparent that formation of angiotensin peptides within the cellular environment may not follow what has been characterized in the circulation or the extracellular compartment. The intertwined relationship between the ACE/Ang II/AT_1 receptor axis and its counterlever ACE2/Ang-(1–7)/mas-R axis is now buttressed on a firm literature and its clinical impact is beginning to be fully appreciated in terms of the possibility that many cases of essential hypertension, particularly those associated with low renin, may be explained by a genetic or acquired deficiency of ACE2 or Ang-(1–7) activity. Research in this field is extending knowledge of disease processes outside the cardiovascular system as altered function of the ACE2/Ang-(1–7)/mas-R axis is being implicated in the pathology of cancer (Altundag et al., 2006; Menon et al., 2007; Rodgers et al., 2002, 2003, 2006; Soto-Pantoja et al., 2009; Zhou et al., 2009), liver disease (Alfany-Fernandez et al., 2009; Carvalho et al., 2007; Herath et al., 2007, 2009; Lubel et al., 2008, 2009; Pereira et al., 2007), blood disorders (Ellefson et al., 2004; Heringer-Walther et al., 2009; Kucharewicz et al., 2002), and autoimmune diseases (Pignone et al., 2007).

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Abbreviations

ACE	angiotensin-converting enzyme
ACE2	angiotensin-converting enzyme 2
ACE2-KO	ACE2-knockout mice
Ang II	angiotensin II
AT_1	type 1 angiotensin II receptor (R)
$AT_{1–7}$	angiotensin-(1–7) receptor
AT_2	type 2 angiotensin II receptor
CHO	Chinese hamster ovary
hrACE2	human recombinant ACE2
LV	left ventricular
MDCK	Madin–Darby canine kidney

Mrg	mas-related gene
rACE2	recombinant ACE2
SHR	spontaneously hypertensive rat
TGF-β_1	transforming growth factor β_1
mas-R	mas receptor

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The Role of Calpain in Diabetes-Associated Platelet Hyperactivation

Abstract

Diabetes mellitus is a major risk factor for vascular diseases and is associated with accelerated atherosclerosis and a high rate of arterial thrombotic complications. A number of studies support the concept that platelets contribute to the pathogenesis and progression of the vascular complications of diabetes. μ-Calpain, a non-lysosomal, Ca^{2+}-dependent cysteine protease, is expressed in platelets and is involved in physiological platelet activation. However, the inappropriate activation of calpain alters platelet function, partially degrades a spectrum of proteins and results in

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hyperaggregability. Changes in the activity of calpain in different cells involved in diabetes-related pathways, or the polymorphism of calpain genes have been associated with the development of type 2 diabetes but their relevance to the diabetes-related vascular complications is not really clear. This review will give an overview of the role of calpain in diabetes and analyze the role of calpain in platelet activation and the changes occurring during the onset of diabetes. Finally, we will discuss future therapeutic possibilities for the improvement of diabetes-associated vascular diseases.

I. Introduction

Diabetes mellitus is a multifactorial disease, the development of which is determined by both genetic and environmental factors. Although the primary problem is related to insulin insensitivity and dysregulated glucose and lipid homeostasis, it is now well accepted that the morbidity and mortality associated with diabetes mostly result from micro- and macro-vascular complications (Beckman et al., 2002). The diabetes-associated acceleration in the development of cardiovascular disease is linked to endothelial dysfunction characterized by a decrease in nitric oxide (NO) bioavailability, reduced prostacyclin production, and a general reduction in the antithrombogenic properties of vascular wall (Schafer et al., 2008).

Platelet function is also markedly altered in diabetic individuals. Indeed, platelets obtained from patients with type 1 or type 2 diabetes are hyperreactive and demonstrate increased adhesiveness as well as exaggerated aggregation and thrombus formation. Different mechanisms have been suggested to be responsible for these phenomena, such as abnormal platelet Ca^{2+} homeostasis, the altered surface expression of glycoprotein (GP) receptors and adhesive proteins, the increased binding of fibrinogen, and alterations in platelet signaling (for review see Randriamboavonjy & Fleming, 2009).

When focusing on aggregation and thrombosis, it is easy to overlook the fact that platelets are a rich source of different lipid mediators (e.g., sphingosine-1-phosphate; S1P), growth factors (e.g., platelet-derived growth factor), and chemokines such as platelet factor 4 (PF4 or CXCL4), and RANTES (regulated upon activation, normally T-cell expressed, and secreted or CCL5). When, as in the diabetic state, platelets are preactivated, a limited degranulation takes place which results in the deposition of cytokines on the endothelial cell surface. For example, increased levels of RANTES can be detected in peripheral blood from diabetic versus control patients (Nomura et al., 2000). The deposition of proteins such as RANTES may be one mechanism by which activated platelets accelerate the progression of vascular disease, i.e., by triggering the arrest of circulating monocytes

(von Hundelshausen et al., 2001). This process requires the association of RANTES with PF4, which can actually occur as a preformed complex stored in platelet α-granules (Gleissner et al., 2008; Zernecke et al., 2008).

For a long time, platelets were considered to be either inactive or active but more or less immune to the disease-associated changes in the proteome that were evident in other cell types. It has since become clear that platelets, despite the absence of the nucleus, contain microRNA, can synthesize proteins on a *de novo* basis and indeed possess active transcription factors such as peroxisome proliferator-activated receptors (PPARs) and nuclear factor κB (Spinelli et al., 2010). Platelet proteins are also subjected to digestion and breakdown, and the limited proteolysis of a spectrum of platelet proteins by the Ca^{2+}-dependent protease, μ-calpain, has been demonstrated in diabetic individuals with poor glycemic control (Randriamboavonjy et al., 2008).

A. The Calpain System

Calpains are a well-conserved family of non-lysosomal, Ca^{2+}-dependent neutral cysteine proteases that contain 16 known genes, 14 of which encode proteins that contain cysteine protease domains and the other 2 genes encode smaller regulatory proteins (Goll et al., 2003; Suzuki et al., 2004). Several calpain isoforms, including calpain 1 (or μ-calpain), calpain 2 (or m-calpain), and calpain 10, are ubiquitously expressed, whereas others such as the calpain 3/muscle-specific p94 and calpain 8/stomach nCl-2 demonstrate tissue-specific expression patterns. μ-Calpain and m-calpain are referred to as "conventional" calpains and usually coexist in most cells. The two isoforms are heterodimers composed of distinct 80 kDa catalytic subunits and an identical 28 kDa regulatory subunit. Characteristically, the enzymes differ in their Ca^{2+} requirement for *in vitro* activation, in that μ-calpain is activated by micromolar concentrations of Ca^{2+} while the activation of m-calpain requires millimolar concentrations of Ca^{2+}.

The calpains were initially believed to be cytosolic proteins; however, several studies have demonstrated the presence of the conventional calpains and calpastatin in mitochondria (Kar et al., 2007, 2010; Ozaki et al., 2007). Moreover, there is also evidence indicating that active proteases can also be found in the extracellular space (Ishikawa et al., 1999). As the calpain proteases exhibit broad substrate specificity and inappropriate regulation can influence many aspects of cell physiology, it is clear that enzyme activity needs to be tightly regulated. The latter is largely the task of calpastatin—an endogenous inhibitor molecule which reversibly binds to several sites on the calpain molecule in a Ca^{2+}-dependent manner (Wendt et al., 2004). In addition to its interaction with calpastatin, calpain activity can be regulated by phosphorylation. Indeed, calpains possess several phosphorylation sites, and the phosphorylation of Ser369 and Thr370 by protein kinase (PK) A

negatively regulates m-calpain activity *in vitro* and *in vivo* (Shiraha et al., 2002; Smith et al., 2003).

Calpains process many different substrates, including molecules involved in numerous signal transduction cascades, cell differentiation, cell cycle progression, apoptosis, membrane fusion, and platelet activation (Saido et al., 1994). However, whereas normal physiological processes are usually triggered by transient calpain activation, the sustained activation of calpain has been associated with pathological effects. Indeed, the "over-activation" of calpain is implicated in the cell death characteristic of several neurodegenerative diseases, including Alzheimer's disease (Huang et al., 2010; Paquet-Durand et al., 2007; Vosler et al., 2008), as well as myocardial infarction (Hernando et al., 2010; Inserte et al., 2005; Sandmann et al., 2002). Although the vast majority of the diseases linked to calpain have been attributed to the prolonged activation of the protease, loss-of-function mutations in the calpain 3 gene can account for limb-girdle muscular dystrophy type 2A (Richard et al., 1995, 2000). Recent findings have linked these mutations to an increased rate of autoproteolytic degradation of the enzyme (Garnham et al., 2009). Polymorphisms in other calpain genes have been linked to disease development. For example, there is convincing evidence that polymorphism of the calpain 10 gene is involved in the etiology of type 2 diabetes (see below).

B. Role of Calpains in Physiological Platelet Activation

Platelets play a fundamental role in hemostasis. Under normal conditions, they come into close contact with the vascular wall but do not adhere to it as the endothelium permanently secretes antiplatelet factors, thus conferring antithrombotic properties upon it (Bunting et al., 1977). Following vascular injury, membrane receptors on platelets facilitate their rolling, adherence, and finally firm attachment to the exposed subendothelial matrix. When shear stress levels are high, thrombus formation is initiated by the concerted binding of GP VI to collagen and GPIb–IX–V complex to von Willebrand factor (Andrews et al., 2003; Massberg et al., 2003; Nieswandt et al., 2003). The actin-binding protein filamin then binds the activated GPIb–IX–V and forms cross-links with F-actin, to create a loose network parallel to the membrane and initiating platelet shape change and adhesion. The interaction of GPIb with von Willebrand factor also triggers intracellular signaling events such as an increase in intracellular Ca^{2+} and the activation of multiple PK pathways, including integrin activation (Du, 2007; Kroll et al., 1991; Ozaki et al., 2005). Platelet activation is further reinforced by the generation of thrombin which activates specific protease-activated seven-transmembrane domain receptors (PAR 1 and/or PAR 3) on the platelet surface. The thrombin-induced Ca^{2+} mobilization together with the activation of PKC are key events in the platelet activation process.

Certainly, the cytoskeletal rearrangement, granule content secretion, arachidonic acid mobilization, phospholipid scrambling, and the activation of the major platelet surface integrin αIIbβ3 (also known as GPIIb–IIIa) are all Ca^{2+}- and PKC-dependent processes.

Secretion of the contents of platelet granules facilitates the transport of receptors such as P-selectin to the platelet surface and promotes the release of secondary mediators of platelet activation such as thromboxane A_2 and adenosine diphosphate (ADP). The latter promotes the recruitment of additional platelets and potentiates hemostasis. Activated PKC and Ca^{2+} bind to and phosphorylate the cytoplasmic tail of the β3-subunit of the αIIbβ3 integrin complex (inside-out signaling), causing a conformational change in the extracellular domain and enabling the binding of circulating fibrinogen. This in turn interacts with activated αIIbβ3 integrin of neighboring activated platelets and mediates platelet aggregation. Fibrinogen initiates the so-called outside-in signaling by stimulating the tyrosine phosphorylation of β3 integrin which allows binding of the cytoskeletal proteins talin and vinculin. The latter associates with α-actinin thereby cross-linking the cytoskeleton to the plasma membrane. In the final stages of activation, phosphatidylserine, which is normally sequestered in the inner leaflet of the plasma membrane, is relocated to the outer leaflet effectively giving platelets a procoagulant surface.

