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Preface

This volume contains selected lectures presented at the 12th International Conference on Advances in Prostaglandin, Leukotriene and Other Bioactive Lipid Research: Basic Science and Clinical Applications which was held in Istanbul, Turkey, on August 25-29, 2002.

This meeting brought together basic and clinical scientists for the purpose of discussing advances in bioactive lipid research with special attention to cancer, cardiovascular diseases, gastrointestinal diseases and respiratory diseases. Topics covered included: the role of leukotrienes and lipoxins in inflammation, the cytochrome P450 pathway, the genetics and genomics of bioactive lipids, lipid peroxidation, apoptosis, angiogenesis, isoprostanes, receptors and inhibitors, cyclooxygenase and lipoxygenase pathways and inhibitors, prostaglandin synthases and receptor signaling, phospholipases and inhibitors. Sessions included plenary lectures with expertise in particular areas, oral presentation on selected topics and general poster sessions. J.M. Drazen (Boston, USA) discussed anti-leukotriene treatment in asthma patients while C. Brink (Paris, France) presented the recent advances in leukotriene receptors. The recent advances in cytochrome p450 pathway described in the session organized by J.C. McGiff (Valhalla, NY, USA). T. Shimizu (Tokyo, Japan) and M. Balazy (Valhalla, NY, USA) gave an update on phospholipases and arachidonic acid peroxydation.

The editors are greatful to the Organizing, Programme and Advisory Committees for their valuable contributions. We greatfully acknowledge the generous financial support provided by Pharmacia/Pfizer, Fako Pharmaceuticals Inc. and Novartis Pharmaceuticals Inc.

We are also greatful to all of the contributors to this volume, in particular to those who delivered their manuscripts by or before the requested deadline.

We dedicate this volume to Professors Alan Bennett and Simonetta Nicosia who passed away since the last gathering. It represents a snapshot of the field including a substantial amount of new data. We hope it will be a useful reference for investigators in both basic and clinical research.

Z. Yazıcı, G. Folco, J.M. Drazen,
S. Nigam, and T. Shimizu

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Chapter #1

LEUKOTRIENES IN ASTHMA

Jeffrey M. Drazen

One of the major reasons for pursuing the chemical structure of the biological material known as slow-reacting substance of anaphylaxis (SRS-A) was that this material was known to be a potent bronchoconstrictor substance in guinea pigs [1] and in isolated human airways [2]. Thirty years ago, the simple concept was that SRS-A was released from sensitized cells following antigen sensitization and challenge, and that the released material transduced a signal at an as yet to be identified receptor leading to smooth muscle constriction and therefore the manifestations of human asthma. It has been almost 25 years since the elucidation of the structure of SRS-A as a mixture of the cysteinyl leukotrienes (LT) [3] and just over 5 years since agents that act on leukotriene pathway have been available as asthma treatments [4,5]. What have we learned about asthma from the use of these agents and what is the role of these agents in the treatment of asthma?

Koch's Postulates

A set of criteria to prove that a substance has a role as a mediator in asthma, similar to the ones that Koch outlined to prove that a microbe was the cause of a disease, have not been formally advanced. Nevertheless, it is reasonable to assume that four conditions need to be met, namely the synthesis, the physiological effects, the recovery, and the effects of inhibition on asthma needed to be demonstrated.

By 1997, when the first agents to treat asthma by inhibiting the synthesis or action of the leukotrienes became available worldwide, the first three of these conditions had been met. It was known that many cell types could produce the cysteinyl leukotrienes but that only those that contained appropriate forms of phospholipase A_2, 5-lipoxygenase activating protein, 5 lipoxygenase and LTC_4 synthase could produce these agents without the

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cooperation of other cells [6]. Chemically produced leukotrienes were shown to be potent constrictors of human airways in vitro [7] and in vivo [8]. On average the leukotrienes were 1000 to 10,000 times more potent than histamine as bronchoconstrictor agonists. In addition, the bronchoconstriction induced by these agents persisted for 3 to 6 times as long as the equivalent degree of airway narrowing induced by inhalation of an aerosol of histamine. An end product of leukotriene metabolism [9], the cysteinyl leukotriene, LTE_4, had been recovered from the urine of patients during both induced and spontaneous asthmatic events [10,11]. Since patients with chronic stable asthma excreted LTE_4 in their urine [12], it seemed likely that there was chronic activation of the cysteinyl leukotriene pathway in patients with asthma. However we learned the most from the results of clinical trials in which agents active on the 5-lipoxygenase pathway were used as both treatments and disease probes.

Continuous Synthesis of the Leukotrienes by patients with asthma

Based on our understanding of the antigen sensitisation and challenge model, it was not unreasonable to think that the leukotrienes were produced only episodically rather than continuously. Studies with zafirlukast, showed that treatment with this $CysLT_1$ receptor antagonist resulted in an increase in the FEV_1 of about 8 percent [13]. Since, treatment with the 5-lipoxygenase inhibitor zilueton resulted in a 10-12 percent improvement in the FEV_1 [14], these data indicate that, in patients with mild-to-moderate chronic persistent asthma, there is continuous activation of the leukotriene pathway. Thus the stimuli that activate the pathway, which are still not known with certainty, must be active nearly continuously.

Population Variation in the Contribution of the Cysteinyl Leukotrienes to the Airway Obstruction of Asthma

In the studies of leukotriene recovery from the urine, there was substantial among subject in the amount of LTE_4 recovered [10,12]. This could reflect variation in the excretion of the leukotrienes, in the face of constant synthesis or variation among subjects in the availability of the leukotrienes to the $CysLT_1$ receptor. Data from a study comparing the effects of montelukast to that of beclomethasone on FEV_1 in patients with mild-to-moderate persistent asthma indicated that there is wide variance in the therapeutic effect of both agents [15]. About one quarter of the subjects improved their FEV_1 as a result of montelukast treatment, by 20 percent or more. Likewise another quarter of the subjects actually had a decrease in the FEV_1 over the treatment period. This variation could reflect among subject variation in the uptake, distribution and metabolism of this $CysLT_1$ receptor antagonist, or it could reflect variance among subjects in the role played by the cysteinyl leukotrienes in the biology of asthma. Since there is similar variance in the treatment response to zileuton [16], an agent with very little

among subject differences in uptake and metabolism [17], it does not seem unreasonable to conclude that there is substantial among subject variance in the contribution of the leukotrienes to the airway obstruction of asthma. There is no simple way to identify subjects with asthma in whom agents active on the leukotriene pathway are likely to be of therapeutic value. Genetic testing for polymorphism in the promoter regions of either the gene encoding 5-lipoxygenase [18] or the gene encoding LTC_4 synthase [19] offers promise, but since the allele frequencies of these genes are low, neither can explain a large fraction of the population variance in treatment response.

Comparative Studies of Agents Acting on the Cysteinyl Leukotriene Pathway and Inhaled Corticosteroids

There have been a number of clinical trials comparing the effects of inhaled steroids and the cysteinyl leukotriene receptor antagonists in asthma [15,20]. In these comparisons, inhaled steroids have been more effective. These observations demonstrate that the sum of the bronchoconstrictor pathways inhibited by corticosteroids, which have multiple potential targets, exceeds that directly attributable to the cysteinyl leukotrienes acting via the $CysLT_1$ receptor.

Additive Studies in which Agents acting on the Leukotriene Pathway are Added to Asthma Treatment

Another clinical trial design is to add an agent active on the leukotriene pathway to the asthma treatment regimen of a patient who is already receiving inhaled corticosteroids. Most [21-23], but not all [24], studies have shown an increment of positive benefit from adding anti-leukotrienes to the therapeutic regimen in such patients. These observations indicate that the spectrum of biological effects of corticosteroids does not include complete suppression of the synthesis or actions of the leukotrienes.

Conclusion

The leukotrienes contribute significantly to the airway obstruction of asthma, but they are not the sole mediator. Although there are likely other moieties that act in concert with the leukotrienes to produce this complex clinical picture, there have been no other successful interventions in which a single pathway is inhibited in either induced or spontaneous asthma [25-28]. Thus the possibility exists that there is no other single mediator pathway that is as critical as the leukotriene pathway to the airway obstruction of asthma.

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Chapter #2

LEUKOTRIENE RECEPTORS: STATE OF THE ART

Charles Brink

The leukotrienes (cysLTs) are lipid mediators derived from the ubiquitous membrane component arachidonic acid. The plethora of biological activities suggested that they activate different receptors. A number of elegant genetic and biochemical studies have been performed to elucidate the nature of these receptors. Results from classical pharmacological and molecular studies indicate that there are two main classes of leukotriene receptors. One based on the biological activities of leukotriene B_4 and related hydroxyacids, referred to as BLT receptors, and a second class identified by the cysteinyl-leukotrienes (cysLTs). Activation of the BLT receptors initially was shown to produce potent chemotactic activities on leukocytes whereas the latter class (CysLT receptors) stimulated smooth muscle as well as other cells. Furthermore, there is now sufficient information from classical pharmacological and biochemical assays as well as molecular investigations to divide both categories into receptor sub-classes, namely, BLT_1 and BLT_2 as well as $CysLT_1$ and $CysLT_2$ (Table 1).

However there are several pharmacological studies, which suggest that other receptor subtypes may also exist and this is particularly evident from the observations concerning principally CysLT receptors, since data with the BLT receptors appear less controversial.

The absolute values for the potencies of cysLTs were markedly similar between the recombinant molecular assays when compared with the classical pharmacological studies for both types of CysLT receptors. Interestingly, the $hCysLT_1$ receptor exhibited a greater ligand variability (LTD_4>LTC_4) when compared with the $CysLT_2$ receptor data (LTD_4=LTC_4). These results suggest that there may be differences in conformational aspects at the ligand-membrane level and/or a more effective coupling to the second messengers at the $CysLT_2$ receptor when compared with the $CysLT_1$ receptor. Furthermore, LTE_4 potencies are markedly different from that of LTC_4 and LTD_4 (Table 1) in spite of the extremely small structural difference

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between these native ligands and appeared to be independent of the assays. Such data support the early work [1,2] that various polar isomers with slight alterations in the structures of the native cysLTs affected both binding and potency of the agonists. Preliminary results based on human isolated pulmonary arterial smooth muscle supporting this notion have recently been published [3]. These preparations failed to respond to LTE_4 while LTC_4 and LTD_4 were equipotent. Since failure of a ligand to activate the CysLT receptor is extremely rare, the data suggest that perhaps another "novel CysLT receptor" exists in the human pulmonary arterial vasculature.

Table 1: Receptors, ligands and tissue distribution

BLT_1 [4]	
Ligands	LTB_4 > 20-OH-LTB_4 = 12-keto-LTB_4 > 12R-HETE > 20-COOH-LTB_4
Distribution	Leukocytes, spleen, thymus, lung
BLT_2 [5]	
Ligands	LTB_4 > 12-epi-LTB_4 > 12S-HPETE >12R-HETE > 20-hydroxy-LTB_4
Distribution	Spleen, liver, ovary, leukocytes
$CysLT_1$ [6,7]	
Ligands	LTD_4 > LTC_4 >>LTE_4
Distribution	Smooth muscle, spleen, peripheral blood leukocytes
$CysLT_2$ [8-10]	
Ligands	LTD_4 = LTC_4>>LTE_4
Distribution	Heart, leukocytes, spleen, kidney, ovary

Walch and co-workers [3] also treated arterial preparations with different antagonists and demonstrated that the selective $CysLT_1$ receptor antagonists (ICI 198615 and MK 571) blocked only the LTC_4 induced contractions and had no effect on the LTD_4 responses. These results show a preferential antagonism for one ligand and suggest that another receptor subtype may be present in the pulmonary vasculature. These antagonists are known to block the contractile effects of all the ligands in human airways [11] with a pK_B of approximately 8.5 whereas in the human pulmonary artery the pK_B was about 7. Such data suggest that there may be a low affinity CysLT receptor in the human pulmonary artery since the pK_B values are at least one order of magnitude different from the values obtained in the human airways. Of considerable interest was that the contractions induced by LTD_4 were not modified by any of the antagonists. These observations support the previous publications, which have shown a cysLT resistant contractile component in the guinea pig lung strip [12], as well as in the porcine [13] and human pulmonary artery [3,14]. These data indirectly suggest that another "novel functional CysLT receptor" may exist.