Several reports have highlighted the importance of calpains in many different steps of the platelet activation process including spreading, aggregation, granule secretion, and integrin signaling (Figure 1). As mentioned above, platelets express both μ-calpain and m-calpain; however, the functional importance of the two isoforms varies between species. For example, while human platelets express both isoforms, murine platelets express predominantly μ-calpain which is localized to focal adhesions (Andrews et al., 2007; Gleissner et al., 2008). Platelet stimulation with thrombin has been shown to directly lead to calpain activation, while other ligands, such as von Willebrand factor, require αIIbβ3 integrin engagement in order to activate the protease. Intriguingly, calpain has been shown to associate with, as well as to cleave β3 integrin, a finding that underlines the potential of protease activation to interfere with outside-in signaling, aggregation, clot retraction, and clot stability. A thorough analysis of calpain substrates in platelets has yet to be performed but the number is >100, and μ-calpain is known to be responsible for the limited proteolysis of a spectrum of cytoskeleton- associated proteins including spectrin, adducin, talin, filamin, vinculin, and cortactin. Other substrates include kinases such as the myosin light chain kinase, PKC, and the tyrosine phosphatase PTP1B (for review see Kuchay & Chishti, 2007). Calpain does not completely degrade these proteins; in fact, stable degradation products can usually be detected. Rather, the function of each of the target proteins is markedly and irreversibly altered.

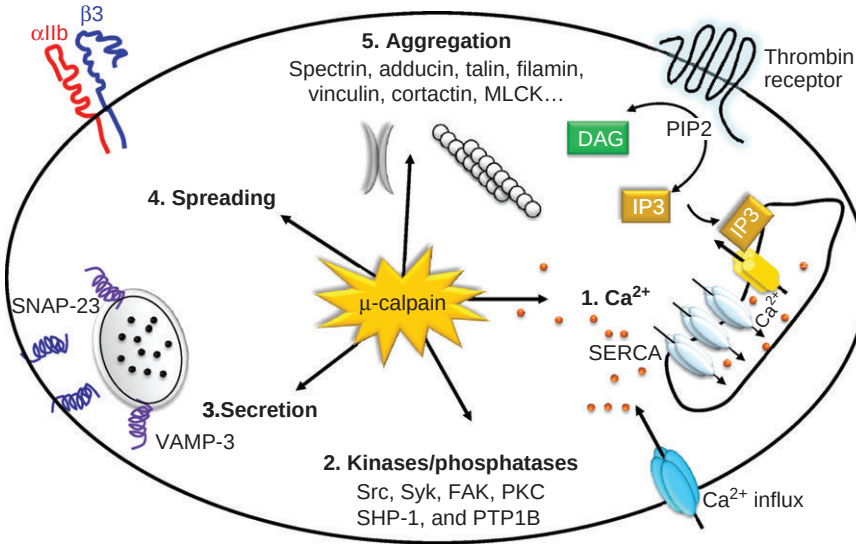

FIGURE 1 Role of calpain in physiological platelet activation. Calpain activation leads to the limited proteolysis of a spectrum of platelet proteins ranging from (1) Ca^{2+}-ATPases (e.g., SERCA-2) that regulate platelet Ca^{2+} signaling, (2) kinases involved in different signaling cascades, (3) proteins involved in platelet degranulation such as the SNARE proteins, and integrins, and (4 and 5) cytoskeleton-associated proteins that are ultimately responsible for platelet shape change, spreading, aggregation, and clot retraction.

The involvement of μ-calpain in platelet secretion has been demonstrated in studies looking at the effect of calpain inhibitors on exocytosis and on the proteins that are part of the secretion machinery. Indeed, α-granule secretion from human platelets can be prevented by pretreatment with calpain inhibitors (Croce et al., 1999). Moreover, different *N*-ethylmaleimide-sensitive factor attachment receptor proteins such as the attachment protein, SNAP-23, and the vesicle-associated membrane protein, VAMP-3, are also calpain substrates (Lai et al., 2003; Rutledge et al., 2002).

The generation of μ-calpain knockout mice (Azam et al., 2001) provided the first direct evidence for a role of the calpains in the regulation of platelet function and expanded information obtained from studies using calpain inhibitors (Croce et al., 1999). However, although μ-calpain knockout mice demonstrated reduced aggregation and attenuated clot retraction, several of the functions related to calpain activation in human platelets were not affected. For example, the time course of proteolysis of known calpain substrates such as talin, filamin, and the β3 integrin cytoplasmic domain did not differ between the wild-type and μ-calpain-deficient platelets (Azam et al., 2001). These observations were explained by assuming that either these proteins are not μ-calpain substrates in murine platelets or m-calpain

compensated the μ-calpain defect. However, no direct evidence of the latter suggestion has been provided.

The role of calpain is not only limited to mechanisms related to early events of platelet activation but also to its procoagulant activity such as membrane blebbing and microparticle formation (Siljander et al., 2001).

C. Role of Platelets in Diabetes-Associated Vascular Complications

Platelets from patients with diabetes demonstrate a number of changes including exaggerated adhesion (Dittmar et al., 1994; Knobler et al., 1998), increased surface expression of GPIb and αIIbβ3 integrin (Tschoepe et al., 1990), increased fibrinogen binding (DiMinno et al., 1985), an increased capacity for thromboxane generation (Davi et al., 1990), and resistance to the antiplatelet effects of exogenously applied NO (Anfossi et al., 1998). Perhaps the best studied defect in platelets from diabetic individuals is disturbed calcium homeostasis (Vicari et al., 1996), but increased platelet volume (Papanas et al., 2004) and altered membrane fluidity (Mazzanti et al., 1997; Watala et al., 1998) have also been described. All of these alterations contribute to enhanced risk of small vessel occlusion and the accelerated development of the atherothrombotic disease in coronary, cerebral, and other vessels.

What are the events that are able to elicit such marked changes in platelet function? It is conceivable that the initial stimulus is a physiological platelet response to the increasingly toxic environment, i.e., hyperglycemia. Certainly, hyperglycemia-dependent metabolic changes have been clearly associated with secondary increases in thromboxane and thrombin generation as well as the activation of PKC (Assert et al., 2001; Gleissner et al., 2008; Pirags et al., 1996). Even a transient increase in plasma glucose seems to affect platelet function and is thought to involve elevated reactive oxygen species (ROS) production.

Endothelium-derived NO production is attenuated during the development of diabetes, and several different groups have reported a decrease in NO output by diabetic platelets (Martina et al., 1998; Oskarsson et al., 1996; Rabini et al., 1998). However, although we and others were able to detect a 130-kDa protein in isolated human platelets using antibodies directed against eNOS and demonstrated the generation of NO by electron spin resonance spectroscopy (Assert et al., 2001; Fleming et al., 2003; Ji et al., 2007), these findings are now considered to be highly controversial. Indeed, it has not been possible to demonstrate the presence of any NO synthase in purified human or murine platelet preparations using modern proteomic methods (reviewed in Naseem et al., 2008). Thus, despite the fact that eNOS derived from endothelial cells is an excellent calpain substrate, it has not

been possible to demonstrate a similar mechanism in platelets (Randriamboavonjy et al., 2008). What are we then to make of the earlier reports of platelet-derived NO generation? To analyze this it is necessary to realize that NO production was initially assayed using indirect methods, i.e., by measuring the accumulation of cyclic GMP. Indeed, more recent data have suggested that some platelet agonists directly affect the activity of the soluble guanylyl cyclase, resulting in an NO-independent increase in cyclic GMP levels (Gambaryan et al., 2008; Ishikawa et al., 1999).

One of the consequences of maintained hyperglycemia is the nonenzymatic formation of advanced glycation endproducts (AGEs). AGEs generated endogenously or provided in the diet have been shown to induce platelet activation (Gawlowski et al., 2009; Hangaishi et al., 1998) via binding to the specific RAGE receptor (Basta, 2008). In patients with diabetes mellitus, these derivates can also potentiate agonist (e.g., serotonin)-induced platelet aggregation (Hasegawa et al., 2002). One of the best characterized consequences of platelet RAGE activation is the generation of ROS (Basta et al., 2005; Yan et al., 1994).

II. Calpains and the Development of Diabetes

Individuals with excessive body weight and showing signs of the metabolic syndrome (also referred to as prediabetes) are at high risk of developing type 2 diabetes. Many of the changes that take place in different cell types during the development of these conditions have also been linked to calpain. For example, calpain activity is required for the isobutylmethylxanthine-induced differentiation of 3T3-L1 fibroblasts/preadipocytes into adipocytes. In addition, either the overexpression of calpastatin or the pretreatment of cells with a calpain inhibitor prevents the expression of adipocyte-specific genes such as C/EBPα (Patel et al., 1999). Interestingly, calpain inhibition failed to affect adipocyte differentiation induced by either dexamethasone or insulin.

The calpain 10 gene (CAPN10) is one of the few genes widely accepted to contribute to diabetes risk and is the first diabetes gene to be identified through a genome scan. For example, three intronic CAPN10 single-nucleotide polymorphisms (UCSNP-43, -19, and -63) have been associated with insulin resistance phenotypes including high glucose levels, high blood pressure, and elevated cholesterol levels (von Hundelshausen et al., 2001). CAPN10 polymorphism is also associated with the increased incidence of cardiovascular diseases including subclinical atherosclerosis (assessed by the carotid artery intima-media thickness) in a population of Mexican-American families with a history of coronary artery disease (Goodarzi et al., 2005, 2009). From detailed studies looking at different single-nucleotide polymorphisms, it seems that CAPN10 affects intima-media thickness

independently of the diabetes-related phenotypes. Moreover, the fact that CAPN10 influenced the risk of developing atherosclerosis as well as that of developing diabetes was taken as evidence that inherited factors may underlie the frequent co-occurrence of these two conditions. At the moment, the molecular mechanism(s) linking calpain 10 to diabetes remain to be fully elucidated, but a deletion/insertion polymorphism in the CAPN10 (single-nucleotide polymorphism-19) has been associated with reduced lipolytic function of the β3-adrenoceptor in adipocytes from obese individuals (Hoffstedt et al., 2002).

It is, however, important to stress that while there are numerous reports of associations between CAPN10 polymorphism and type 2 diabetes as well as insulin sensitivity, insulin secretion and also different aspects of adipocyte biology and microvascular function, these findings have not always been linked with the same polymorphisms (Turner et al., 2005).

One characteristic of type 2 diabetes is the apparent insensitivity to higher than normal (at least in early stages) circulating concentrations of insulin. There are many reports of diabetes-associated changes in insulin receptor expression/activity and signaling events. One key mediator of the biological activation of insulin is the insulin receptor substrate (IRS)-1, the tyrosine phosphorylation of which allows the binding and activation of the phosphatidylinositol 3-kinase (PI3-K) and the subsequent activation of the Akt, atypical PKCs and mammalian target of rapamycin pathways that are involved in the anabolic actions of insulin. In addition to tyrosine phosphorylation, insulin increases the serine phosphorylation of several sites within IRS-1, some of which can be classified as "inhibitory," i.e., phosphorylation shuts down insulin signaling. Interestingly, several inducers of insulin resistance also promote the phosphorylation of these inhibitory sites through the activation of several kinases including *c*-Jun N-terminal kinases, extracellular signal-regulated kinases, PKCθ, and inhibitory-κB kinase β (IKKβ) (Tanti et al., 2009). Chronic exposure of adipocytes to insulin not only affects the phosphorylation of the IRS-1 but also stimulates the degradation of the protein (Rice et al., 1993). Interestingly, calpain has also been implicated in this process, at least in adipocytes (Smith et al., 1996; Zhang et al., 2003), even though more recent studies suggest that the more likely degradation mechanism involves the proteasome (Satoh et al., 2008). There is another possible interaction between IRS-1 and calpain at the level of tyrosine phosphatase, PTP1B. The latter phosphatase, which is also regulated by IKKβ, downregulates insulin receptor and IRS-1 tyrosine phosphorylation and thus attenuates insulin signaling (Tanti et al., 2009). PTP1B is, however, also a μ-calpain substrate (Trumpler et al., 2009), and the activity and expression of PTP1B are markedly increased in platelets from μ-calpain knockout mice (Kuchay et al., 2007).