In HUVECs the concentrations of SKF 104353 used to inhibit the LTD_4 induced vWF were approximately 2 μM (estimated IC_{50}) [15] and therefore different from the pK_B values obtained in human airways (SKF 104353, 8.5). In addition, the inhibition of LTD_4 enhancement of EGF induce proliferation of HASMC [16] was also observed at low affinities for several $CysLT_1$

antagonists (pobilukast, 30 μM) and in the case of zafirlukast (1 μM) no inhibitory activity was observed, suggesting that a higher concentration of this compound may be necessary to observe inhibition. Further support for the existence of a low affinity mast cell treated with IL-4 did not fit the classical CysLT receptor profile $CysLT_1$ receptor is derived from the work of Porreca and co-workers [17] who showed that the LTD_4-induced vascular smooth muscle cell proliferation could be inhibited with high concentrations of MK 571 (2-20 μM). Recently, Mellor and co-workers [18] demonstrated that the LTC_4 response in mast cells treated with IL-4 did not fit the classical CysLT receptor profile. In summary the data highlighted in this presentation suggest that other functional receptors exist. Molecular techniques must now be used to address these observations.

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Chapter #3

LTC_4 PRODUCTION BY EOSINOPHILS IN ASTHMATIC SUBJECTS WITH ALTERNATIVE FORMS OF ALOX-5 CORE PROMOTER

Ömer Kalaycı, Michael Wechsler, Ben Galper, Chris Hong, Elliot Israel, Jeffrey M. Drazen and Craig M. Lilly

Introduction

Pharmacogenetics is the use of genetic information to determine who will (or will not) respond to a given treatment. In order to have a pharmacogenetic basis, there should be variability in the treatment response. Although there are data on the variability of the treatment response to all classes of medications that are used in the treatment of asthma- i.e., glucocorticoids, teophyllin, β_2 agonists and leukotriene modifiers- pharmacogenetic associations have been described only for β_2 agonists and leukotriene modifiers [1].

Studies on β_2 agonists have shown that polymorphisms at the β_2 receptor may influence airway responses to regular inhaled β agonist treatment and the acute response to β agonists [2,3].

Pharmacogenetic studies of leukotriene modifiers, on the other hand, have investigated the effect of polymorphisms of two enzymes that participate in leukotriene biosynthesis- Leukotriene (LT) C_4 synthase and 5-lipoygenase (ALOX-5)- on the response to agents that inhibit leukotriene biosynthesis or block their effect at the receptor level. LTC_4 synthase catalyses the formation of cysteinyl leukotrienes from LTA_4. It has been suggested that -444C variant of the LTC_4 synthase creates an additional histone H4 transcription factor-2 binding motif and is associated with an enhanced LTC_4-S transcription rate as compared with the -444A. Accordingly, some clinical studies have suggested that the presence of -444C variant is associated with a better response to leukotriene modifiers [4].

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ALOX-5 is the enzyme required for the production of both the cysteinyl leukotrienes and LTA_4. Transcription of the ALOX-5 gene is partly regulated by the Sp-1 Egr-1 binding motifs (GGGCGG) in the promoter region [5-7]. Normal individuals (wild type-WT) have five Sp-1/Egr-1 binding motifs. Mutations consisting of addition of one or the deletion of one or two of Sp-1/Egr-1 binding motifs are associated with decreased transcriptional activity as shown by transfection studies using promoter reporter constructs. Subsequently, it was shown that asthmatic individuals harboring the mutant forms of the ALOX-5 core promoter do not improve upon treatment with a drug that inhibits ALOX-5 [8]. Although this observation clearly indicated a pharmacogenetic effect of the promoter sequence on the treatment response, the biological basis of this effect remains to be determined. In order to provide a biological explanation to this question, we hypothesized that eosinophils from asthma patients with the variant forms of the ALOX-5 core promoter produce less LTC_4 than WT patients.

Material and Methods

Genotyping: Asthmatic individuals, who were being followed at Asthma Research Center of Brigham and Women's Hospital, were genotyped to the ALOX-5 core promoter using ABI 3100 capillary technology. Patients harboring the wild type (WT) ALOX-5 core promoter (with five Sp1/Egr-1 tandem binding motifs), and those homozygous for one of the deletional mutant forms (MT) (three or four binding motifs) and heterozygous patients (HT) (three/five or four/five binding motifs) were identified.

Eosinophil isolation and stimulation: Neutrophils were isolated from heparinized peripheral blood by density gradients and with anti CD-16 antibody from Pharmingen (San Diego, CA) followed by coating with pan mouse IgG coated beads purchased from Dynal (Oslo, Norway). Eosinophils were then separated by negative selection on a magnetic rack. Eosinophils ($1x10^6$/ml) were incubated in HBSS containing 50 mM L-serine and studied under 3 conditions: 1) unstimulated, 2) stimulated with 1 μM Ca ionophore A23187 (Sigma, St Louis, MO), and 3) stimulated with Ca ionophore A23187 and 10 μM Zileuton. LTC_4 was measured by EIA in the supernatants according to the instructions of the manufacturer (Cayman Chemicals, Ann Arbor, MI).

Statistics: Three groups were compared using ANOVA or ANOVA on ranks depending on the normality of data distribution

Results

The genotype and allele frequencies of the study population are shown in Table 1 and 2.

Table 1. Genotype frequencies of the ALOX-5 core promoter variants

Genotype	n	Frequency
55	218	0.57
44	8	0.02
33	4	0.01
34	9	0.02
35	25	0.07
45	105	0.28
46	3	0.01
56	9	0.02
Total	381	1

Table 2. Allele frequencies of the ALOX-5 core promoter variant

Allele	Frequency
3	0.06
4	0.18
5	0.75
6	0.01

From this cohort, ten WT, four MT, and six HT patients constituted the study population. Median LTC4 levels at baseline did not show any difference among the WT (26 pg/ml; interquartile range, 19-45), MT [30(17-42)] and HT [41(16-79)] groups (p=0.9 by ANOVA). Although Ca-ion robustly stimulated LTC4 production, no differences were detected among the WT [109±17 (mean±SD) ng/ml], MT (109±23 ng/ml) and HT (110±21 ng/ml) (p=1, by ANOVA on ranks). Zileuton effectively suppressed >99% of LTC4 production in all groups. There was no significant difference in the amount of LT production after Zileuton treatment.

Discussion

Our findings suggest that there are no significant differences in LTC_4 production by eosinophils from asthmatic subjects with alternative genotypes at baseline or after near maximal stimulation with Ca ionophore. Although Zileuton inhibited >99% of leukotriene production, it failed to bring about a difference among different genotypes.

There may be several reasons why we have not been able to show any significant difference in the leukotriene production by eosinophils from asthmatic individuals with different genotypes at ALOX-5 core promoter. First of all, because of the relatively low frequency of the mutant alleles, we could detect only 12 MT individuals in our study population. Therefore, we have not been able to conduct our study in well balanced populations with equal age, race and gender distribution. This may have had a major impact on the LT production especially at baseline. Maybe even more importantly is the fact that all the patients involved in this study were taking varying doses of inhaled corticosteroids. Although it is well known that corticosteroids do not effect the *in-vivo* LT production, the fact that the symptoms of the patients are controlled by various doses of corticosteroids suggests that the patients had varying degrees of disease severity and thus were at different

states of disease activation at the time of sampling. It is highly likely that these factors may have affected the baseline LT production by eosinophils.

Another factor that could be responsible for the lack of a difference in LT production by eosinophils from patients with alternative genotypes may be the possible activation of eosinophils during the long isolation procedure (approximately 8 hours) or by the culturing conditions used in our study.

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Chapter #4

IL-4-INDUCED LIPID MEDIATORS CLASS SWITCHING IN HUMAN NORMAL MONONUCLEAR PHAGOCYTES

Angelo Sala, Mirella Profita, Liboria Siena, Peter M. Henson, Robert C. Murphy, Alessandra Paternò, Anna Bonanno, Loredana Riccobono, Angela Mirabella, Giovanni Bonsignore and A. Maurizio Vignola

Introduction

Leukotrienes (LT) are important molecules arising from arachidonic acid, knokn to play an important role in inflammatory reactions [1]. Their synthesis is limited to a restricted number of cell involved in the inflammatory response, such as mast cells, neutrophils, eosinophils and monocyte/macrophages.

Activated monocytes can promote the bronchial inflammatory response by releasing numerous mediators [2], including leukotrienes [3], and may contribute to the development of inflammatory processes such as asthma [4], and chronic obstructive pulmonary disease (COPD). Within the asthmatic airways macrophages have been shown to be in close contact with inflammatory cells, such as mast cells, basophils and Th2 cells [5], that are able to release several immunomodulatory cytokines that may in turn affect macrophage functions and activities.

Among the cytokines potentially playing a role in the inflammatory response IL-4 is known to regulate the expression of 15-lipoxygenase as well as 15(S)-HETE production in human monocytes [6]. Interestingly 15(S)-HETE may exert several immuno-regulatory functions, which may be relevant in asthma and chronic bronchitis [7]. 15(S)-HETE is a potent mucosecretagogue in human airway [8], and it has been reported to possess chemotactic activity for neutrophils, contributing to the recruitment of these cells in the airways.

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Interestingly, aside from its pro-inflammatory actions, 15(S)-HETE may also modulate the inflammatory response through the inhibition of leukotriene production in a variety of cell types [9].

The ability of IL-4 to induce the expression of 15-LO, and the potential effect of 15(S)-HETE on leukotriene biosynthesis prompted us to study the effect of IL-4 on LT production by human monocytes. We provided evidence that exposure of human monocytes to IL-4, as in inflamed airways, causes a shift in lipid mediator profile from LT to 15-LO metabolites production.

Methods

Mononuclear cells were isolated from buffy coats by density gradient centrifugation using Ficoll-Hypaque cushions [6] and purified by adhesion to cell culture dishes.

Monocytes were incubated in 6-well plates for 24, 48 and 72 hrs with IL-4 (10 ng/ml) and challenged with A23187 (5 μM) for 15 min. Alternatively monocytes were challenged with opsonized zymosan (100 particle per cell, 1 h at 37°C).

At the end of the incubation, the cell supernatant were added with the appropriate internal standard (PGB_2 for RIA/RP-HPLC, d_4-LTB_4 for LC/MS/MS) and stored under an argon atmosphere at –80°. Radioimmunoassay (RIA) of 15(S)-HETE and LTB_4 were performed after RP-HPLC as previously described [10]. Samples from monocytes activated with opsonized zymosan were extracted on LC18 SPE cartridges, analysed by RP-HPLC directly interfaced into the of a triple quadrupole MS (Sciex API-3000, Perkin Elmer, Thornton, Canada), and arachidonic acid metabolites detected by selected reaction monitoring.

Results and Discussion

Treatment with IL-4 for 24, 48 and 72 hrs significantly increased the amount of 15(S)-HETE observed after stimulation with the Ca^{++} ionophore A23187, resulting in over a 10-fold increase after 72 hours of incubation (Figure 1A). Concomitant measurement of LTB_4 production showed a progressive decrease over IL-4 incubation time, reaching a minimum after 72 hrs, where LTB_4 production was less that 15% of that observed in the absence of IL-4 pretreatment (Figure 1B). In order to test if the effect of IL-4 on LTB_4 could be observed in the presence of a more physiological stimulus, control and IL-4-treated (72 hours) monocytes were challenged with opsonised zymosan. LC/MS/MS analysis of supernatants showed the presence of 15(S)-HETE in IL-4-treated cells only, where LTB_4 production was also significantly inhibited (45±11%, n=3, p=0.05); when compared to controls (LTB_4 production: 0.44±0.24 ng/2x10^6 cells).