Calpains have also been suggested to play an important role in insulin secretion, in particular in the intracellular trafficking of insulin-containing

granules in pancreatic cells (Sreenan et al., 2001; Zhou et al., 2003). Certainly, short-term exposure to calpain inhibitors increases glucose-induced insulin secretion in murine pancreatic islets. Longer calpain inhibition results in the impairment of mitochondrial fuel metabolism and the exocytosis of insulin (Zhou et al., 2003). On the basis of such studies, it is tempting to suggest that calpain activation may also be involved in the development of beta-cell failure. However, neither the mechanism leading to the decrease in calpain activity in diabetic pancreatic cells nor the mechanism linking calpain to granule exocytosis has been investigated in detail.

Which calpain isoform is the most important physiologically and which has the closest links to diabetes? This is currently difficult to state definitively as the majority of studies performed to date made extensive use of calpain inhibitors and, for the most part, failed to determine the identity of the calpain isoform involved.

III. Role of Calpain in the Diabetes-Associated Platelet Hyperactivation

Given that calpains play an important role in many of the pathways involved in platelet activation, and the fact that platelet Ca^{2+} levels are elevated in diabetes (Li et al., 2001), it comes as no surprise that there is evidence linking calpain overactivation to type 2 diabetes.

A direct antiplatelet effect of insulin has been demonstrated by many groups, and although we and others have observed an insulin-induced attenuation of the thrombin-induced Ca^{2+} response and platelet aggregation as well as the release of ADP (Fleming et al., 2003; Randriamboavonjy et al., 2004), reports from groups assessing the same responses are inconsistent. Part of the controversy may be attributed to the fact that responses to insulin are highly heterogeneous. Indeed, clear populations of "responders" and "nonresponders" have been identified in several studies (Grill et al., 1987; Randriamboavonjy et al., 2004) and can be related to numerous factors including physical condition (King et al., 1988; Randriamboavonjy et al., 2004). Studies in which an effect of insulin has been documented and that were aimed at addressing the molecular mechanisms that underlie the anti-aggregatory effects of insulin have not yet managed to completely clarify the events involved. Thus, although insulin has been reported to stimulate the AMP-activated PK and Akt in a PI3-K-dependent manner (Fleming et al., 2003), as well as to decrease platelet Ca^{2+} and attenuate agonist-induced platelet activation (Ishida et al., 1996), these events have not been definitively linked to the activation of the insulin receptor. Indeed, authors specifically addressing the expression of the insulin receptor on washed human platelets (Rauchfuss et al., 2008) have failed to demonstrate its presence.

Platelets do, however, express high levels of functional receptors for the insulin-like growth factor (IGF)-1 which, when activated, leads to the tyrosine phosphorylation of the IRS proteins and their association with the p85 subunit of PI3-K (Hers, 2007).

As outlined above, calpains, in particular μ-calpain, seem to play a major role in the regulation of many aspects of platelet function. As Ca^{2+} levels are higher in diabetic than in healthy individuals under basal conditions as well as following stimulation with thrombin, diabetes seems to result in ideal conditions for protease activation. Time and poor glycemic control are prerequisites for μ-calpain activation in platelets as protease activation and substrate degradation were only clearly detectable in patients with hemoglobin A1c >7.5 (Randriamboavonjy et al., 2008).

To date, only three proteins have been reported to be degraded by μ-calpain in platelets from diabetic patients in the absence of additional stimuli, platelet endothelial cell adhesion molecule (PECAM-1/CD31), sarco-endoplasmic Ca^{2+}-ATPase (SERCA)-2 (Randriamboavonjy et al., 2008), and the S1P receptor, S1P2 (Randriamboavonjy et al., 2009). Currently, large-scale proteomic analyses aimed at identifying novel calpain substrates in diabetic individuals are in progress but it is clear even now that the targets cleaved by the overactivated calpain are pathophysiologically relevant.

A. PECAM-1

PECAM-1 negatively regulates platelet function, and platelets from PECAM-$1^{-/-}$ mice are hyperresponsive to stimulation with collagen or thrombin and demonstrate enhanced aggregation, secretion, and adhesion to these agonists (Falati et al., 2006; Patil et al., 2001). In humans, a negative relationship exists between the surface expression of PECAM-1 and variation in platelet responses to stimulation. Indeed, recent observations indicate that in addition to its inhibitory effect on GPVI signaling, PECAM-1 can inhibit multiple signaling pathways, and although each effect on its own is relatively small, their combination results in a more pronounced inhibition (Jones et al., 2009).

While full-length PECAM-1 (130 kDa) is readily detectable in platelets from nondiabetic/healthy subjects using an antibody directed against the C-terminus of the protein, little or no PECAM-1 could be detected in platelets from subjects with type 2 diabetes. Using a second PECAM-1 antibody, it became evident that diabetes was not associated with the loss of PECAM-1 protein but rather with its C-terminal cleavage and the generation of two degradation products: ~124 and 118 kDa (Randriamboavonjy et al., 2008). In *in vitro* studies, calpain was able to reproduce this effect and calpain inhibition prevented PECAM-1 cleavage initiated by peroxynitrite.

B. SERCA

In resting platelets, the cytosolic Ca^{2+} concentration is maintained at approximately 100 nmol/L, both by limiting Ca^{2+} influx and by actively pumping it out of the cytosol. One factor that clearly contributes to the disturbed platelet $[Ca^{2+}]_i$ in diabetic subjects is a marked reduction in Ca^{2+}-ATPase activity (Rosado et al., 2004). Of the multiple Ca^{2+}-ATPases expressed in human platelets, the activity of SERCA-2 has been reported to be regulated by tyrosine nitration or glutathiolation (Adachi et al., 2002, 2004), and this may represent a potential link between diabetes, oxidative stress, dysregulation of Ca^{2+} handling, and platelet activation. Indeed, the tyrosine nitration of SERCA-2 is more pronounced in platelets from diabetic patients compared to that from healthy individuals. Tyrosine nitration was accompanied by a decrease in SERCA-2 activity and could be mimicked by *in vitro* application of peroxynitrite (Randriamboavonjy et al., 2008). SERCA-2 is also a μ-calpain substrate and is degraded in diabetic platelets. Thus, platelet Ca^{2+} dysregulation seems to involve the potentially reversible posttranslational modification and inhibition of the Ca^{2+}-ATPase. If diabetes is maintained, however, the Ca^{2+}-induced activation of calpain results in SERCA degradation.

C. S1P2

S1P is a bioactive lipid mediator that is generated and stored in a number of different cell types such as erythrocytes, leukocytes, endothelial cells, and platelets. Platelets lack the S1P-degrading lyase and in addition to releasing large amounts of S1P following activation, they are also one of the main sources of the circulating mediator (Yatomi et al., 1995, 1997). Consistent with the notion that diabetic platelets continually secrete their contents, plasma obtained from diabetic patients contains significantly higher levels of S1P than that from healthy individuals (Randriamboavonjy et al., 2009).

Although they generate it in large amounts, platelets are not viewed as an important target for S1P, and currently, any effects of S1P on platelets are controversial (Yatomi et al., 1995). It is, however, important to note that S1P can elicit platelet shape change and aggregation (Hashizume et al., 1992), has been reported to inhibit the platelet aggregation induced by other platelet agonists (Nugent et al., 2000), and the S1P-related compound sphingosylphosphorylcholine is known to inhibit platelet function (Altmann et al., 2003). Platelets from healthy humans express functional S1P2 receptors, and stimulation with exogenous S1P elicits an increase in intracellular Ca^{2+} and platelet aggregation (Randriamboavonjy et al., 2009). However, platelets from diabetic patients demonstrated an attenuated aggregability to S1P as well as decreased levels of S1P2 protein. The latter was found to be a

target of μ-calpain. The degradation of S1P2 in diabetic platelets and the accompanied desensitization of S1P response may represent an auto-protective mechanism against exaggerated platelet activation in diabetes.

D. Other Substrates

While a relatively large number of cytosolic signaling proteins are known to be μ-calpain substrates, almost nothing is known about secreted proteins. It is, however, tempting to speculate that the overactivation of μ-calpain may also alter the platelet secretome and thus influence other cell types. Two possibilities exist (i) that calpain degrades secreted proteins—most probably those not stored in granules and (ii) that secretion is altered by the fact that μ-calpain degrades SNARE proteins. Just such a phenomenon may account for the finding that platelets from patients with cardiovascular risk factors, particularly type 2 diabetes, failed to promote the adherence and migration of endothelial progenitor cells while those from healthy donors did (Dernbach et al., 2008). However, the identity of the factor(s) involved has yet to be resolved.

What determines whether or not a protein is a calpain substrate? One theory was that calpain substrates could be identified on the basis of regions rich in the amino acids proline (P), glutamate (E), serine (S), and threonine (T) and that this "PEST sequence" represents a putative site for proteolytic degradation (Rechsteiner et al., 1996; Rogers et al., 1986). This hypothesis needs to be readdressed as many of the calpain substrates identified do not possess a PEST sequence, and it seems that an alternative sequence of events such as posttranslational modification play a greater role in determining susceptibility to cleavage. The ability of a protein to bind calmodulin confers a strong likelihood that it is a substrate for calpain (Wang et al., 1989). Indeed, both calmodulin and calpain have been implicated in regulating the intracellular proteolysis of the cytoplasmic domain of PECAM-1 in platelets (Naganuma et al., 2004; Turner et al., 2005; Wong et al., 2004). An additional modification that can influence the calpain-mediated cleavage of proteins is phosphorylation, and several proteins are reportedly protected from degradation by calpain when they are phosphorylated (Chen et al., 1989; Elvira et al., 1993). There are, of course, other proteins whose sensitivity to calpain is increased by phosphorylation (Wu et al., 2007). Whether or not additional posttranslational modifications such as nitration, glutathiolation, or glycoxidation can determine the susceptibility of proteins to calpain remains to be clarified.

E. Microparticles

Microparticles are intact membrane vesicles released from cell membranes upon activation or apoptosis. Platelets are known to produce

microparticles, and there is evidence that platelet-derived microparticle (PMP) formation is a Ca^{2+}- and calpain-dependent mechanism (Pasquet et al., 1996). PMPs are known to play an important role in hemostasis due to their procoagulant properties but their role in various clinical conditions remains controversial. Certainly, PMPs can be detected in blood from healthy individuals, and their levels are known to be elevated in diabetes (Koga et al., 2006; Nomura et al., 1993) as well as in atherosclerosis (Nomura et al., 1995). Although the increase in circulating PMPs in patients with thrombotic thrombocytopenic purpura has been attributed to elevated calpain activity in the plasma (Kelton et al., 1992), there is no direct evidence linking increased calpain activity in platelets with the elevated circulating PMPs in diabetes.