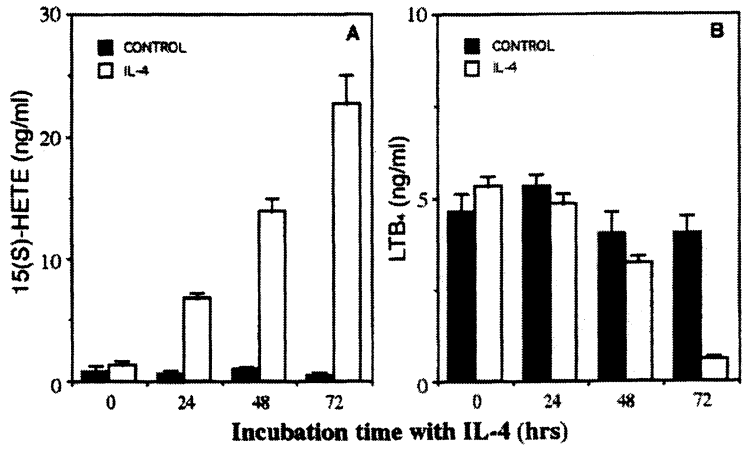

Figure 1. Monocytes ($2x10^6$ cells/ml) were cultured in the presence or absence of IL-4 for 24, 48, 72 hours, and activated with A23187 (5 μM). 15(S)-HETE (panel A) and LTB_4 (panel B) were quantitated in supernatants by specific RIAs after RP-HPLC separation. Data are expressed as mean ± SE of eight consecutive experiments. Statistical analysis was performed using Student's t-test for unpaired samples.
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The results of this study showed that IL-4 induced expression of 15-LO in human monocytes is accompanied by a significant inhibition of LTB_4 production upon challenge with either the Ca^{++}-ionophore A23187 or a physiologically relevant stimulus such as opsonized zymosan.

The potential for IL-4 to significantly change the profile of arachidonic acid metabolites from the production of leukotrienes to that of 15(S)-HETE raises important questions on the biological relevance of such an effect. Recently we have shown that 15(S)-HETE is present in high concentration in induced sputum obtained from chronic bronchitis patients, and that its concentration inversely correlate both with the percentage of neutrophils recovered and the concentration of LTB_4 in induced sputum [9]. Hence, the ability of IL-4 to increase the expression of 15-LO by human monocytes/macrophages, and to concomitantly inhibit the production of a potent chemotactic factor such as LTB_4, may play an important role in modulating the neutrophilic influx following monocyte activation within the *milieu* of inflamed airways.

Recently, Levy and coll. showed that treatment of polymorphonuclear leukocytes with PGE_2, mimicking an inflammatory setting, resulted in the expression of 15-LO accompanied by a similar shift from the production of pro-inflammatory LTB_4 to the synthesis of the anti-inflammatory 5-LO/15-LO metabolite lipoxin A_4 [11], and suggested that the expression of 15-LO may signal the end of the acute phase of the inflammatory response. The results of our studies, taken together with the data obtained by Levy and

coll., may provide the molecular basis for events playing an important role in the resolution of the acute inflammatory response.

Acknowledgements

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Chapter #5

LIPOXINS AND ASPIRIN-TRIGGERED LIPOXINS IN AIRWAY RESPONSES

Bruce D. Levy, George T. De Sanctis, Pallavi R. Devchand, Eugene Kim, Kate Ackerman, Birgitta Schmidt, Wojciech Szczeklik, Jeffrey M. Drazen, and Charles N. Serhan

Leukotrienes (LT) and prostaglandins (PG) have been implicated as pro-inflammatory mediators in asthma [1], while lipoxins (LX) are a distinct class of eicosanoids that carry unique counter-regulatory actions [2]. Analyses of the time course of appearance of eicosanoids during acute inflammation has revealed the early coordinate appearance of LT and PG with leukocyte recruitment followed by LX during resolution [3]. LX's are generated in human tissues, including airways [4], and can interact with at least two classes of receptors, $CysLT_1$ and LXA_4 receptors (designated ALX) [5]. LX's, aspirin-triggered 15-epimer-LX's and stable, longer-acting analogs of these compounds can promote resolution of cytokine-driven acute inflammation [5], inhibit LTC_4-stimulated airway hyper-responsiveness in human asthmatics [6] and block LTD_4-initiated constriction of airway smooth muscle *in vitro* [7] .Of note, aspirin-tolerant asthmatic individuals have a decreased biosynthetic capacity for LX's [8]. In view of these intriguing findings, we determined the impact of LX's in an experimental murine model of asthma [9].

Airway lipoxin formation after antigen challenge

After systemic sensitization to ovalbumin (OVA, 10 μg i.p.), male Balb/c mice (5-7 weeks) were exposed to aerosolized OVA (6%, 25 min) on four consecutive days. 24 hours after the last aerosol, bronchoalveolar lavage (BAL) was performed. After allergen sensitization and aerosol challenge, high levels of both CysLT (138.95+/-27.30 pg CysLT/ml, mean +/- SEM, n=9) and PGE_2 (1117+/-103.83 pg PGE_2/ml, mean +/- SEM, n=5) were present in BAL fluids. LXA_4 was identified (15.01+/-3.26 pg LXA_4/ml, mean +/- SEM, n=5) at levels similar to LTB_4 (6.42±2.28 pg LTB_4/ml, mean

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+/- SEM, n=9), but in 10- to 100-fold lower concentrations than CysLT's and PGE_2, respectively. LXA_4 was not detected in BAL fluid from non-immunized mice. The relatively low levels of endogenously generated LX's after allergen challenge may have reflected the timing of sample acquisition, but it is also possible that sensitization adversely impacted LX production during subsequent challenge.

Lipoxin A_4 dampens airway hyper-responsiveness and inflammation

To determine if increased circulating levels of LXA_4 would protect mice from OVA-induced airway hyper-responsiveness, we administered a LXA_4 analog (15-epi-16-*p*-F-phenoxy-LXA_4-me (LXa), 10 μg i.v.) that resists metabolic inactivation [10]. When given 1 h prior to OVA aerosol, LXa significantly decreased methacholine-initiated bronchoconstriction (Figure 1). The ED_{200} to methacholine for mice given LXa approximated the responses of control mice. LXa administration alone did not affect airway hyper-reactivity *in vivo*. Because LXA_4 inhibits LTC_4-stimulated airway hyper-responsiveness in human asthmatics [6], blocks LTD_4-initiated constriction of airway smooth muscle *in vitro* [7], and this LX analogue competes for binding with high affinity at the LTD_4 receptor, cysLT1 [11], pharmacological levels of LXA_4 may have direct protective effects on airway smooth muscle.

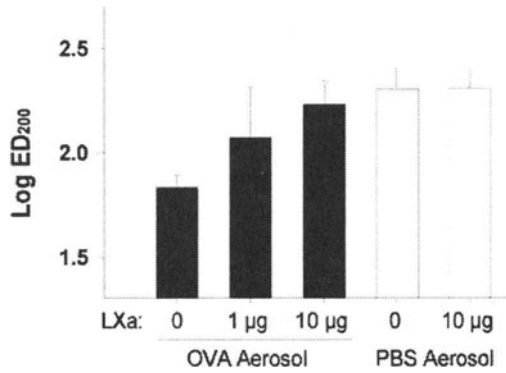

Figure 1. Regulation of airway hyper-responsiveness by LXa. OVA sensitized mice were treated with LX analog or vehicle prior to OVA aerosol challenge. Airway reactivity was determined by methacholine-dependent change in lung resistance and calculation of the effective dose of methacholine resulting in a 200% increase in lung resisitance (ED_{200}). Results are expressed as mean ± SEM ($n \geq 6$).

Administration of LXa significantly reduced tissue eosinophils, lymphocytes and vascular injury [9]. In BAL fluids, there was dose-dependent inhibition of total leukocytes, eosinophils and lymphocytes. LXa also decreased levels of T_H2 cytokines IL-5 and IL-13 in BAL fluids from

OVA sensitized and challenged animals [9]. This inhibition was selective, as levels of IL-12 and TNF were not similarly reduced. LXa also regulated lipid mediator formation, as levels of PGE_2 and CysLT's, but not LTB_4 were decreased [9]. These results indicate that administration of LXA_4 mimetics can significantly inhibit allergic pulmonary inflammation, including leukocyte infiltration and formation of specific peptide and lipid mediators. Moreover, the capacity for LXa to inhibit these processes that characterize asthma pathobiology raises the likelihood that endogenous LX's serve in health as pivotal regulators of airway and allergic inflammation.

Leukocyte expression of human LXA_4 receptors decreases airway inflammation

Leukocyte and airway epithelial cell expression of LXA_4 receptor (ALX) is increased in animals sensitized and aerosol challenged with antigen [9]. To directly assess the regulation of allergic pulmonary inflammation by ALX, we analyzed the responses of mice with expression of human ALX targeted to murine leukocytes (using a component of the CD11b promoter) [9]. After OVA sensitization and aerosol challenge, human ALX transgenic animals

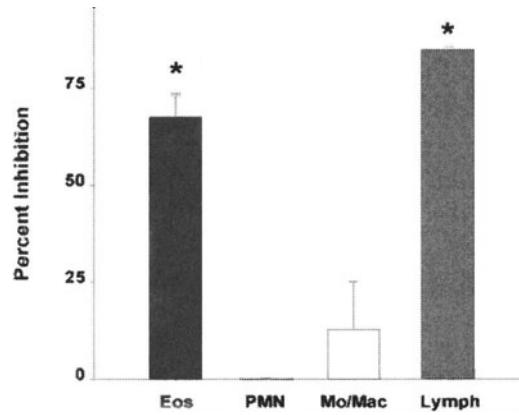

Figure 2. Airway inflammation is decreased in allergen-sensitized human ALX transgenic mice. Non-transgenic and human ALX-tg mice were sensitized and aerosol challenged with OVA. BAL fluids were obtained, leukocytes enumerated and identified after Wright-Giemsa stain. Percent inhibition in cell number was determined. Results are expressed as mean $\pm$ SEM ($n=4$, $d\geq 2$). *$P < 0.05$ by Student's t-test.

(ALX-tg) displayed a clear decrease in pulmonary leukocytic infiltrates. BAL from allergen challenged ALX-tg mice had reduced numbers of total leukocytes, eosinophils and lymphocytes (63%, 68% and 85% inhibition, respectively) (Figure 2). Levels of pro-inflammatory peptide and lipid mediators were also decreased in ALX-tg mice, including IL-13 (77%

inhibition), IL-5 (71% inhibition) and CysLT (74% inhibition). Allergen sensitization in the ALX-tg animals was reduced as evidenced by total serum IgE levels. Of interest, significant differences in airway hyper-responsiveness were not observed in hALX-tg. Together, these results indicate that ALX signaling regulates allergic pulmonary inflammation.

Summary

Here, we have demonstrated potent inhibition of allergen-mediated pulmonary inflammation and airway hyper-responsiveness by a novel dual-pronged action of LX's. Moreover, these results indicate that LX's play pivotal and previously unappreciated roles in regulating allergy and pulmonary inflammation.