IV. Effect of Antidiabetics on Calpain Function

A. Metformin

Metformin is an oral antidiabetic drug of the biguanide class and is the first-line drug of choice for the treatment of type 2 diabetes. Above and beyond its antihyperglycemic effect, metformin has been conclusively shown to improve oxidative stress, preserve antioxidant function, and reduce platelet activation in type 2 diabetes as well as to prevent the onset of cardiovascular complications (Formoso et al., 2008). The fact that metformin alone (or even better in combination with lifestyle changes and weight loss) reduces oxidative stress suggests that it should be able to influence calpain activation; however, no studies have addressed this specific question as yet.

B. Thiazolidinediones

The thiazolidinediones are a class of antidiabetic drugs known to effectively decrease blood glucose by improving sensitivity to insulin. Although the thiazolidinediones are reported to elicit several pleiotropic effects, relatively little is known about their direct action on platelet function. In diabetic humans, troglitazone reduces platelet-dependent thrombus formation (Osende et al., 2001), and rosiglitazone decreases the percentage of P-selectin-positive platelets in nondiabetic patients with coronary artery disease (Sidhu et al., 2004). Relatively, little is known about the mechanism by which PPAR agonists can affect platelet function, but the potential importance of platelet PPARs became evident following reports that PPARβ activation inhibits platelet aggregation and can synergize with NO (Ali et al., 2006). It now seems that PPARγ agonists can exert similar effects and reduce collagen-induced Ca^{2+} mobilization and P-selectin exposure.

Furthermore, PPARγ was found to associate with the kinase Syk and the transmembrane adapter protein LAT after platelet activation, and PPARγ ligands inhibited the tyrosine phosphorylation of multiple components of the GPVI signaling pathway (Moraes et al., 2010).

In addition to the acute effects on platelet signaling, PPARγ agonists have been shown to increase the expression of SERCA-2 expression in megakaryocytes and to restore platelet Ca^{2+} signaling and reduce calpain activation in platelets from patients with type 2 diabetes (Randriamboavonjy et al., 2008). Consistent with these effects, diabetic patients treated with rosiglitazone demonstrated normal expression levels of the full-length PECAM-1 (Randriamboavonjy et al., 2008) and S1P2 (Randriamboavonjy et al., 2009). It is therefore conceivable that despite the risk of heart attacks reported to be associated with rosiglitazone treatment, use of a low dose of thiazolidinediones which specifically target platelets may damp the contribution of platelets and platelet-derived products to the development of vascular diseases associated with type 2 diabetes.

V. Conclusion: Calpain as a Platelet Drug Target?

Drugs used to treat diabetes are mostly directed at glycemic control and restoration of insulin sensitivity. However, a reduction in the mortality associated with diabetes has been achieved through the combination of oral antidiabetic drugs and antiplatelet therapy. Despite the efficacy of the treatment, a considerable number of patients continue to experience recurrent atherothrombotic events, which are attributed to the resistance to antiplatelet drugs such as aspirin or clopidogrel. Dose modification, combination therapy, or the use of newer agents have been proposed to solve these limitation problems (reviewed in Angiolillo, 2009).

The facts that (i) calpain plays a key role in platelet activation and (ii) that calpain activity is elevated in diabetic platelets make it tempting to suggest the Ca^{2+}-activated proteases as promising therapeutic targets to prevent thrombotic complications in diabetic patients. Given the arguments presented above, it may seem that calpain inhibition could have adverse effects, preventing physiological calpain activation, perhaps even promoting the diabetic phenotype (by interfering with the actions of calpain 10) and platelet aggregation. However, calpain 10 is regulated differently than the other isoforms and is, for example, unable to bind calpain small unit which is the target of calpastatin. Moreover, non-isoform-specific calpain inhibitors have been successfully used *in vivo* in different animal models of ischemia–reperfusion injury (Neuhof et al., 2008; Shimazawa et al., 2010; Yoshikawa et al., 2010) as well as in humans, in the latter case, however, only in the treatment of some neuronal diseases (Mangas et al., 2010). The beneficial effect of an

inhibitor that targets the conventional calpain isoforms on diabetes-associated thrombotic complications could be achieved by the use of low doses to specifically target circulating cells.

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Abbreviations

ADP	adenosine diphosphate
AGEs	advanced glycation end products
C/EBPα	CCAAT/enhancer-binding protein-α
Ca^{2+}	calcium
CAPN10	calpain 10 gene
CCL5	chemokine (C-C motif) ligand 5 or RANTES
CXCL4	chemokine (C-X-C motif) ligand 4 or platelet factor 4
IGF	insulin-like growth factor
IKKβ	inhibitory-κB kinase β
IRS	insulin receptor substrate
NO	nitric oxide
PAR	protease-activated receptor
PECAM-1	platelet endothelial cell adhesion molecule-1
PF4	platelet factor 4
PI3-K	phosphatidylinositol 3-kinase
PK	protein kinase
PMP	platelet-derived microparticle
PPARs	peroxisome proliferator-activated receptors
PTP1B	protein tyrosine phosphatase-1B
RAGE	receptor for advanced glycation endproducts
RANTES	regulated upon activation, normally T-cell expressed
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
S1P2	sphingosine-1-phosphate receptor 2
SERCA	sarco-endoplasmic Ca^{2+}-ATPase
SNAP-23	*N*-ethylmaleimide-sensitive factor attachment receptor proteins-23
SNP	single-nucleotide polymorphisms
VAMP-3	vesicle-associated membrane protein-3

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Tissue Factor in Cardiovascular Disease: Pathophysiology and Pharmacological Intervention

Abstract

Tissue factor (TF) is the major trigger of the coagulation cascade and thereby crucially involved in the maintenance of vascular hemostasis. By binding factor VIIa, the resulting TF:VIIa complex activates the coagulation factors IX and X ultimately leading to fibrin and clot formation. In the vessel wall, TF expression and activity is detectable in vascular smooth muscle cells and fibroblasts and, at a much lower level, in endothelial cells and can be induced by various stimuli including cytokines. In addition, TF is found in the bloodstream in circulating cells such as monocytes, in TF containing

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microparticles, and as a soluble splicing isoform. Besides its well-known extracellular role as a trigger of coagulation, TF also functions as a transmembrane receptor, and TF-dependent intracellular signaling events regulate the expression of genes involved in cellular responses such as proliferation and migration. TF indeed appears to be involved in the pathogenesis of neointima formation and tumor growth, and increased levels of TF have been detected in patients with cardiovascular risk factors or coronary artery disease as well as in those with cancer. Therefore, pharmacological or genetic inhibition of TF may be an attractive target for the treatment of cardiovascular disease and cancer. Different strategies for inhibition of TF have been developed such as inhibition of TF synthesis and blockade of TF action. Clinical applications of such strategies need to be tested in appropriate trials, in particular for evaluating the advantages of targeted versus systemic delivery of the inhibitors.

I. Introduction

The coagulation cascade represents a complex system of proteases maintaining and restoring vascular integrity after vessel injury. Known as a key trigger of the coagulation cascade, tissue factor (TF) has more recently been discovered to act as a mediator in the pathogenesis of cardiovascular disorders beyond its role in coagulation. In this chapter, we will review the many faces of TF in the pathophysiology of cardiovascular disease and resulting therapeutic approaches.

II. TF: Structure and Function

A. TF Protein Structure

The *TF* (thromboplastin, CD142) gene located on chromosome 1 at p21–22 spans a 12.4 kb region and contains six exons encoding a single transmembrane polypetide chain composed of 263 amino acids (Kao et al., 1988; Mackman et al., 1989; Morrissey et al., 1989; Scarpati et al., 1987). The TF protein is divided into three domains: an extracellular (219 amino acids), a membrane-spanning (23 amino acids), and a cytoplasmic (21 amino acids) region (Breitenstein et al., 2010b). TF is considered as a class I integral membrane protein since the amino-terminus of the protein is located extracellularly while the cytoplasmatic tail contains the carboxy-terminus. Mobility studies on sodium dodecyl sulfate gels reveal that the molecular weight of the fully glycosylated protein is about 45,000 kDa (Broze et al., 1985). The extracellular domain provides two high-affinity sites for the binding of factor VII to TF. This binding is crucial for induction

of the conformal changes required to induce the catalytic activity of factor VII (Edgington et al., 1991; Rehemtulla et al., 1991). In addition, anchoring of the TF protein to the membrane via the hydrophobic transmembrane domain has been demonstrated to be essential for procoagulant activity of the TF protein. However, the nature of the anchoring domain does not seem to play a role (Paborsky et al., 1991). Indeed, deletion of the cytoplasmatic tail is not paralleled by an impaired TF procoagulant activity (Paborsky et al., 1991). Therefore, recent studies focussed on possible non-hemostatic functions of this domain. Experiments performed mainly in fibroblasts and cancer cell lines revealed that phosphorylation of the cytoplasmatic tail activates intracellular signaling pathways and induces cellular responses which are described in the following chapter.

In 2003, an alternatively spliced (as) isoform of TF mRNA lacking the fifth exon and exhibiting a frameshift mutation in the carboxy-terminal region was characterized (Bogdanov et al., 2003). This TF protein lacks membrane anchorage and is detectable in the plasma as a soluble TF protein. While the alternatively spliced tissue factor protein has been described in different species and cell types, its contribution to the TF procoagulant activity in plasma as well as thrombus formation *in vivo* remains barely understood (Bogdanov et al., 2006; Mackman, 2007).

B. TF: Key Trigger of Coagulation

In intact vessels, blood circulates as a fluid due to the physiological balance between natural procoagulant and anticoagulant factors. The clotting cascade defines a complex system of zymogens and enzymatic reactions designed to limit blood loss and ultimately restore vascular integrity by formation of a clot following vessel injury. Initially, initiation of coagulation was thought to be triggered by two distinct pathways: the intrinsic, or contact activation pathway, and the extrinsic, or TF pathway. Even though the two pathways are independently activated, the distinction between both of them has been blurred since they share a final common pathway and, in addition, there is crosstalk between the two systems. The intrinsic pathway is activated when blood or plasma comes in contact with subendothelial connective tissue or negatively charged surfaces and involves circulating factors such as factor XII, factor XI, factor IX, prekallikrein, and high molecular weight kininogen (Gailani & Renne, 2007). However, it has been questioned whether this pathway is required for maintenance of normal hemostasis, since congenital deficiency in the involved factors, for example, factor XII, does not result in an increased bleeding tendency or clinically apparent pathologies in humans.