Acknowledgements

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Chapter #6

POTENTIAL ROLE OF CYSTEINYL LEUKOTRIENES IN TRAFFICKING AND SURVIVAL OF HEMATOPOIETIC PROGENITOR CELLS

Andreas M. Boehmler, Claudio Denzlinger, Lothar Kanz and Robert Möhle

In the clinical setting of a stem cell transplantation, progenitor and stem cells within the bone marrow (BM) can be mobilized into the donors' peripheral blood by administration of hematopoietic growth factors. Since these cells retain the ability to repopulate the hematopoietic compartment of the bone marrow (a process referred to as 'homing'), they can account for hematopoietic recovery within the patient [1]. Thus both mobilization to the peripheral blood and homing back to the bone marrow crucially depend on the extensive migratory capabilities of hematopoietic stem and progenitor cells (HPC).

Our current understanding of the factors and mechanisms involved in the regulation of hematopoietic stem cell trafficking is mainly limited on the role of particular proteins and peptides (adhesion molecules, cytokines, chemokines), while the function of other compounds therein, i.e. non-peptide mediators such as lipids, still remains elusive. However, a variety of biological functions are regulated at the cellular level by both peptides as well as by lipid mediators such as leukotrienes, with the latter playing a dominant role in inflammation-related processes [2]. Notably, extravasation of mature leukocytes at sites of inflammation and the process of progenitor cell homing depend at least in part on similar mechanisms, as the hematopoietic microenvironment bears some features resembling inflamed tissues, such as expression of certain adhesion molecules [3]. In addition, production of lipid mediators, particularly leukotrienes, has been observed in the normal bone marrow; nevertheless, their potential function within the hematopoietic microenvironment is unknown [4,5].

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Addressing the question in how far cysteinyl leukotrienes such as LTD4 might affect hematopoietic progenitor cells, we could show in previous studies that $CD34^+$ hematopoietic progenitors derived from bone marrow as well as from peripheral blood highly express the CysLT1 receptor, as does the $CD34^+$ positive leukemic cell line KG1a [6]. In an *in vitro* model of transendothelial migration [7], the presence of 50 nM LTD4 in the lower chamber of the transwell system resulted in a 2-fold increase in the number of cells migrating through monolayers of the bone marrow endothelial cell line BMEC-1. Additional experiments on the influence of LTD4 on progenitor cell migration demonstrated that LTD4 had a sustained effect on the cytoskeleton of $CD34^+$ cells, and that these cells responded by chemotaxis towards a gradient of LTD4 [6].

Since HPC trafficking is regulated at least in part at the level of the bone marrow endothelium, a series of *in vitro* assays was set up to further characterize potential effects of LTD4 on progenitor cell adhesion. Here, preincubation of $CD34^+$ cells derived from peripheral blood with 1 μM LTD4 rapidly upregulated β1-integrin-mediated adhesion of HPC to endothelial monolayers (HUVEC) in a time-and dose-dependent manner by up to 81.1 ± 12.6 % (mean ± SEM). Similar results were seen on primary bone marrow endothelium isolated from donor aspirates, immobilized VCAM-1, and fibronectin. Both immature and lineage-committed progenitors responded to LTD4, as determined by cobblestone-area forming cell assays on FBMD-1 stromal feeders as well as in methylcellulose colony assays. The triggering effect of LTD4 on cell adhesion could be blocked by the CysLT1 receptor antagonist MK-571, demonstrating the specificity of the observed results.

Within the niches of the bone marrow microenvironment, survival and proliferation of hematopoietic progenitors depend on a close relationship between hematopoietic and stromal cells. Serum-free liquid cultures were set up to address a potential role of cysteinyl leukotrienes within this compartment as mediators of HPC proliferation and/or differentiation. As seen in the adhesion experiments, LTD4 augmented cytokine-induced expansion of hematopoietic progenitors in a dose-dependent manner by up to 69.47 ± 0.3 % (mean ± SEM) in cultures containing 100 nM LTD4 compared to cytokines alone. In this system, the effect of LTD4 on HPC proliferation was dependent on the presence of IL-3. Characterisation of expanded cells again revealed an effect of LTD4 also on more primitive progenitors. Moreover, LTD4 evenly triggered proliferation of all analyzed lineage-committed subpopulations, i.e. treatment of cells with the leukotriene did not cause a shift in differentiation patterns.

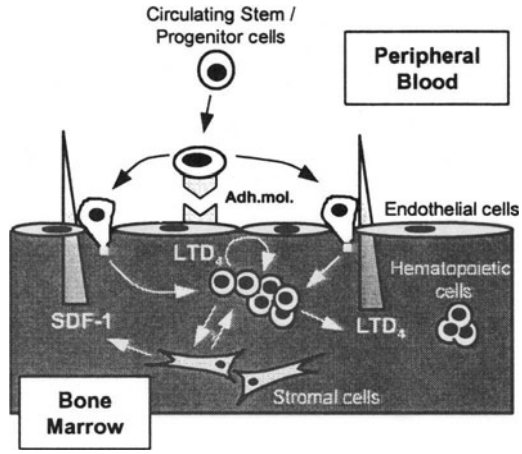

Figure 1. Proposed model illustrating a potential function of cysteinyl leukotrienes such as LTD4 within the hematopoietic system. Similar to the chemokine SDF-1, LTD4 could modulate progenitor cell trafficking via a transendothelial gradient, resulting in activation of adhesion molecules of the integrin family expressed by immature hematopoietic cells and also modulating progenitor cell chemotaxis. Within the bone marrow microenvironment, LTD4 could act as autocrine or paracrine factor regulating proliferation and cell-cell-interactions.

Conclusions

Trafficking and survival of immature hematopoietic cells are regulated by a complex network of cellular interactions mediated by adhesion molecules, cytokines, and chemotactic factors. Our recent studies provide convincing evidence that in addition to proteins, also nonpeptide lipid mediators such as LTD4 not only modulate chemotaxis of immature hematopoietic cells, but also can strongly influence HPC trafficking and proliferation. It therefore seems feasible to propose a function of leukotrienes within the hematopoietic system similar to cytokines such as stromal cell-derived factor-1 (SDF-1) (Figure 1). Moreover, the rather short *in vivo* range of LTD4 makes it an ideal candidate mediator for the regulation of the spatial distribution of cells in the bone marrow.

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Chapter #7

COVALENT BINDING OF LEUKOTRIENE A_4 TO NUCLEOSIDES, NUCLEOTIDES, AND NUCLEIC ACIDS

Joseph A. Hankin and Robert C. Murphy

Introduction

The chemical reactivity of leukotriene A_4 (LTA_4) is widely appreciated following the detailed studies of its stability in aqueous solutions, solutions containing organic solvents, and solutions containing albumin [1]. The half-life of LTA_4 is less than one second unless a stabilizing substance is present in aqueous buffers. This has led to the concept that intracellular LTA_4 exists only for a brief period of time before it is either nonenzymatically hydrolyzed to 6-trans-LTB_4 isomers or converted by LTA_4 hydrolase or LTC_4 synthase into the biologically active leukotrienes, LTB_4 and LTC_4, respectively [2]. The reactivity of LTA_4 is due to its electrophilic character and within the cell various nucleophiles are present including nucleic acids which could, in theory, donate electrons into the conjugated triene epoxide moiety of LTA_4.

LTA_4 was found to react with nucleosides and nucleotides at basic pH to form multiple products characterized as a mixture of 5,6 and 5,12 adducts as well as several other products, suggesting multiple sites of attack by the nucleoside base [3]. Interest in this chemical reactivity of LTA_4 with nucleosides stems from the known site where LTA_4 is synthesized within the cell, namely close to the DNA storage site, the nucleus [4]. Thus, newly synthesized LTA_4 could have access to nucleic acids present in relatively high concentrations at this site.

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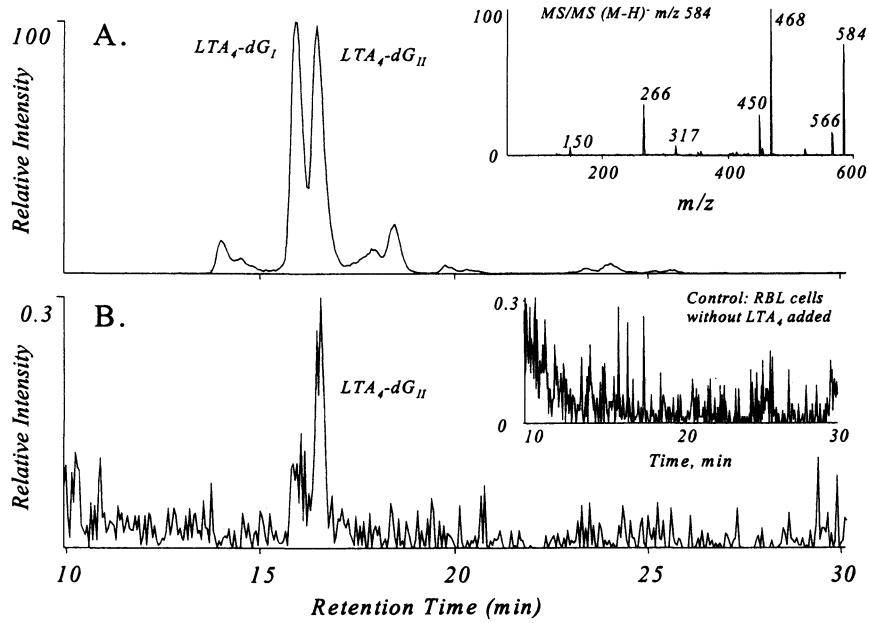

Figure 1. **(A)** LTA_4-dG products formed from isolated DNA detected using MRM (MS/MS) for *m/z* 584 → 468. **Inset:** Collision induced dissociation of the precursor ion at m/z 584 of LTA_4-dG_{II} obtained from the reaction of LTA_4 with dG. **(B)** LC/MRM (*m/z* 584 → 468) from DNA isolated from RBL-2H3 cells after addition of LTA_4 and calcium ionophore. **Inset**: LC/MRM of the control cell experiment with no evidence of adduct formation.

Reaction of LTA_4 with DNA Nucleosides

Incubation of the deoxyguanosine (1.5 mM) with LTA_4 (0.25 mM), prepared immediately before hand, led to the production of numerous products (data not shown). The profile of products was very similar to that also observed for the reaction of LTA_4 with the RNA-nucleoside base, guanosine [3]. The mass spectrum of the major LTA_4-deoxyguanosine adduct (LTA_4-dG_{II}) (Figure 1A inset), as illustrated by the collision induced decomposition (CID) of the molecular anion at *m/z* 584.3, revealed a series of product ions, the most abundant corresponding to the loss of the neutral, unsaturated deoxyribose (*m/z* 468) as typically observed for nucleosides [3]. There was evidence for ions corresponding to the arachidonic acid portion of the adduct from the product ion observed at *m/z* 317 as well as a specific nucleoside product ion corresponding to loss of the lipid portion of the adduct as a neutral species (*m/z* 266). LTA_4 was found to react with all the DNA-deoxynucleosides studied; the most facile reaction being with deoxyguanosine (data not shown).

Reaction of LTA_4 with Nucleic Acids *In Vitro*

Since LTA_4 was shown to react with nucleosides present in both RNA and DNA, experiments were carried out to study whether or not DNA or RNA would participate in the reaction with LTA_4 to form covalent adducts. DNA and RNA (0.5 mg/mL) were reacted with LTA_4 (6 nmol) for 1 hr at room temperature. Following these incubations the RNA and DNA were enzymatically digested to nucleosides and LTA_4-nucleoside adducts [5,6]. Following digestion, samples were purified by reversed phase solid phase extraction and then analyzed by LC/MS/MS using specific ion monitoring of abundant transition ions for suspected LTA_4 adducts of guanosine (RNA) and deoxyguanosine (DNA). The ion transitions recorded correspond to the most abundant fragment ions in collisional activation of the molecular ions as seen for the deoxyguanosine adduct to LTA_4 in Figure 1A. Adducts were quantified by mass spectrometry using isotopically labeled internal standards.

For both nucleic acids, adducts with LTA_4 could be readily detected using mass spectrometry. The yield of DNA and RNA adduct increased as the quantity of DNA and RNA in the reaction mixture increased from 25-400 μg. In all cases, the total quantity of adduct was higher for the incubation with RNA compared to an equal quantity of DNA, suggesting that the nucleoside bases present in RNA were more reactive than the nucleosides present in DNA.

Reaction of Native DNA with LTA_4

In order to investigate whether or not DNA packaged within the nucleus of cells could be a substrate for the reaction with LTA_4, experiments were carried out with RBL-2H3 cells using conditions to maximize the half-life of LTA_4. For these experiments, RBL-2H3 cells (50×10^6) were suspended in 5 mL HBSS supplemented with BSA (3 mg/mL). LTA_4 was added at 5°C under conditions previously determined to allow LTA_4 to transport into cells [7]. The cells were also stimulated by addition of 2 μM calcium ionophore A23187 and 2 μM arachidonic acid followed by a 20 min incubation. Control experiments were carried out with an equivalent number of cells incubated under identical conditions with LTA_4 or A23187 alone added to the cells or with neither. The DNA from these cells was extracted, quantified by UV spectroscopy, and enzymatically digested (see above) into individual nucleosides for analysis by LC/MS/MS techniques. The addition of LTA_4 to RBL-2H3 cells generated substantial quantities of LTC_4 and LTB_4 present in the supernatant, indicating that LTA_4 had access to the intracellular compartments of the RBL-2H3 cells. These leukotrienes were analyzed by

LC/MS/MS techniques as previously described [8]. Substantial quantities of nonenzymatic hydrolysis products of the exogenously added LTA_4 were also present in the supernatant. Under conditions of addition of LTA_4 with stimulation by A23187, a measurable quantity of LTA_4-dG adduct was found in the DNA extracted from the cells after enzymatic digestion and purification of the adduct (Figure 1B). The control experiments that had no LTA_4 added or no calcium ionophore added to the cells indicated no production of adduct. The quantity of adduct formed in three different experiments was found to range between 2-4 adducts in 1 x 10^8 base pairs of DNA. There was no evidence for the production of other nucleoside adducts with LTA_4 detected in these samples using LC/MS/MS techniques and multiple reaction monitoring specific for each nucleoside adduct. Furthermore, the cytosol from cell incubations did not contain any LTA_4 adducts of guanosine or deoxyguanosine or any deribosylated bases from DNA or RNA that may have come from depurination reactions.

An interesting feature of these experiments was that the activation of the RBL-2H3 cells was critical for the formation of LTA_4 adducts with DNA present in the nucleus of the RBL-2H3 cells. The elevation of intracellular calcium therefore appeared to be an important step, driving the access of LTA_4 to target DNA. The reason for a requirement for cell activation in order to observe LTA_4 adduct formation with DNA is not at present understood, but the requirement for exogenous LTA_4 stems from the fact that RBL-2H3 cells produce very low levels of endogenous LTA_4 when stimulated with the calcium ionophore.

In summary, it is clear that LTA_4 can covalently bind with nuclear DNA packaged within the cell. The covalent modification of DNA is known to alter significant events within the cell including major toxicological sequelae as well as suppression of protein synthesis. These experiments raise the possibility that unexpected chemical reactions may play a role *in vivo* in regulating cellular events in those cells expressing 5-lipoxygenase and generating LTA_4.

Acknowledgements

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Chapter #8

FIRM ADHESION OF NEUTROPHILS TO CEREBRAL VASCULAR ENDOTHELIUM "IN VIVO": A ROLE FOR CYS-LEUKOTRIENES

Chiara Carnini, Gabriela Constantin, Barbara Rossi, Marco De Curtis and Giancarlo Folco

Introduction

Interactions between neutrophils (PMNL) and endothelial cells (EC) are involved in pathological alterations of cerebral blood vessel functions, leading to circulatory disturbances such as stroke [1]. The adhesive process induces PMNL activation, coupled to local bioavailability of oxygen free radicals, proteases, cytokines and arachidonate metabolites of the 5-lipoxygenase pathway, such as leukotriene A_4 and B_4 (LTA_4, LTB_4). This contributes to alterations of vasomotor reactivity, occlusion of microvasculature and induction of vascular permeability. The ensuing PMNL emigration through the blood brain barrier represents a critical event in the initiation of cerebral inflammation. Therefore it is clear that the PMNL-EC interaction may be a target for therapeutic interventions. Significant reductions in volume of the lesions and improved physiological functions were found in rats subjected to transient MCA occlusion and treated with anti-ICAM antibody, as well as with selective PMNL depletion in a murine model of reperfused stroke.

The interaction between EC and PMNL represents a unique site of biosynthesis of cysteinyl-leukotrienes (cys-LT), according to a process termed "transcellular biosynthesis". The unstable intermediate LTA_4, made available by activated PMNL, can be captured by the juxtaposed EC and converted to cys-LT. This causes increased vascular permeability, edema, PMNL diapedesis, creating a vicious cycle that sustains and enhances inflammation. While transcellular biosynthesis of cys-LT has been largely documented in the coronary vascular bed [2], little is known about a direct involvement of cys-LT in brain damage. We have recently obtained preliminary evidence "in vitro" that the activation of human PMNL within

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the cerebral vasculature of an isolated guinea-pig brain (with a functionally-intact endothelium), triggers cys-LT formation and causes brain edema [3].

Since PMNL adhesion to EC represents a prerequisite for cys-LT formation, we asked whether *in situ* biosynthesis of cys-LT may play a role in the regulation of adhesive phenomena in cerebral microcirculation *in vivo*.

We used a newly developed model of intravital microscopy in mice, allowing analysis of the interactions (rolling and firm adhesion) between PMNL and endothelium in brain microvasculature. Using this *in vivo* model and the highly specific leukotriene biosynthesis inhibitor MK886, we demonstrate that cys-LT play a role in the regulation of firm adhesion of PMNL to the endothelium in inflamed cerebral venules.

Methods

Human PMNL were purified as described [2]. For intravital microscopy experiments cells were incubated with Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF, Sigma) 1 nM and orange CMTMR for 30 min. at 37°C. The pellet was resuspended in 0.4 ml D-PBS, pH 7.2, before animal injection. Whenever necessary, PMNL were incubated with MK886 (1μM). Intravital microscopy experiments were performed as described [4]. $2x10^6$ cells /condition were injected into the carotid artery at a flow rate of 0.13-1.0 μl/s. Video analysis was performed on postcapillary venules. Vessel diametre (D), hemodynamic parameters and the velocities of rolling were determined. PMNL that remained stationary on venular wall for at least 30 s were considered adherent. At least 60 consecutive cells/venule were examined.

Results and Discussion

The evaluation of PMNL interactions with the vascular endothelium was carried out in a total of 7 different cerebral venules, with internal diameter (D) between 35 and 80 μm, from 4 different animals; the same observation field was kept for control and MK886-treated cells. Rolling PMNL constitute a small (13.2±4.73 x±ES, n=7) fraction of the total cell number which is not influenced by the hemodynamic parameters.

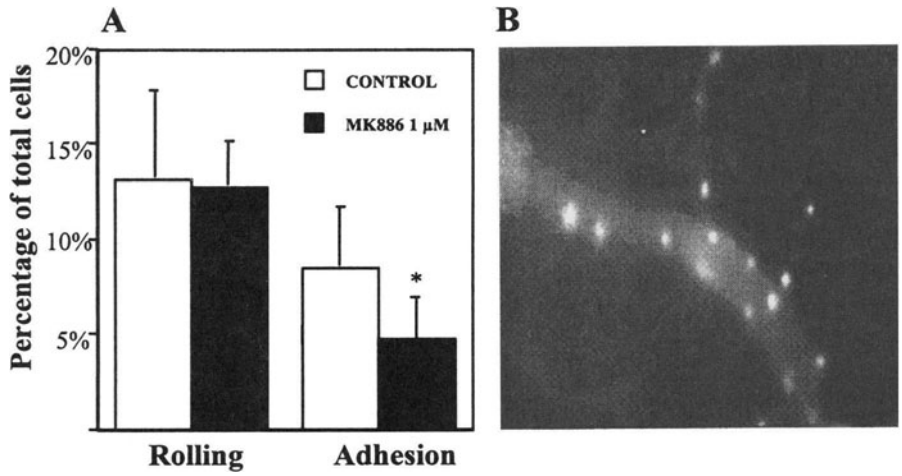

Figure 1: Panel A: Percentage of rolling and sticking PMNL in mouse cerebral venules: effect of cell treatment with the cys-LT synthesis inhibitor compound MK886 (1 μM). Since the same observation field was kept for control and MK886 treated cells, data were analysed using a paired t-test. *$p<0.05$. Panel B: A micrograph showing fluorescently labelled PMNL arrested in inflamed brain venules.

The total fraction of rolling PMNL was unaffected by the cysLT synthesis inhibitor, compond MK886 (1 μM), with significant intervenular variability. Leukocytes were considered as stickers when firm adhesion was kept for at least 30 s. In control conditions, the percentage of sticking cells over the total number of cells was 8.45±3.19, x±ES, n=7 and was significantly reduced by PMNL treatment with MK886 (4.75±2.12, x±ES, n=7) (Figure 1A). The reduction in the fraction of sticking PMNL after MK886 treatment represents a constant feature in all considered venules.

The present results indicate that firm adhesion of PMNL to cerebral vascular endothelium involves a leukotriene-sensitive mechanism, as demonstrated by the capacity of a leukotriene synthetase inhibitor, compound MK886, to reduce significantly the number of sticking PMNL. The molecular mechanism of leukotriene-sensitive PMNL adhesion to endothelium is largely unknown. However, McIntyre et al. [5] have demonstrated that cys-LT can stimulate human EC to synthesize platelet-activating factor (PAF) and bind neutrophils. PAF elicited by cys-LT remains associated with EC, acting as a signaling-tethering molecule that contributes to firm adhesion. It is possible to hypothesize that the interaction between primed PMNL and EC leads to transcellular synthesis of cys-LT, resulting in new synthesis of PAF and its translocation to the EC surface. Furthermore, when PAF is expressed by cys-LT-activated EC it acts as a signal that, by inducing juxatacrine activation of PMNL, starts a loop of

amplification of cys-LT synthesis by neutrophil-EC(transcellular) interactions, leading to a significant bioavailability of inflammatory mediators specifically localized at the site of stimulation. This may ultimately lead to perivascular PMNL accumulation, vasogenic cerebral edema and blood-brain barrier breakdown and represent a significant therapeutic target.