Coagulation through the TF pathway is activated when blood interacts with a TF expressing cell surface, such as it occurs after endothelial erosion, plaque rupture, or other vascular injury (Fig. 1). The initial step consists in

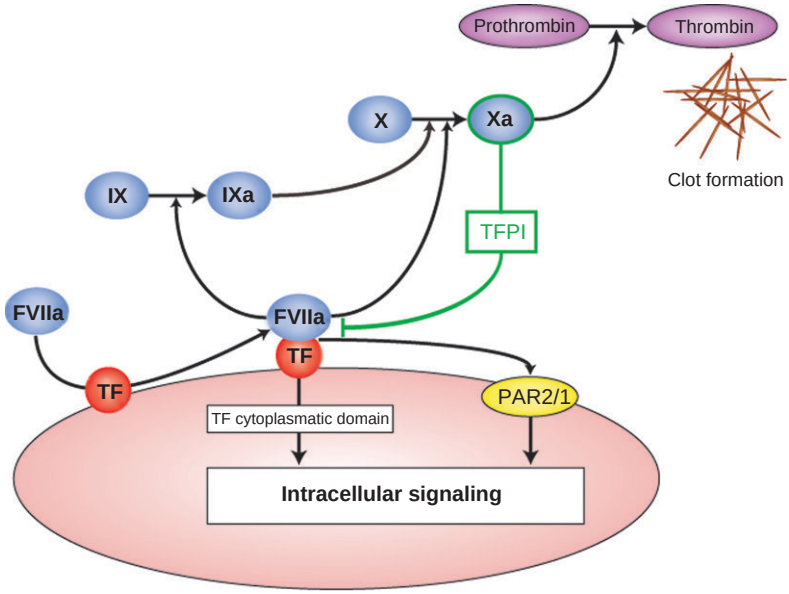

FIGURE 1 TF exerts extracellular effects as main trigger of coagulation as well as intracellular effects on signal transduction. Binding of FVIIa to TF leads to the activation of FIX and FX. This results in thrombin generation and clot formation. The endogenous inhibitor TFPI binds to FXa thereby suppressing TF:FVIIa activity. Besides induction of the coagulation cascade, formation of the TF:FVIIa complex activates intracellular signaling pathways involved in the pathogenesis of cardiovascular disease.

the reversible binding of the zymogen factor VII (FVII) or the activated FVII (FVIIa) to membrane-bound TF (Mackman, 2009; Mackman et al., 2007), resulting in the formation of both TF:FVII and TF:VIIa complexes. Only the latter, however, possesses the enzymatic activity required for further activation of downstream proteases. Once bound to TF, the inactive FVII is converted to FVIIa via limited proteolysis by several plasma proteases, such as XIIa, Xa, Ixa, or thrombin, generating a positive feedback loop (Steffel et al., 2006b). The TF:FVIIa complex is indeed a potent activator of the coagulation cascade and is composed of TF acting as a cofactor with regulatory functions, and the serine protease FVIIa exerting catalytic functions (Breitenstein et al., 2010a). The TF:VIIa complex activates factor IX to factor IXa which in association with VIIIa catalyzes the conversion of factor X to Xa. In addition, factor X is directly converted to factor Xa by the TF:FVIIa complex. Factor Xa in association with factor Va and divalent calcium forms the so-called prothrombinase complex which cleaves prothrombin to form thrombin (Breitenstein et al., 2010a and b). Thrombin exerts direct effects on coagulation through conversion of fibrinogen to fibrin, activation of platelets, and activation of various proteases of the coagulation

cascade such as FVII, FXI, and FV representing an important auto-feedback loop. Besides its well-known role in clot formation, the functions of thrombin extend from coagulation to vascular remodeling by stimulating proliferation of vascular smooth muscle and endothelial cells as well as secretion of growth factors ultimately leading to restoration of vascular integrity (Ferrara & Davis-Smyth, 1997; Maragoudakis et al., 2002; Tsopanoglou et al., 2002, 2004).

C. TF: A Signaling Receptor

The tight link between homeostasis and progression of vascular alterations such as inflammation or atherosclerosis provides evidence for complex interactions between the coagulation cascade and the vascular pathophysiology. Mainly because of its similarity to class II cytokine receptors recent studies suggested a non-hemostatic role of TF in different biological processes through induction of intracellular signaling events (Bazan, 1990). The following chapter provides an overview of TF-mediated cell signaling (Fig. 1).

Evidence for TF:VIIa-induced signaling is derived from experiments in which FVIIa binding to TF was shown to induce intracellular calcium transients in different types of TF expressing cells (Camerer et al., 1996; Rottingen et al., 1995). This observation was followed by studies demonstrating that binding of factor VIIa to TF induces the activation of the three major mitogen-activated protein (MAP) kinase family members p38 MAP kinase, p42/p44 MAP kinase, and *c*-Jun N-terminal kinase (JNK) (Camerer et al., 1999; Poulsen et al., 1998). In fibroblasts, the TF:FVIIa-induced activation of Src-like kinases and phosphoinositide 3 (PI3)-kinase leads to activation of the downstream Rho-like guanosine triphosphatases Rac and Cdc42 resulting in cytoskeletal reorganization (Versteeg et al., 2000).

Hence, by activating different kinases involved in signal transduction, the TF:VIIa complex regulates the transcription of numerous genes involved in physiological and pathophysiological processes such as cell migration, cell growth, or apoptosis. Indeed, binding of factor VIIa to TF induces activation of key transcription factors such as early growth-response protein 1 (Egr-1) or nuclear factor kappa B and the activation of the RNA polymerase A (Camerer et al., 1999; Pendurthi et al., 1997). Moreover, microarray analysis of the gene expression profiles revealed increased mRNA levels of growth factors (i.e., connective tissue growth factors CCN1 and CCN2, fibroblast growth factor 5) and inflammatory cytokines (i.e., interleukin-1β, interleukin-8) following factor VIIa and TF interaction (Camerer et al., 2000; Pendurthi et al., 2000).

Interestingly, induction of signal transduction events was demonstrated to depend on the catalytic activity of factor VIIa, because inactivated factor VII or anti-TF antibodies blunt these effects (Camerer et al., 1996, 1999;

Pendurthi et al., 2000; Poulsen et al., 1998). Consistent with this observation, the TF:VIIa complex initiates intracellular signaling via G protein-coupled protease-activated receptors (PARs) (Riewald & Ruf, 2002; Siegbahn, 2000). Currently, four different PARs have been characterized: PAR1, PAR2, PAR3, and PAR4. There is growing evidence that TF:VIIa signaling is mediated mainly by PAR2 and to a lesser extent by PAR1, while PAR3 and PAR4 do not seem to be involved. For instance, in fibroblasts derived from PAR1 knockout mice, the FVIIa-induced response only occurred when TF and PAR2 were co-expressed (Camerer et al., 2000). Moreover, data obtained in a carcinoma cell line demonstrate that antibodies targeting PAR2, but not PAR1, blunt TF:VIIa-induced IL-8 expression and smooth muscle cell migration (Marutsuka et al., 2002).

Even though not essential for TF:VIIa-induced activation of MAP kinases and gene expression, the cytoplasmic domain of TF is implicated in cytoskeletal organization and cell migration (Bromberg et al., 1995; Mueller & Ruf, 1998). Protein kinase C-mediated phosphorylation of the cytoplasmatic domain at the serine residues Ser253 and Ser258 enables the interaction of the cytoplasmatic tail with the actin-binding protein 280 thereby regulating actin filament rearrangement (Ott et al., 1998; Zioncheck et al., 1992). Studies performed in mice lacking the cytoplasmatic tail of TF demonstrated the crucial role of the cytoplasmatic domain for vascular smooth muscle migration and vascular remodeling after injury *in vivo* (Ott et al., 2005).

Since increasing evidence suggests an essential role of TF as an important signal transducer beyond its well-known functions in coagulation, developing specific inhibitors that suppress either TF:VIIa-mediated signaling or its coagulant function may offer interesting therapeutic approaches for the treatment of TF-associated diseases.

III. TF in Cardiovascular Disease

A. TF Distribution

1. Vascular TF

Under normal hemostatic conditions, TF is only barely expressed in endothelial cells (Fig. 2). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (Holy et al., 2009; Steffel et al., 2005b) or interleukin-1β (Napoleone et al., 1997) enhance TF expression. This induction of TF protein is mainly mediated by the MAP kinases p38, extracellular signal-regulated kinase (ERK), and JNK which activate transcription factors such as nuclear factor kappa B, activator protein-1, or early growth-response gene product-1, all of them involved in transcription of the TF gene (Bavendiek et al., 2002; Mackman, 1997; Mechtcheriakova et al., 2001).

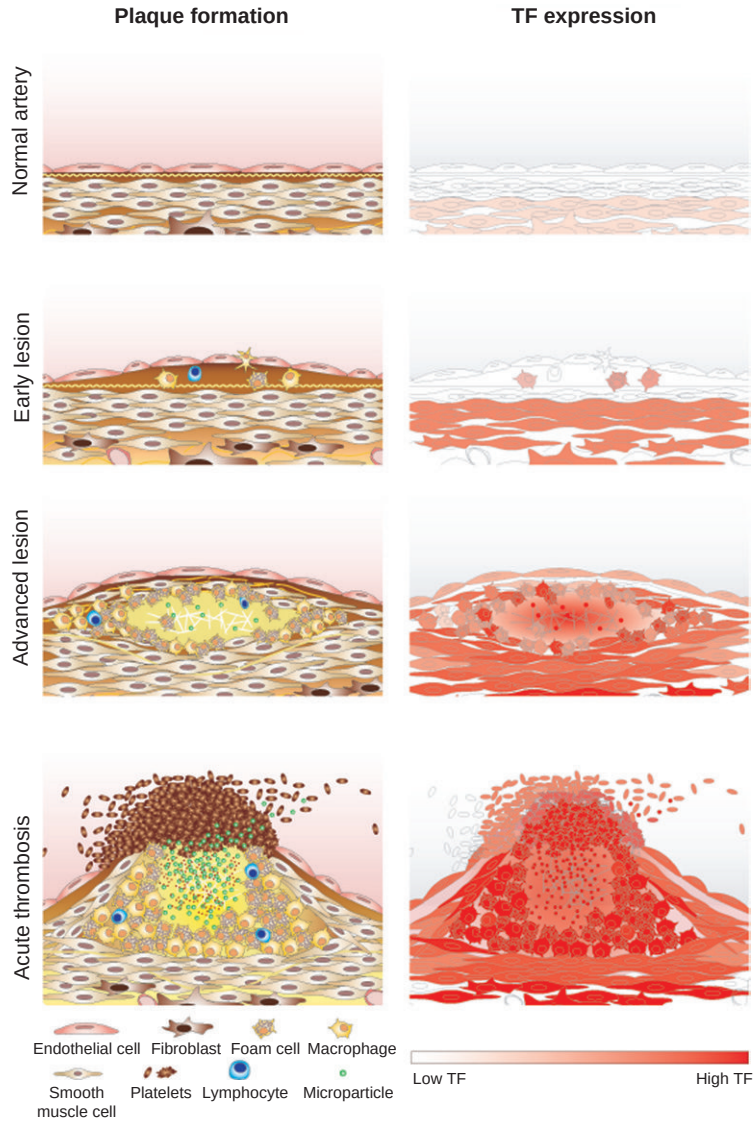

FIGURE 2 Expression of TF in different cell types during the pathogenesis of atherosclerotic lesions. During plaque progression, the inflammatory environment triggers TF expression in endothelial cells, vascular smooth muscle cells, and macrophages/foam cells. On plaque rupture, the procoagulant material including TF-containing microparticles located in the necrotic core is released into the blood, leading to rapid initiation of coagulation, thrombus formation, and vessel occlusion.

Depending on the stimulus, induction of TF expression occurs via activation of either all three MAP kinases (Holy et al., 2009; Steffel et al., 2005a, 2006a and b) or only a single pathway (Mechtcheriakova et al., 2001). Protein kinase C and the Rho-kinase pathway have also been shown to mediate the induction of endothelial TF expression (Eto et al., 2002; Mechtcheriakova et al., 2001; Zhang et al., 2007). Whereas the MAP kinase, Rho-kinase, and protein kinase C pathways regulate TF expression in a positive manner, the PI3k pathway exerts a negative regulatory effect on TF expression (Blum et al., 2001; Steffel et al., 2006b). Indeed, downstream targets of PI3k are able to regulate TF expression at both the transcriptional and the translational level. Akt and glycogen synthase kinase 3β regulate TF transcription (Eto et al., 2005), whereas the mammalian target of rapamycin and p70S6 kinase inhibits TF translation (Guba et al., 2005; Steffel et al., 2005b). An additional mechanism affecting the regulation of TF protein expression is stabilization of TF messenger RNA. A recent study in endothelial cells demonstrated that a decrease in TF promoter activity may be counteracted by an increased TF mRNA stability leading to an overall enhanced TF protein expression (Holy et al., 2009).

In contrast to endothelial cells, vascular smooth muscle cells constitutively express TF providing a hemostatic barrier after vascular injury (Breitenstein et al., 2008; Camici et al., 2006; Schecter et al., 1997; Wilcox et al., 1989). Similar to endothelial cells, TF expression in vascular smooth muscle cells is enhanced by several receptor ligands such as CD40 ligand, histamine, thrombin, bacterial endotoxin, oxidized low-density lipoprotein (LDL), or C-reactive protein (Camici et al., 2006; Kamimura et al., 2004; Llorente-Cortes et al., 2004; Taubman et al., 1993; Wu et al., 2008). Even though not investigated to the same extent as in endothelial cells, the MAP kinase and phosphoinositide 3-kinase pathways regulate TF expression also in vascular smooth muscle cells.

While the intracellular TF pool is only released following cellular damage, biologically active TF is located at the cell surface (Camera et al., 1999; Schecter et al., 1997). Discrepancies between TF protein expression and activity have been described and seem to be due to the role of a functionally inactive so-called encrypted form of TF at the cell surface. Encrypted TF enables a cell to rapidly increase TF activity in response to stimulatory factors without contribution of *de novo* protein synthesis. In fact, de-encryption of TF has been observed following changes such as intracellular calcium levels or changes structure of TF (Wolberg et al., 1999). Hence, the procoagulant effect induced by a stimuli is determined by the relative contribution of TF protein synthesis, structure, and cellular localization.

2. *Blood-Borne TF*

More recently, TF has been detected at low levels in plasma and urine, and these levels increase in several inflammatory disease conditions such as

atherosclerosis, disseminated intravascular coagulation, glomerulonephritis, sepsis, or diabetes.

Monocytes are thought to represent a major source of the plasmatic TF pool. Monocytes constitutively express TF and its expression is enhanced after exposure to numerous inflammatory mediators (Cai et al., 2007; Cermak et al., 1993; Guha & Mackman, 2002; Lewis et al., 1995; Luyendyk et al., 2008; Mach et al., 1997). Stimulation of monocytes with bacterial lipopolysaccharide induces TF expression *in vitro* and *in vivo*, and it has been shown that TF expression by hematopoietic cells contributes to activation of coagulation in endotoxemic mice (Pawlinski & Mackman, 2010; Pawlinski et al., 2010). Elevated levels of TF have been measured in plasma during septic states and may account for the thrombotic complications encountered in those patients (Osterud & Bjorklid, 2001; ten Cate, 2000). Endotoxin-induced TF expression is mediated via MAP kinases (Guha & Mackman, 2002; Lewis et al., 1995) and the consecutive activation of the transcription factors Egr-1, c-Fos/c-Jun, and nuclear factor kappa B. In addition, endotoxin also increases TF mRNA stability (Brand et al., 1991).

During atherosclerotic plaque formation, monocytes infiltrate the intimal layer where they transform into macrophages and foam cells (Hansson, 2005). This represents a key step in the inflammatory alterations associated with atherosclerosis. In this inflammatory environment, cytokines such as TNF-α are released and induce expression of TF. During the early stages of atherogenesis, enhanced TF expression is indeed observed in monocytes and at later stages also in foam cells (Steffel et al., 2006b).

TF has also been described in circulating granulocytes. Eosinophils, neutrophils, and basophils have been found to express TF (Kambas et al., 2008; Maugeri et al., 2006a, 2006b; Moosbauer et al., 2007; Muller et al., 2003). Both the transcription and the translocation of TF to the cell surface are enhanced upon stimulation of these cells with granulocyte/macrophage colony-stimulating factor or platelet-activating factor (Moosbauer et al., 2007). These findings may explain the pro-thrombotic state associated with hypereosinophilia (Cugno et al., 2009).

Even though TF-containing platelets have been described by several groups (Falati et al., 2003; Muller et al., 2003; Panes et al., 2007; Perez-Pujol et al., 2005; Zillmann et al., 2001) the role of platelet-derived TF remains debatable (Butenas et al., 2005). In platelets, TF is encountered in different compartments including the membrane, the matrix of α-granules, and the canalicular system; it is translocated to the cell surface upon activation by different agonists. Since TF messenger RNA is not found in human megakaryocytes, the source of platelet-derived TF remains a matter of debate. One hypothesis suggests that TF-containing microparticles derived from activated monocytes, leucocytes (Rauch et al., 2000), or endothelial cells (Aras et al., 2004; Camera et al., 2003) fuse with the platelet membrane

through a receptor-mediated process (Falati et al., 2003; Rauch et al., 2000) and thereby deliver TF into platelets. However, other studies demonstrated the presence of a spliced TF mRNA in activated human platelets confirming an increased TF protein synthesis and activity in ADP-stimulated platelets (Camera et al., 2003).

TF-containing microparticles have been well characterized in plasma. Besides endothelial and vascular smooth muscle cells (Abid Hussein et al., 2003; Lechner et al., 2007; Moosbauer et al., 2007; Schecter et al., 2000), monocytes and platelets are the main source of TF-containing microparticles (Hron et al., 2007; Siddiqui et al., 2002). Despite studies demonstrating the role of microparticle-derived TF in the thrombin generation *in vitro* (Sturk-Maquelin et al., 2003), doubts remain about its contribution to thrombus formation *in vivo* (Biro et al., 2003; Day et al., 2005).

The full-length form of TF mRNA contains six exons and splicing of fifth exon results in a frameshift mutation generating a soluble alternatively spliced TF protein lacking the transmembrane domain (Bogdanov et al., 2003). Apart from plasma, alternatively spliced TF has been found in several tissues and cell types including the lung, placenta, vascular smooth muscle cells, and keratinocytes. Whereas it remains unclear whether alternatively spliced TF exerts pro-coagulant activity (Szotowski et al.,2005), more recent reports have revealed a role of alternatively spliced TF in angiogenesis by promoting endothelial cell migration and differentiation (Censarek et al., 2007; He et al., 2008).

3. *Cardiac TF*

In contrast to skeletal myocytes, cardiac myocytes express the full-length form of TF under basal conditions (Hartzell et al., 1989; Mackman et al., 1993). Studies performed in mice expressing low levels of TF revealed a potential role of TF in the maintenance of cardiac hemostasis, since these mice had increased cardiac hemorrhages and hemosiderin deposition ultimately leading to cardiac fibrosis and a reduction in left ventricular function as compared to wild-type animals (Pawlinski et al., 2002). Phagocytosis of hematin derived from hemorrhaging erythrocytes is thought to play a key role in this process. Interestingly, mice lacking factor IX had normal hearts, underlining the specific role of the TF:VIIa complex in hemostatic protection of the heart.

B. TF in Cardiovascular Disease

1. *TF in Thrombus Formation*

Although TF is critically involved in initiation of the coagulation cascade, questions regarding the role and contribution of the different TF pools in arterial thrombus formation remain open.

Under normal hemostatic conditions, TF is constitutively expressed in the vascular smooth muscle layer of the media and in fibroblasts present in the vessels as well as in the adventitia surrounding the vessels (Drake et al., 1989; Fleck et al., 1990). Hence, disruption of blood vessel integrity promotes the interaction between circulating FVII and the vascular TF pool leading to initiation of the clotting cascade (Fig. 2). Moreover, the release of inflammatory triggers following vascular injury or plaque rupture induces the expression of TF in endothelial cells as well as in circulating cells thereby potentiating a full coagulation response (Steffel et al., 2006b). In contrast to the initiation phase of coagulation, the mechanisms promoting the propagation of clot growth are not as evident yet.

Blood-borne TF may contribute to the propagation of the clotting reaction. This consideration is based on the fact that the maintenance of coagulation requires a constant activity of TF as a cofactor and that the formed fibrin clot isolates and prevents the initially formed TF:VIIa complexes from reacting with circulating new inactive coagulation factors (Hathcock & Nemerson, 2004). This hypothesis is supported by the observation that TF is detected at very low levels in the blood unable to trigger a coagulant response, whereas the TF concentration reaches functionally significant levels on the surface of a forming thrombus (Bogdanov et al., 2003; Chou et al., 2004; Jesty & Beltrami, 2005). In addition, TF has been found embedded in murine (Day et al., 2005; Hoffman et al., 2006) and human (Wysokinski et al., 2004) thrombi. The role of blood-borne TF in coagulation is strengthened by an *in vitro* study demonstrating that the sub-picomolar TF concentrations found in blood cause an increase in thrombin formation under flow, but not under no-flow conditions (Okorie et al., 2008).

On the other hand, recent experiments performed in mathematical as well as whole blood models revealed different stages of TF requirement in the clotting process and showed that the propagation of coagulation is TF independent once thrombin has started to be formed (Orfeo et al., 2005). By abrogating TF activity with specific antibodies targeting either VIIa or TF, three phases in the clotting process could be distinguished: a first short (<10 s) period of absolute TF:VIIa dependence; a second period (10–240 s) of partial TF:VIIa dependence that decreases with the progress of the reaction, and a period which is TF:VIIa independent beginning after 2 min. In addition, this study revealed that the initial activation of procoagulant proteases by the TF:VIIa complex is sufficient to maintain a pool of activated procoagulant catalysts able to maintain a sustained coagulation response in a TF-independent manner.

Since venous thrombosis is not always associated with vessel damage, circulating TF may play an important role in this pathology. In a model of jugular vein thrombosis inhibition of TF reduced thrombus growth in the uninjured vein (Himber et al., 2003). However, in another study,

hematopoietic cell-derived TF did not affect venous thrombosis in an inferior vena cava thrombosis model in the mouse (Day et al., 2005). Hence, the exact role of the different TF pools and their contributions to the propagation of clot formation remains controversial and further studies are definitely required.

2. *TF in Neointima Formation and Atherosclerosis*

Recent studies implicate that TF and TF:VIIa signaling may also play a role in the pathogenesis of atherosclerosis. The TF:VIIa complex has been shown to be a strong chemotactic stimulus for vascular smooth muscle cells (Sato et al., 1996). Inhibition of TF:VIIa by overexpression of TF pathway inhibitor leads to a consecutive reduction of vascular smooth muscle cell migration and impairs vascular remodeling in the mouse *in vivo* (Jang et al., 1995; Sato et al., 1999; Singh et al., 2001). Since TF:VIIa-mediated cell signaling induces a number of gene products involved in cell proliferation and migration, it is very likely that similar effects occur in TF expressing cells within atherosclerotic lesions. TF:VIIa signaling indeed enhances the expression of the connective tissue growth factors CCN1 and CCN2 in fibroblasts and vascular smooth muscle cells. Similar to TF, CCN1 and CCN2 mRNA levels are highly expressed in atherosclerotic lesions of apoE$^{-/-}$ mice (Siegbahn et al., 2000) and in human atherosclerotic plaques (Oemar & Luscher, 1997; Oemar et al., 1997). Since CCN1 regulates proliferation of vascular smooth muscle cells and neointimal hyperplasia in a rat carotid artery balloon injury model (Matsumae et al., 2008), it is conceivable that by inducing the expression of CCN1 and CCN2, TF:VIIa promotes plaque progression via enhanced extracellular matrix accumulation and vascular smooth muscle cell proliferation. In fibroblasts binding of factor VIIa to TF leads to an enhanced activation of platelet-derived growth factor-BB-stimulated chemotaxis (Siegbahn et al., 2000) providing an additional explanation for the value of TF:VIIa inhibition in preventing neointima formation (Jang et al., 1995). Finally, a role of CCN1 and CCN2 in monocyte and platelet adhesion to the subendothelial matrix after endothelial injury has also been described (Jedsadayanmata et al., 1999; Schober et al., 2002).