Acknowledgements

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Chapter #9

THE CYP P450 ARACHIDONATE MONOOXYGENASES: ENZYMATIC RELAYS FOR THE CONTROL OF KIDNEY FUNCTION AND BLOOD PRESSURE

Jorge H. Capdevila, Kiyoshi Nakagawa and Vijaykumar Holla

During P450-dependent metabolism, arachidonic acid (AA) is: a) hydroxylated at its ω or ω-1 carbons to 19- and 20-hydroxyeicosatetraenoic acids (HETEs)(AA ω/ω-1 hydroxylase), or b) epoxidized at any one of its four olefins to 5,6-, 8,9-, 11,12-, or 14,15-epoxyeicosatrienoic acids (EETs)(AA epoxygenase). The demonstration that many of these P450-derived eicosanoids, were formed endogenously by several rat, rabbit, and human tissues, and that they were present in human and rat plasma and/or urine, established the P450 AA monooxygenase as a formal metabolic pathway, and stimulated an extensive functional characterization of its metabolites [1-7]. During the last 15 years, the list of biological activities attributed to the P450-eicosanoids has increased at a rapid pace, and led to an extensive *in vitro* characterization of their vasoactive properties, roles in cell and organ ion transport, and of their participation in hormone signaling cascades [1-7]. In male Sprague-Dawley rats, AA ω/ω-1 hydroxylation and epoxidation account for 70-80, and 20-30% of the total kidney microsomal monooxygenase activity, respectively, and 20-HETE and 11(R), 12(S)-EET (88% optical purity) are recovered as the major ω-hydroxylase and epoxygenase metabolites, respectively [3]. The high degree of structural homology displayed by the members of a P450 gene family [8] has complicated the identification of the P450 isoforms responsible for the metabolism of endogenous AA pools [3,5,9-11]. Nevertheless, extensive enzymatic, biochemical, immunological, and functional evidence indicates that CYPs of the 2 and 4 gene families are responsible for AA metabolism, and that, in rat kidney, CYPs 2C and 4A are the predominant and functionally significant AA epoxygenases and ω/ω-1 hydroxylases, respectively [3,9-19]. For example, CYPs, 2C23, 2C24, and 2C11 are

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expressed in the rat kidney in decreasing order of abundance [17] but, of these, only CYP 2C23 metabolizes AA with a regio- and enantioselectivity that matches that of the microsomal enzymes [16,17]. Furthermore, as shown in Figure 1, antibodies raised against CYP 2C23 inhibited better than 95% of the microsomal epoxygenase activity present in rat kidney microsomes, and had no effect on microsomal AA ω/ω-1 hydroxylation. The recognition of CYPs 2C and 4A as endogenous AA epoxygenases and ω/ hydroxylases, as well the early proposal of a role for these isoforms in the pathophysiology of experimental hypertension [1,4-7], open the way to the introduction of molecular approaches for the study of the functional roles of the AA monooxygenases, and the characterization of P450-isoform/gene-dependent phenotypes of renal dysfunction.

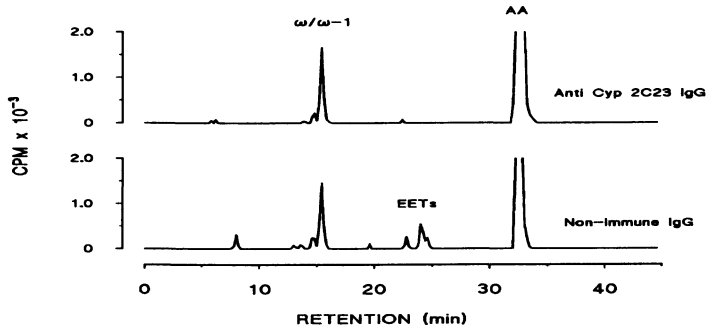

Figure 1. Selective inhibition of the rat kidney microsomal AA epoxygenase by anti-CYP 2C23 antibodies: Microsomes, isolated from the kidney of adult male Sprague Dawley rats [1] mg/ml) were incubated with either non-immune rabbit IgG or anti-CYP 2C23 IgGs (at a ratio of 5 mg of IgG/nmol of microsomal P450). After 15 min at 23°C, the microsomal fractions were incubated with [1-^{14}C] AA (100 M final concentration), and in the presence of 1mM NADPH, and an NADPH regenerating system [17]. Organic soluble products were extracted, resolved by reversed phase HPLC and quantified as described [17]. Shown are the HPLC-radiochromatograms of organic soluble products generated after a 15 min incubation at 35° C.

Based on studies using rat models of genetically controlled spontaneous (the SHR/WKY model), and salt sensitive hypertension (the Dahl model), pro and antihypertensive roles were proposed for products of the AA epoxygenase and ω/ω-hydroxylases [1-7]. Early studies demonstrated that EETs (5,6-EET, in particular) inhibited Na^+ reabsorption in the distal nephron [3], and that the kidney CYP 2C23 AA epoxygenase was induced by excess dietary salt, suggesting a role for the enzyme in the control of body salt and fluid homeostasis [3,5,17]. Subsequently, is was shown that: a) clotrimazole inhibition of the salt regulated renal epoxygenase caused a type of hypertension that was inhibitor-dependent and salt sensitive, and b) compared to normotensive Dahl salt resistant rats, hypertensive salt loaded Dahl salt sensitive rats showed decreased AA epoxygenases activity, an

reduced urinary excretion of epoxygenase metabolites [19]. More recently, the EETs have been identified as endothelium-derived relaxing factor (EDHF) [2,20,21], and their antihypertensive activity, as vasodilators of the renal microcirculation, have been reported [22-24]. Nephron-segment specific pro- and antihypertensive effects have been identified for 20-HETE, a product of the CYP 4A AA ω/ω-1 hydroxylases [1,4,6,7]. In the proximal tubule and TALH segment, synthetic 20-HETE promotes natriuresis and functions as an anti-hypertensive molecule [7]. In the renal microvasculature, 20-HETE has been described as a vasoconstrictor and a pro-hypertensive mediator [4,6,7].

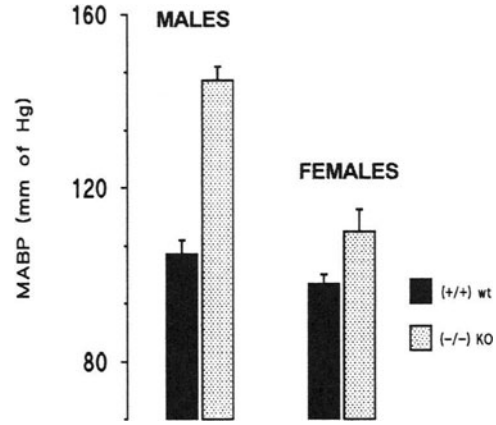

Figure 2. Disruption of the Cyp 4a14 gene raises mean arterial blood pressures in a sexually dimorphic fashion: The blood pressures of conscious adult Cyp 4a14 (-/-) and wild type male and female mice were measured by means of a right carotid artery catheter exactly as described [25]. Shown are averages ± SE calculated from groups of 40 (-/-) or 38 (+/+) males and of 20 (-/-)or 14(+/+) female mice, respectively.

Conclusive evidence for an important role of CYP 4A isoforms in renal physiology and blood pressure control was provided by studies showing that disruption of the murine Cyp 4a14 gene caused a type of hypertension that, like most human hypertension, was sexually dimorphic, and more severe in males [25]. As shown in Figure 2, lack of a Cyp 4a14 gene product(s) raises the mean arterial blood pressure of male and female Cyp 4a14 (-/-) mice by 38 and 14 mm of Hg, respectively [25]. Hypertensive Cyp 4a14 (-/-) mice show increases in plasma androgen levels, and in renal 20-HETE synthase activity [25]. Castration reduced kidney microsomal 20-HETE biosynthesis, and normalized the blood pressure of hypertensive Cyp 4a14 (-/-) mice, while on the other hand, androgen administration raised systemic blood pressures and microsomal 20-HETE biosynthesis, regardless of animals genotype [25]. Northern blot analyses showed that either disruption of the

Cyp 4a14 gene, or androgen administration caused the upregulation of Cyp 4a12, an active, androgen sensitive, renal 20-HETE synthase [25], and provided a convenient explanation to paradoxical effects of Cyp 4a14 disruption in renal AA ω-hydroxylase activity. Based on the known hemodynamic effects of 20-HETE [1,4,6,7], and the increased renovascular resistance and impaired afferent arteriole autoregulatory capacity of Cyp 4a14 (-/-) mice [25], we proposed that androgen mediated increases in the renal biosynthesis of vasoconstrictor 20-HETE were responsible for the hypertensive phenotypes of Cyp 4a14 (-/-) and androgen-treated wild type mice [25]. The pro-hypertensive effects of androgens were confirmed by administering 5-α-dehydrotestosterone to male or female Sprague-Dawley rats. In these animals, treatment with DHT (40 mg, daily for 14 days) raised the systolic blood pressure of male and female rats by 29 and 57 mm of Hg, respectively, caused parallel increases in 20-HETE biosynthesis, CYPs 4A8 (the rat homologue of murine Cyp 4a12) and 4A2/4A3 expression [26], and decreased the kidney levels of CYP 4A1 mRNAs (Figure 3).

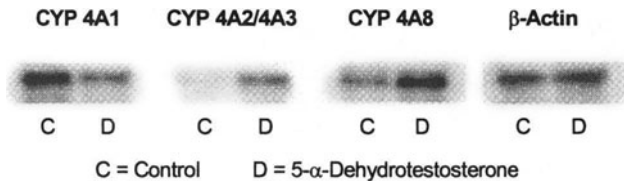

Figure 3. Nucleic acid hybridization analysis of total RNAs extracted from the kidneys of control (C) and 5-α-dehydrotestosterone treated (D) female rats: Samples of total RNA were isolated and fractionated by agar electrophoresis, transfer to nitrocellulose membranes, and hybridized to [^{32}P]labeled CYP P450 specific DNA probes coding for portions of the 3'-end untranslated segment of CYPs 4A1, 4A2/4A3, and 4A8 as described [14]. After high stringency washes, the membranes were exposed to X-ray films. RNA loadings were normalized using a β-actin probe.

The characterization of hypertensive Cyp 4a14 knockout mice showed that the pressure effects of this gene were apparently independent of the intrinsic AA monooxygenase activity of its encoded protein but, rather associated with changes in the regulation of alternate AA ω/ω-1 hydroxylases (Cyp 4a12)[1-7,19]. Based on the known pro-hypertensive properties of 20-HETE [1,4,6,7], and the above described gene-dependent, androgen mediated, control of 20-HETE renal expression and activity, we concluded that blood pressure regulation by kidney CYP 4A proteins involves a combination of transcriptional and hemodynamic mechanisms that determine the levels and site of expression of CYP 4A ω-hydroxylases and, ultimately, the level and site(s) of 20-HETE biosynthesis. Support for this conclusion was provided by the demonstration that dissected rat micro-

vessels, the target organ for most of the pro-hypertensive effects of 20-HETE, posses an androgen regulated CYP 4A8 20-HETE synthase [26]. A similar combination of EET-functional and epoxygenase-regulatory mechanisms were suggested by experiments done using P450 inhibitors [19], or the Dahl rat model of salt sensitive hypertension [19]. These studies indicated that induction of the kidney AA epoxygenase and increased renal EET biosynthesis prevented the development of salt sensitive hypertension [19], presumably by inhibiting distal Na^+ reabsorption and facilitating the excretion of excess dietary salt [19]. Similarly studies with SHR rats implicate changes in CYP 4A gene expression in the animals hypertensive phenotype since: a) the developmental phase of hypertension in these animals is accompanied by the upregulation of 20-HETE synthase activity and CYP4A2 expression [1,4,6,27,28], and b) antisense nucleotide inhibition of CYP 4A expression normalizes the blood pressures of hypertensive SHR rats [29,30].

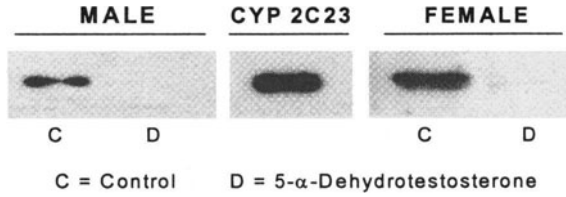

Figure 4. Anti-CYP 2C23 immunoreactive proteins present in kidney microsomes isolated from control (C) and 5-α-Dehydrotestosterone treated male and female rats: Samples of rat kidney microsomes (35 μg each), or purified recombiant CYP 2C23 (7 pmol) were fractionated by SDS- PAGE electrophoresis [17], and transfer by electroblotting into PVDF membranes as described [14,17]. After blocking, the mebranes were exposed to rabbit anti-CYP 2C23 IgGs. Immunoreactive proteins were visualized using an anti-rabbit IgG coupled to horseradish peroxidase, and a SuperSignal Substrate Western Blotting kit (Pierce).