Inflammation is tightly linked to the development of atherosclerosis (Hansson, 2005). Elevated levels of inflammatory cytokines known to induce TF expression such as TNF-α and interleukins are observed in early atherosclerotic lesions. In the early stages of atherogenesis, TF mRNA and antigen are expressed in macrophages (Thiruvikraman et al., 1996; Wilcox et al., 1989), and as the lesions progress, TF is increasingly expressed in other cell types such as endothelial cells and smooth muscle cells (Fig. 2). Within the necrotic core (Marmur et al., 1996), it is predominantly associated with TF-containing microparticles released by foam cells, monocytes, lymphocytes, and smooth muscle cells; in addition, it is detected

in the extracellular space (Mallat et al., 2000). During plaque rupture, the content of the core comes in contact with the circulating blood, and TF present within the plaque as well as expressed by activated endothelial and smooth muscle cells triggers thrombus formation leading to an acute vascular event. Indeed, higher levels of TF are measured in atherosclerotic plaques of patients suffering from an acute coronary syndrome as compared to patients exhibiting stable angina (Annex et al., 1995).

3. TF in Inflammation

In response to inflammatory mediators, TF transcription can be induced in different cell types. In the context of myocardial ischemia/reperfusion injury, which is characterized by an extensive inflammatory response, TF expression is found to be highly upregulated in cardiomyocytes and blockade of TF activity consequently reduced myocardial infarction area (Chong et al., 2003). On the other hand, TF expressed on these cells appears to regulate the inflammatory response. During sepsis, the TF pathway induces a lethal inflammatory exacerbation independent of thrombin generation and clot formation (Creasey et al., 1993; Taylor et al., 1991). Moreover, under these conditions, inhibition of the TF:VIIa complex is paralleled by a decrease in interleukin secretion and a reduction in inflammatory infiltrations in several tissue (Miller et al., 2002). This finding is confirmed by the observation that mice suffering from sepsis and expressing low TF levels display reduced interleukin levels and an increased survival compared with control mice (Pawlinski et al., 2004). Studies performed in PAR2-deficient mice suggest that the pro-inflammatory TF:VIIa signaling response is likely mediated via PAR2 (Pawlinski et al., 2004). However, it is worth noting that deficiency in PAR1 or PAR2 alone has no effect and that a reduction in inflammation and mortality is only observed in the context of a combined inhibition of thrombin generation and PAR2 receptor, indicating a tight link between coagulation and inflammation.

4. TF in Angiogenesis

First evidence demonstrating a role of TF in embryonic angiogenesis arises from studies performed in TF knockout mice showing embryonic lethality around day 10.5 after conception as a result of hemorrhage of the yolk sac vasculature (Carmeliet et al., 1996; Toomey et al., 1996). In contrast, mice lacking only the cytoplasmic tail of TF show normal embryonic development (Melis et al., 2001). Furthermore, mice deficient in the intracellular TF domain exhibit enhanced angiogenesis in a murine xenograft model (Belting et al., 2004). Experiments in mice lacking PAR2 and the cytoplasmic tail of TF demonstrated that the enhanced angiogenic phenotype is PAR2 dependent, since PAR2 signaling leads to protein kinase C α activation and downstream phosphorylation of the cytoplasmic TF tail. In diabetic patients, phosphorylation of the intracellular TF domain and

expression of PAR2 are tightly associated with pathological proliferative neovascularization in the retina (Belting et al., 2004). These observations suggest a suppressive role of the dephosphorylated intracellular TF domain, which is lost following PAR2-dependent phosphorylation of TF and may play a role in the development of vascular disorders such as diabetic retinopathy.

TF upregulation in the endothelium has been described in the context of tumor progression and vascularization (Abdulkadir et al., 2000; Koomagi & Volm, 1998). Tumor cell transfection with antisense TF, injection of TF blocking antibodies, or inactivated factor VIIa have been demonstrated to inhibit tumor growth and metastasis in murine xenograft models (Mueller & Ruf, 1998; Mueller et al., 1992; Zhang et al., 1994). In this context, a tight correlation between the expression of TF and the proangiogenic molecule vascular endothelial growth factor (VEGF) expression has been described in several tumors, and both TF and VEGF are upregulated in tumor tissues (Abe et al., 1999; Bromberg et al., 1999; Shoji et al., 1998). TF can indeed upregulate VEGF and, in return, VEGF upregulates endothelial TF expression via Egr-1(Mechtcheriakova et al., 1999; Takano et al., 2000).

5. *TF in Acute Coronary Syndromes*

High levels of TF have been detected in coronary atherectomy specimens from patients with unstable angina or myocardial infarction (Ardissino et al., 1997). In line with this, plasma TF levels are elevated in patients with acute coronary syndromes compared with controls (Misumi et al., 1998; Soejima et al., 1996) and correlate with an unfavorable outcome in these patients (Soejima et al., 1999). In addition, patients with unstable angina or non-ST-elevation myocardial infarction and a high TIMI score (≥ 4) exhibit increased TF plasma levels as compared to those with a low TIMI score (<3) (Lee et al., 2005). At the same time, another study revealed that inducible myocardial ischemia during dobutamine stress testing increases plasma levels of biomarkers, notably TF (Ikonomidis et al., 2005). In line with this observation, increased TF mRNA, antigen, and activity are observed in ischemic cardiomyocytes isolated from both rabbits and mice after in situ coronary artery ligation (Chong et al., 2003).

Percutaneous transcoronary angioplasty and coronary stent implantation has become the gold standard in the treatment of culprit lesions in acute coronary syndromes. The local inflammatory reaction following balloon dilatation and stent implantation (Brunetti et al., 2007) induces the release of TF from the dissected plaque. Thus, elevated levels of TF positively correlate with the incidence of restenosis after percutaneous interventions, presumably due to promigratory effects of TF on vascular smooth muscle cells (Tutar et al., 2003). Despite the reduction in restenosis following deployment of drug eluting stents (DES) when compared to bare metal stents, stent thrombosis rates have not been decreased with the use of DES

and may even be higher (Iakovou et al., 2005). One possible explanation may be that the drugs eluting from the stents which are intended to inhibit restenosis by reduction of vascular smooth muscle cell migration and proliferation exert unwanted biological effects on these or other cell types in the vasculature, in particular endothelial cells. The drugs applied for coating the first- and second-generation stents, like rapamycin, everolimus, or zotarolimus, enhance TF expression in vascular cells and accelerate arterial thrombus formation via inhibition of the mTOR/p70S6K pathway (Camici et al., 2010; Steffel et al., 2005b). Similarly, paclitaxel, another widespread stent coating drug, was shown to increase endothelial TF expression and activity through activation of the MAP kinase JNK (Stahli et al., 2006). Hence, these findings may raise important issues in the context of DES design since coating a stent with a drug exerting not only antiproliferative but also antithrombotic properties may offer new options in the interventional treatment of vascular disease.

6. *TF and Cardiovascular Risk Factors*

Diabetes mellitus is associated with an increase in plasmatic TF activity (El-Ghoroury et al., 2008), TF antigen levels (Lim et al., 2004), and TF containing microparticles (Diamant et al., 2002). Consistent with this, high glucose increases TF antigen and activity in monocytes derived from healthy subjects (Stegenga et al., 2006; Vaidyula et al., 2006). A similar effect of high glucose on TF expression was also observed in human endothelial cells (Boeri et al., 1989). In diabetes, hyperglycemia induces the formation of advanced glycation end products (AGE), which upregulate TF expression in endothelial cells and monocytes via nuclear factor kappa B (Bierhaus et al., 1997a, 1997b; Kislinger et al., 2001). Not surprisingly, higher levels of AGE and TF were observed in the vasculature of diabetic mice, and blockade of the receptor for AGE significantly reduced the increased TF expression in the aorta of these mice (Kislinger et al., 2001). Ligands of the peroxisome proliferator-activated receptor (PPAR) family have been introduced in the treatment of metabolic disorders, in particular insulin resistance (Lalloyer & Staels, 2010). Besides their regulatory effect on genes involved in lipid metabolism and lipid peroxidation, PPAR agonists also exert anti-inflammatory and anti-thrombotic properties in vascular cells (Israelian-Konaraki & Reaven, 2005; Marx et al., 2004). In line with these observations, application of oral antidiabetics such as the PPAR-γ agonist rosiglitazone inhibits TF protein expression (Golledge et al., 2007) in these patients.

Patients with hyperlipidemia display an increased risk of developing thrombosis (Durrington, 2003). Elevated LDL levels are associated with an increased TF plasma activity (Sambola et al., 2003). Oxidized LDL triggers TF protein synthesis in monocytes (Wada et al., 1994), endothelial cells (Fei et al., 1993), and vascular smooth muscle cells (Penn et al., 1999). In contrast, high-density lipoproteins reduce the thrombin-induced TF

expression in endothelial cells through PI3K pathway activation (Viswambharan et al., 2004). Moreover, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) reduce TF expression in monocytes (Markle et al., 2003), endothelial cells (Eto et al., 2002), and vascular smooth muscle cells (Brandes et al., 2003). These findings have been confirmed by studies performed in $ApoE^{-/-}$ mice in which simvastatin and rosuvastatin treatment reduced TF antigen expression in atherosclerotic lesions independent of their lipid-lowering effects (Bea et al., 2003; Monetti et al., 2007). Fibrates, which are also in use as lipid-lowering drugs in the treatment of hyperlipidemia, also exert pleiotropic effects on vascular TF expression beyond their lipid-lowering properties (Marx et al., 2001).

In contrast to normotensive subjects, hypertensive patients display increased plasma levels of TF antigen (Felmeden et al., 2003), which can be lowered by an appropriate antihypertensive management (Koh et al., 2004). Several mechanisms are thought to mediate this enhanced TF expression observed in arterial hypertension. For instance, the chronic exposure to shear stress induces the activation of transcription factors and consequently the expression of TF in endothelial cells (Dielis et al., 2005). On the other hand, angiotensin II increases TF expression in monocytes (He et al., 2006), endothelial cells (Dielis et al., 2005), and vascular smooth muscle cells via the angiotensin II type 1 receptor. Consistent with this observation, ACE inhibitors and angiotensin II type 1 receptor antagonists reduce TF plasma activity in hypertensive patients and inhibit endotoxin-induced TF expression in monocytes (Napoleone et al., 2000).