The mechanism(s) by which Cyp 4a14 gene product(s) control plasma androgen levels are yet to be defined however, ample precedent supports a role for the kidney androgen receptor in regulating renal Cyp 4A and 2C expression [31-33]. Furthermore, in male and female rats, the androgen-mediated increases in systemic blood pressure, and in the levels of kidney CYP 4A8 transcripts [26], are accompanied by a marked decrease in the levels of renal CYP 2C23 epoxygenases protein (Figure 4) and diminished microsomal EET biosynthesis. The androgen-mediated counter-regulation of renal CYPs 4A ω-hydroxylases and 2C epoxygenases, suggest that its effects on blood pressure results from coordinated, nephron site specific, increases in the biosynthesis of pro-hypertensive 20-HETE, and decreases in anti-hypertensive EET formation. Finally, in mice, activation of PPARα up-

regulates the expression of Cyps 4a10 and 4a14 (but not 4a12) [34], and the counter-regulation of rat Cyp 4A and 2C isoforms by PPARα ligands is published [35], Nevertheless, the pressure effects of PPRA ligands in mice are yet to be fully defined, although, they have been characterized as anti-hypertensive in rats [36,37]. The studies summarized suggest that blood pressure regulation by renal P450s involves combinations of regulatory (transcriptional) and functional (tubular and hemodynamic) components, and that the organ balance of pro- and anti-hypertensive mediators and thus, its functional status, is dictated by: a) the nephron segment specific expression and regulation of the corresponding genes, b) the enzymatic properties of the encoded proteins, and c) the expression and activities of ancillary enzymes responsible for EET and/or HETE metabolism, disposition or activation.

Acknowledgements

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Chapter #10

CYTOCHROME P450-DERIVED EICOSANOIDS ARE MEDIATORS OF OCULAR SURFACE INFLAMMATION

Michal Laniado-Schwartzman and Michael W. Dunn

Injury to the corneal surface evokes an inflammatory reaction which includes the release of arachidonic acid (AA) and, subsequently, the production of eicosanoids which have been implicated as inflammatory mediators. These eicosanoids are produced by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 monooxygenases (CYP). We have identified CYP as a primary inflammatory pathway in the corneal epithelium where it metabolizes AA to 12-hydroxyeicosanoids, primarily 12(R)-hydroxy-5,8,10,14-eicosatetraenoic acid [12-(R)-HETE] and 12(R)-hydroxy-5,8,14-eicosatrienoic acid [12(R)-HETrE]; both metabolites exhibit biological activities that are typical of inflammatory mediators including increased membrane permeability, vasodilation, chemotaxis and angiogenesis. Indeed, numerous studies provided evidence that these metabolites are critical tissue-derived mediators of ocular surface inflammation.

Relationship between corneal epithelial 12-HETE and 12-HETrE synthesis and ocular surface inflammation

We have developed two models of corneal surface injury and assessed the production of 12-HETE and 12-HETrE and the severity of the inflammatory response. One model was that of closed eye contact lens wear. Eye closure renders the ocular surface hypoxic and creates an environment that has been described as a state of subclinical inflammation characterized by corneal swelling, conjunctival vasodilation, and significant levels of polymorphonuclear leukocytes (PMNs) in the tear film [1]. These changes are attributed to reduced oxygen and carbon dioxide exchange leading to corneal hypoxia and subsequent acidosis [2]. Contact lens wear in addition to eye closure increases hypoxia and, thereby, can significantly enhance the anterior surface inflammatory response [1]. Using this model of closed eye

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contact lens wear in rabbits, we showed a time-dependent increase in the capacity of the corneal epithelium to produce 12-HETE and 12-HETrE (Figure 1). This increase was significantly correlated with the in situ inflammatory response including corneal thickness, limbal vasodilation and neovascularization (Figure 1) [3].

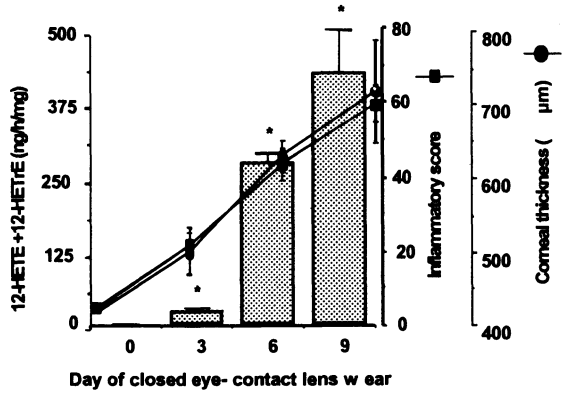

Figure 1. Synthesis of 12-HETE and 12-HETrE correlates with the inflammatory response in hypoxic injury. Rabbit eyes were fitted with two hydrogel contact lenses and the lids were sutured. At the indicated time, sutures were removed and eyes examined. Corneal thickness, inflammatory score and corneal epithelial synthesis of 12-HETE+12-HETrE for each time point were measured as described [3]. The results are expressed as the mean±SEM (n=5-6). Day 0=control, no eye closure or hydrogel contact lens. The results demonstrated significant correlation between inflammatory score ($r = 0.963$, $p=0.04$, $n=4$), corneal thickness ($r = 0.971$, $p=0.03$, $n=4$) and the production rates of 12-HETE + 12-HETrE over the extent of closed eye-soft contact lens wear.

The other injury model used is the corneal alkali burn, which is a well established model for the study of anterior surface inflammation, neovascularization and wound healing processes. We showed that alkali burning caused a time dependent increase in the corneal synthesis of 12-HETE and 12-HETrE resulting in a 40-fold increase over controls at day 7 post-burn. Both enantiomers of these metabolites were found to be formed; however, the biologically active R enantiomers were the predominant [4]. More importantly, total arachidonic acid metabolism in our study correlated with the area of neovascularization, particularly, the synthesis of 12(R)-HETrE ($p<0.02$) (Figure 2). In view of these observations and the known potent direct and indirect angiogenic properties of 12(R)-HETrE, there is reason to consider that this eicosanoid is responsible, at least in part, for the corneal neovascularization. Corneal neovascularization is part of the reparative process following an alkali burn. The presence of new vessels

lessens the chances of corneal perforation which has dire consequences for the eye [5].

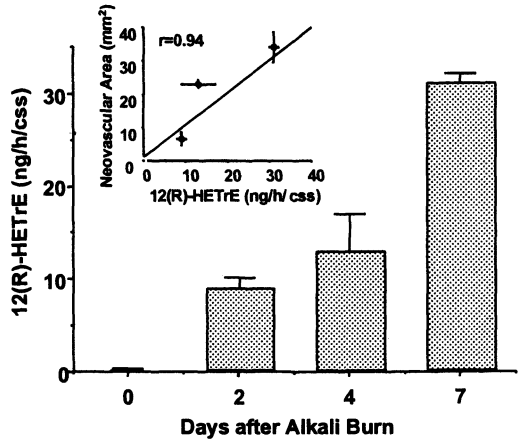

Figure 2. Alkali burn injury: Relationship between 12(R)-HETrE synthesis and corneal neovascularization in vivo. A 10 mm alkali burn was performed as described (4). Area of neovascularization was calculated using image analysis software and expressed in mm^2. Corneal epithelial scrapings were incubated with ^{14}C-AA, metabolites extracted, purified and derivatized prior to chiral analysis. Results are expressed as ng/h/corneal surface scrapings (css) and are the mean±SE (n=3, $p<0.01$ by ANOVA).

In order to assess the role of these eicosanoids as mediators of the inflammatory response in vivo, we used stannous chloride to inhibit the epithelial CYP-dependent synthesis of 12-HETE and 12-HETrE using the closed eye contact lens wear model of hypoxic injury. Treatment of the lenses with stannous chloride (100 μg/ml) significantly attenuated by day 7 the inflammatory score (56% decrease), corneal thickness (17% decrease), and 12-HETE/12-HETrE synthesis (77% and 71% decrease, respectively). These results further substantiated the involvement of 12-HETE and 12-HETrE in the inflammatory response following injury to the cornea [6,7].

Whether this pathway is functioning in the human corneal surface as an inflammatory pathway is yet to be established. However, a recent report by our laboratory demonstrated that these metabolites are present in human tears [8]. More importantly, their levels and in particular those of the 12-HETrE, are markedly increased in tears from inflamed eyes of different etiologies. In control tears (n = 10), 62±13 pg/μl of 12-HETrE was detected: the mean level of 12-HETrE increased to 448±200 pg/μl in tear film from inflamed eyes (n = 38). Thus, the human eye produces detectable amounts of 12-HETrE which is released into the tear film. Moreover, the levels of 12-HETrE in the tear film correlated with the severity of the inflammation. The increased levels of 12-HETrE associated with ocular surface inflammation

suggest that this eicosanoid may contribute to inflammation of the ocular surface in humans.

The tremendous increase (100-300 fold increase from day 0 to day 9) in the production rate of 12-HETE and 12-HETrE by the inflamed corneal epithelium seen in vitro may be a consequence of enzyme induction; however, in vivo a combination of other factors may be operative. For example, the rate limiting step for eicosanoid synthesis is believed to be the release of arachidonic acid from cellular lipids. Mechanical trauma such as shear stress as well as hypoxia are potent stimuli for the release of arachidonic acid in many tissues [9]. Furthermore, hypoxia, the principal contributor to the adverse effects of contact lens wear, has shown to be a potent inducer of CYP isozymes [10,11]. In addition, the level of reducing equivalents, particularly NADPH, is increased in the corneal epithelium under stressful conditions, e.g., contact lens wear [12]. Thus, the mechanical stress and hypoxia placed upon the epithelium via hydrogel lens wear in a closed eye, provides an adequate stimulus for arachidonate release and subsequent 12-HETE and 12-HETrE synthesis [3]. Thus, not only may these stimuli activate the release of substrate, hypoxia may also induce the metabolic enzyme(s) CYP as well as increase the levels of essential cofactors necessary for this enzymatic reaction. To this end, incubation of corneal organ cultures under hypoxic conditions (2% O_2) resulted in a marked increase in the capacity of the corneal epithelium to produce 12(R)-HETE and 12(R)-HETrE [13]. Moreover, a CYP protein, whose expression was markedly increased following hypoxic injury in vitro and in vivo, was isolated, cloned and identified as CYP4B1 [14,15]. Inhibition of CYP4B1 by immunoprecipitation reduced the synthesis of 12-HETe and 12-HETrE in the corneal epithelium suggesting that this CYP contributes to the synthesis of these proinflammatory eicosanoids [14]. Moreover, CYP4B1 mRNA levels in the corneal epithelium increased during hypoxic injury in vivo. The pattern of expression of CYP4B1 corresponded well with the progression of the anterior surface inflammatory response including corneal thickness and inflammatory score determined as well as with the rate of synthesis of the inflammatory and angiogenic eicosanoid, 12-HETrE [15].

The inflammatory and angiogenic activities of 12-HETrE

During inflammation, microvessel endothelial cells play a key role in the overall orchestration of the vascular responses of the inflammatory process. In acute inflammation, microvessels dilate and increase their permeability; the endothelium, in response to endogenous and circulating factors, regenerates to replace injured or dying cells. In chronic inflammation, neovascularization becomes the hallmark of the vascular response possibly to provide new vessels through which inflammatory cells enter the site of injury [16,17]. A wide variety of disorders of the cornea culminate in angiogenesis and a considerable amount of information has accumulated about the circumstances under which newly formed blood vessels sprout and

extend into the cornea. Vascularized corneas are clinically significant because they are associated with markedly diminished visual acuity. Diverse mediators have been implicated in the process including PGs, vasoactive amines, epithelial angiogenic factors, and components of leukocytic extracts [18].