IV. Therapeutic Implications

The fate of a forming thrombus is determined by the balance of numerous factors, including vessel wall, coagulation system, and aggregating platelets. In addition to the classical antithrombotic agents, drugs targeting TF and the TF:FVIIa complex have been developed, since targeting the initiation phase of thrombosis may prevent thrombus formation more efficiently. Inhibition of TF involves mainly two different strategies. The first one interferes with the synthesis of TF in vascular cells, which may occur at both the transcriptional and the post-transcriptional level. The second and up to now therapeutically most advanced approach involves direct inhibition of TF:VIIa activity. Considering the non-hemostatic pro-migratory and pro-proliferative properties of the TF:VIIa complex, inhibition of TF may also interfere with physiological and pathological remodeling responses. Despite promising experimental studies suggesting an effective reduction in thrombosis with a lower bleeding risk as compared to other coagulation inhibitors such as heparin or direct thrombin inhibitors, approaches to inhibit TF have not yet been applied successfully in humans. The following

chapter reviews current and potential TF-targeting strategies in the context of cardiovascular disease.

A. Inhibition of TF Production

Inhibition of TF production can be achieved by genetic and pharmacological intervention at both the transcriptional and the post-transcriptional level (Fig. 3). Hairpin ribozymes degrade TF mRNA and decrease TF induction in vascular smooth muscle cells (Cavusoglu et al., 2002). Antisense oligonucleotides hybridize to the complementary TF mRNA sequence and thereby inhibit translation of the TF protein (Stephens & Rivers, 1997). Dimethyl sulfoxide, used for long-term storage of hematopoietic stem cells, inhibits arterial thrombus formation by impairing TF activity (Camici et al., 2006). Further, cardiac glycosides such as digoxin and ouabain inhibit TF translation by lowering the intracellular potassium concentration through impaired Na^+/K^+-ATPase activity (Stahli et al., 2007). Even more interesting, the class III anti-arrhythmic drug amiodarone

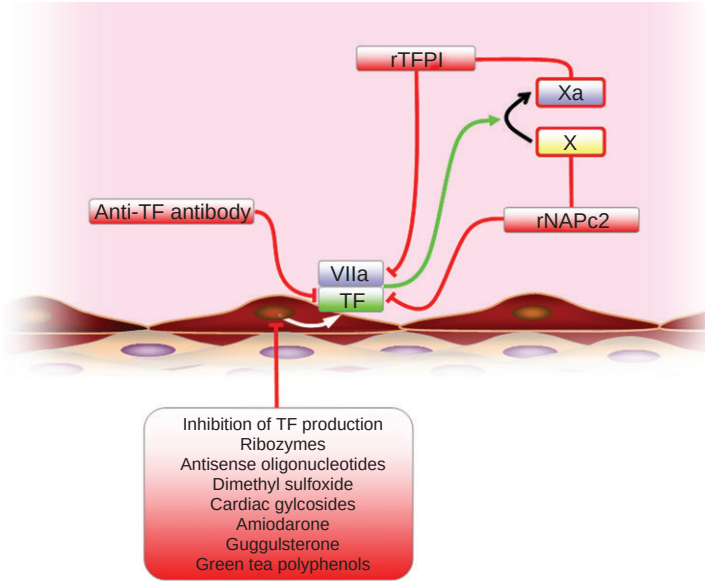

FIGURE 3 Different approaches for inhibition of TF. TF synthesis can be targeted at the transcriptional and the post-transcriptional level. Anti-TF antibodies target the FVIIa binding site, thereby inactivating the TF protein. Recombinant tissue factor pathway inhibitor (rTFPI) interferes with the activity of the TF/FVIIa complex by binding to the active site of factor Xa (FXa), leading to formation of an inhibitory complex. rNAPc2 interferes with the TF/FVIIa complex by binding to FXa or FX before formation of a quaternary inhibitory complex with TF/FVIIa.

inhibits the translation of the TF protein in human vascular cells and impairs arterial TF expression as well as arterial thrombus formation in mice *in vivo* (Breitenstein et al., 2008). These pleiotropic effects of amiodarone may at least in part account for the beneficial action of the drug on prognosis of patients with heart failure and coronary artery disease (Breitenstein et al., 2008). More recently, the phytosterol guggulsterone as well as the green tea polyphenol epigallocatechin-3-gallate, at concentrations detected in plasma after oral ingestion, were observed to inhibit TF induction through inhibition of MAP kinase activation in human vascular cells and to reduce TF expression in the carotid artery of mice *in vivo* (Gebhard et al., 2009; Holy et al., 2009).

These studies demonstrate that both synthetic and naturally occurring drugs can inhibit the induction of TF in human vascular cells as well as thrombus formation *in vivo*. Such effects may at least in part account for the established beneficial actions of the above-mentioned drugs on the prognosis of patients with cardiovascular disease. Since these studies focussed mainly on the antithrombotic effects of TF inhibition, the impact of those drugs on TF-dependent angiogenesis and neovascularization requires further assessment. Clinical trials assessing the application and the safety of these drugs in the context of TF inhibition, thrombus prevention, vascular remodeling, and angiogenesis should be performed. Ideally, the drugs would have to be applied in a targeted local manner in order to achieve a maximal effect in the vessel territory at risk for thrombosis while reducing systemic bleeding complications.

B. Anti-TF Antibodies

Administration of anti-TF antibodies (Fig. 3) delays thrombus formation in a rabbit carotid thrombosis model and reduces the infarction area following ligation of coronary arteries (Erlich et al., 2000; Ragni et al., 1996). A clinical trial (PROXIMATE-TIMI 27) performed in 26 subjects suffering from stable coronary disease tested the safety of a chimeric monoclonal antibody targeting human TF at the factor X binding site (Sunol-cH36/ALT836) (Morrow et al., 2005). Safety analysis focussed on overt or occult bleeding. Sunol-cH36/ALT836 was administrated as an intravenous bolus (0.03, 0.06, 0.08, 0.1, 0.3 mg/kg) to three to eight subjects per dose. Sunol-cH36/ALT836 exhibited dose-dependent anticoagulant effects. No major bleeding was observed in any of the groups. Spontaneous minor bleedings occurred in a dose-dependent manner and were related to platelet-mediated bleeding without thrombocytopenia. More recently, application of Sunol-cH36/ALT836 after endarterectomy in chimpanzees reduced local thrombus formation and improved vessel patency after 30 days without increasing bleeding time or surgical blood loss, indicating that

early inhibition of coagulation with TF inhibitory antibodies reduces arterial thrombus formation effectively after vascular injury (Jiao et al., 2010).

Considering the role of TF signaling in tumor angiogenesis, growth, and metastasis, inhibition of TF may also translate into anti-neoplastic effects. Indeed, administration of a monoclonal anti-TF antibody (CNTO 859) reduced breast cancer metastasis and tumor growth in xenograft models (Ngo et al., 2007).

C. Recombinant TF Pathway Inhibitor

TF pathway inhibitor (TFPI) is the physiological inhibitor of TF activity and the balance between TF and TFPI is critical for thrombus formation (Pedersen et al., 2005). By binding the active site of FXa, TFPI interferes with the activity of the TF:VIIa complex (Fig. 3). Administration of recombinant TF pathway inhibitor (rTFPI) inhibits thrombus formation at the site of balloon-induced arterial injury and reduces plaque thrombogenecity in humans (Badimon et al., 1999; Harker et al., 1996; St Pierre et al., 1999). In addition, local adenoviral overexpression of TFPI in arteries increases the resistance to thrombus formation (Nishida et al., 1999) and reduces intimal hyperplasia in injured vessels (Zoldhelyi et al., 2001). Despite these positive actions, application of rTFPI may be limited because of its apoptotic effects observed in cultured endothelial and vascular smooth muscle cells, which may contribute to instability of atherosclerotic plaques (Fu et al., 2008; Hamuro et al., 1998). Moreover, a randomized controlled study including 1754 patients with severe sepsis and high international normal ratio found no effect on all-cause mortality (Abraham et al., 2003). Therefore, additional trials are needed with patients exhibiting cardiovascular diseases other than severe sepsis and being in a better general condition.

D. Nematode Anticoagulant Protein

Recombinant nematode anticoagulant protein c2 (rNAPc2), isolated from the saliva of the hematophagous hookworm *Ancylostoma caninum*, is a factor Xa-dependent small protein inhibitor of the TF:VIIa complex which binds to a site on factor Xa that is distinct from the catalytic center (exo-site) (Bergum et al., 2001). In a double-blind placebo-controlled trial, rNAPc2 was found to be safe and well tolerated at doses ranging from 0.3 to 5 μg/kg body weight in preventing thrombin generation during coronary angioplasty in combination with aspirin, clopidogrel, and heparin (Moons et al., 2003). A more recent phase II clinical study (ANTHEM-TIMI-32) demonstrated that in patients with non-ST-elevation acute coronary syndrome managed with standard antithrombotics and an early invasive

approach, additional proximal inhibition of the coagulation cascade with rNAPc2 was well tolerated and not associated with an increase in major or minor bleedings as compared with a placebo treatment (Fluture et al., 2007; Giugliano et al., 2007). A novel finding in the ANTHEM-TIMI-32 trial was that the doses of rNAPc2 suppressing thrombin generation through 48 h were associated with a $\geq 50\%$ reduction in ischemia on continuous ECG. This provides a link between the mechanism of action of this drug and the hypothesized benefit in patients with plaque rupture and vessel wall injury. However, the trial was not powered for such an assessment and larger trials are required to confirm the hypothesis that rNAPc2 reduces clinical events. Since rNAPc2 has a half-life of 50–60 h, a inhibition of thrombin generation by rNAPc2 may improve the current discharge regimen after ACS. However, immune responses after administration may limit its clinical application, since rNAPc2 is a non-human protein. Anti-rNAPc2 immunoglobulins were indeed observed in one subject out of 20 patients receiving rNAPc2. Although these antibodies were not biologically active and without clinical relevance, the fact that they occurred in a high percentage of patients will have to be considered in future trials.

V. Conclusion

Major progress has been achieved in decrypting the biological effects and clinical relevance of TF in the context of cardiovascular disease. TF is a transmembrane glycoprotein, the main trigger of coagulation, and, beyond this, an intracellular signal transducer. Several signaling pathways, specific for both the cell type and the stimulus, regulate TF induction and its cellular distribution. The discovery of blood-borne TF has launched a new debate about its *in vivo* relevance, since the relative contribution of vessel wall as compared to blood-borne TF for thrombus formation and propagation remains unclear.

Elevated levels of TF activity are present in patients with cardiovascular risk factors and in subjects suffering from coronary artery disease. TF expression is upregulated in atherosclerotic plaques, and cellular TF and extracellular TF contained in microparticles come in contact with the blood during endothelial erosion or plaque rupture. Therefore, TF contributes critically to the development of acute vascular events such as myocardial infarction or stroke. On the other hand, TF may promote the progression of atherosclerosis by enhancing vascular smooth muscle cell migration and proliferation.

Since targeting the initiation phase of the clotting cascade may be more effective in preventing thrombus formation than at later stages, several strategies have been developed to inhibit TF expression or activity. Despite promising experimental results, the clinical benefit of these new approaches

in comparison with current antithrombotic regimens remains to be demonstrated in large-scale trials. Since the major part of TF activity is cell associated and induced by vascular inflammation, a targeted local inhibition of TF may well prove to be the most effective approach with a minimal effect on the systemic balance of thrombosis and bleeding.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Abbreviations

asTF	alternatively spliced tissue factor
Egr-1	early growth-response protein 1
ERK	extracellular signal regulated kinase
JNK	*c*-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
PAR	protease-activated receptor
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3 kinase
PPAR	peroxisome proliferator activated receptors
rNAPc2	recombinant nematode anticoagulant protein c2
TF	tissue factor
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

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