The biological activities ascribed to 12(R)-HETrE (i.e., vasodilation, chemotaxis and angiogenesis) and its induced synthesis in inflamed tissues strongly indicate an important role for this compound in both the acute and chronic phases of the inflammatory response. It is produced by the corneal epithelium in response to injury, displays potent inflammatory properties [19,20,21], is a mitogen for microvessel endothelial cells [22], and is angiogenic in vitro and in vivo [21]. Following hypoxic and chemical injury to the corneal epithelium 12(R)-HETrE is produced at a rate of 60-100 pmol/hr/mg which is sufficient to elicit its effect on the adjacent capillary endothelial cells of the limbal vessels [3,4]. It is readily released into the incubation media from injured rabbit corneas [23] and its levels increase dramatically in tears from inflamed human eyes [8].

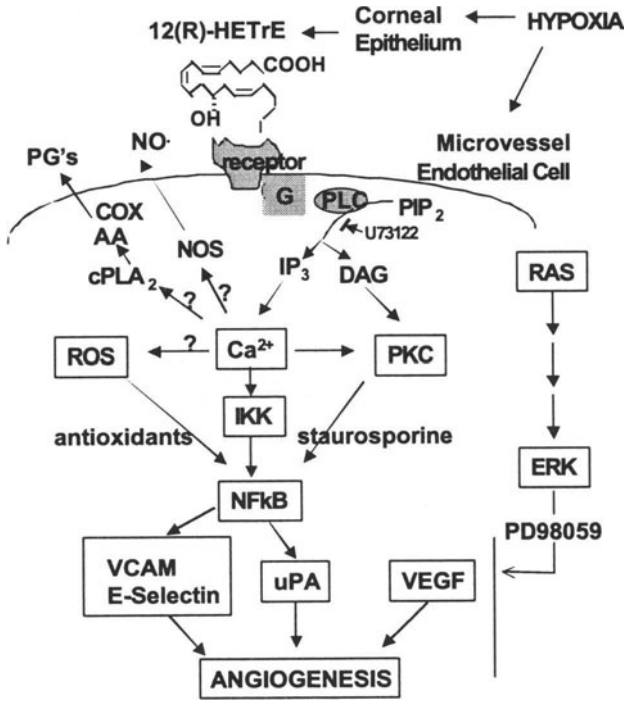

Figure 3. Postulated signal transduction mechanisms underlying 12(R)-HETrE's angiogenic activity in microvessel endothelial cells.

The aforementioned findings suggest that 12(R)-HETrE, a corneal epithelial-derived angiogenic factor whose synthesis is induced in response

to injury, acts in a paracrine manner on the limbal vessels to activate endothelial cells via a specific receptor/binding site into the angiogenic phenotype. The cellular mechanisms underlying 12(R)-HETrE's angiogenic activity are still unclear. We have identified several of the events involved in the signal transduction of endothelial cells stimulated by 12(R)-HETrE including the demonstration of a putative receptor in limbal microvessel endothelial cells (24), a PLC-IP_3-mediated increase in intracellular Ca^{2+} concentration $[Ca^{2+}]i$ (Figure 3), a PKC-dependent NFκB activation [25], increased c-fos, c-jun and c-myc oncogene expression [26] and increased VEGF mRNA via activation of Erk1/2 MAPK pathway [27]. Each of 12(R)-HETrE's post receptor signaling steps, including Ca^{2+}, NFκB, MAPK, oncogenes and VEGF, have been implicated in the transformation of endothelial cells from a quiesced to an angiogenic phenotype [28]. Furthermore, our studies also indicate a role for this mediator in inflammatory responses in the skin [29], a tissue where its production has been reported [30,31]. It is possible that this eicosanoid is produced in a tissue/injury specific manner to aid with the orchestration of the inflammatory/angiogenic responses.

Acknowledgements

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Chapter #11

REGULATION OF RENAL MICROVASCULAR 20-HYDROXYEICOSATETRAENOIC ACID (20-HETE) LEVELS

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Introduction

The kidney possesses a large capacity to generate cytochrome P450 (CYP) dependent arachidonic acid (AA) metabolites, chiefly the ω and ω -1 hydroxylase-derived metabolites, 19- and 20- hydroxyeicosatetraenoic acids (HETEs), and lesser amounts of epoxyeicosatrienoic acids (EETs), primarily 11,12 EETs [1]. Angiotensin II (AII) increases renal synthesis of HETEs and EETs, which modulate and mediate the actions of the peptide in the renal vasculature and the nephron [2]. Our studies have identified preglomerular microvessels (PMGV) as a potentially important site of interactions involving AII and CYP-AA products [3,4]. The "complex control mechanisms" governing the renal circulation, are funneled into the PMGV. PGMV are the effector component of key renal regulatory mechanisms, autoregulation of renal blood flow and tubuloglomerular feedback [5]. Renal autoregulation and tubular glomerular feedback are mediated by 20-HETE through constriction of PGMV, particularly afferent arterioles [6].

Results and Discussion

During a 5 min incubation, AII (5 nM) increased HETE release from PGMVs by two-fold. Stimulation of HETE release by AII was specific as several vasoactive agonists including phenylephrine (1μM), bradykinin (1μM) and endothelin -1 (1μM) did not release HETEs. We found,

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unexpectedly, that AT_2 receptors mediated AII-induced release of 20-HETE as the release was blocked by the AT_2 receptor antagonist, PD 123319 and was unaffected by losartan (DUP 753), an AT_1 receptor antagonist. Further, release of 20-HETE in response to stimulation of AT_2 receptors was linked to activation of PLC as U-73122, a PLC inhibitor, reduced 20-HETE levels [3]. Thus, an AT_2 -PLC effector unit is associated with synthesis of a vasoconstrictor product, 20-HETE, in a key renovascular segment.

The AT_2 receptor, which in most tissues expression diminishes rapidly after birth, is upregulated by sodium depletion and AII [7,8]. As Na^+ deprivation induces COX-2 expression/activity in the renal cortex and AII stimulates release of 20-HETE from PGMV, we used a stimulus, low dietary salt, to activate the renin-angiotensin-system (RAS) and COX-2 and thereby explore potential interactions involving 20-HETE and COX-2. The capacity of COX to metabolize 20-HETE to prostaglandin analogs, e.g., 20-OH $PGF_{2\alpha}$ and 20-OH PGE_2, may be critical to modifying the renal vascular and tubular actions of the eicosanoid. Rats were placed on either a low salt (0.05%) or 1% salt diet for 7 days. On the 8^{th} day PGMV (0.5mg protein) were isolated and incubated for 5 minutes at 37°C in Tyrodes solution to which NADPH (1mM) was added in the presence or absence of indomethacin (10 mM). The CYP-AA metabolites were quantitated by negative chemical ionization GC/MS. Rat PGMV produced primarily 20-HETE with lesser amounts of 19-HETE and negligible amounts of 16-, 17-, and 18-HETEs. Low salt intake increased formation of 20-HETE by PGMV that was masked unless microvessels were preincubated with indomethacin (10 μM). The diminished 20-HETE levels are presumably due to increased COX-2 activity and expression in response to low salt intake, COX-2 then serving as a metabolic (biosynthetic) pathway for 20-HETE, giving rise to prostaglandin analogs of 20-HETE that differ from untransformed 20-HETE.

As an association between hypertension and increased synthesis of 20-HETE has been reported [9], and as upregulation of AT_2 receptors occurred in response to AII, we infused a pressor dose of AII for two weeks to determine whether: 1) renal PGMVs responded to *in vivo* challenge with AII as they did to *in vitro* exposure to AII; and 2) AII produced sustained elevation in 20-HETE synthesis over a two-week period. Microvascular 20-HETE release was increased more than two-fold in AII treated groups compared to controls. The enhanced release was evident at 3 days, the earliest time point studied, and was not further enhanced after 14 days of treatment, although blood pressure continued to rise. Thus, *in vivo* AII treatment stimulated microvascular 20-HETE production, an effect that was independent of the magnitude of the pressor action of the peptide. Our results show that, indeed, sodium depletion enhances 20-HETE formation in PGMV which requires inhibition of COX to be discerned, suggesting that

increased COX serves as a metabolic pathway for 20-HETE, forming vasodilator prostaglandin analogs, a function mediated by COX-2 that is expressed in response to low salt intake. The capacity of COX to metabolize 20-HETE to prostaglandins e.g., 20-OH $PGF_{2\alpha}$ and 20-OH PGE_2 may be critical to modifying the renal vascular and tubular actions of AII as, for example, in states of Na^+ deprivation or abnormalities of Na^+ homeostasis such as hepatic cirrhosis, heart failure and diabetic and hypertensive nephropathy. We propose that the induction of cortical COX-2 [10] will result in preservation of renal hemodynamics in large part because vasodilator prostaglandin analogs of 20-HETE (20-OH PGE_2 and 20-OH PGI_2) are generated, thereby, keeping unmetabolized vasoconstrictor 20-HETE levels low. In response to low salt intake, the pivotal role of COX in regulating 20-HETE levels in the kidney becomes evident when COX is not inhibited, resulting in PGMV 20-HETE levels falling by as much as 80%. This mechanism serves to modulate 20-HETE-induced constriction of PGMV under conditions of extracellular volume contraction produced by sodium depletion. We propose that the major adverse effects of non-steroidal anti-inflammatory drugs result from the direct vasoconstrictor action of unmetabolized 20-HETE.

Acknowledgments

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Chapter #12

CYCLOOXYGENASE ACTIVITY IS REGULATED BY THE HEME OXYGENASE SYSTEM IN MICROVESSEL ENDOTHELIAL CELLS

Nader G. Abraham and Rafal Olszanecki

Introduction

Cyclooxygenase(s) catalyzes the oxygenation and peroxidation of arachidonic acid to prostaglandin endoperoxide H_2, the immediate precursor of prostaglandins and thromboxane. Two COX isoforms encoded by two related genes have been identified; COX-1 is constitutively expressed and is considered to generate prostaglandins for normal physiological functions whereas COX-2 is, in most tissues, an inducible enzyme expressing rapidly and transiently in response to a variety of stimuli [1]. Both COX isoforms are hemeproteins [2. Heme binds to the COX apoenzyme with a stoichiometry of approximately one heme molecule per each subunit [3]. It is well documented that the heme prosthetic group of COX is essential for the expression of catalytic activity [3]. Accordingly, the possibility arises that variations in the cellular levels of heme impact on the amount of catalytically active COX present in cells.

The cellular level of heme is regulated by the rate of its synthesis and degradation. Heme degradation occurs almost exclusively by oxidative cleavage of the α-meso carbon bridge of heme, eventually leading to the formation of equimolar amounts of biliverdin, iron and carbon monoxide (CO). The heme oxygenase-1 (HO-1) system controls the rate-limiting step in heme degradation [4]. HO-1, the inducible isoform, plays a critical role in the regulation of cellular heme levels [4] which, in turn, may impact on the expression of catalytically active COX isoforms [5,6] Previous studies have documented induction of vascular and renal HO-1 in response to Ang II in vivo [7], hypoxia and inflammation [5,6,8,9].

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Results

Effect of HO induction and inhibition on prostaglandin levels in rabbit endothelial cells

Under control conditions, the levels of PGE_2 in the culture media of rabbit endothelial cells were approximately 20 times higher than those of 6-keto-$PGF_{1\alpha}$, which is in agreement with previous reports of prostanoid synthesis in cultured endothelial cells derived from microvessels [10]. The addition of heme (10 μM) to the culture medium increased the levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 by 62 and 50%, respectively (Table 1), which is indicative of enhanced prostaglandin synthesis. Interestingly, SnMP (10 μM), an inhibitor of HO activity, did not significantly affect basal or heme-stimulated prostaglandin levels in the culture medium. Hence, PGE_2 levels were 30,130±2,152 and 33,241±2,890 pg/ml in the culture medium of untreated cells and cells treated with SnMP (n=6, p=0.50), respectively. Similarly, 6-keto-$PGF1_\alpha$ levels in untreated cells and cells treated with SnMP were 1,645±103 and 1,845±103 pg/ml, respectively (n=6, p=0.20). Moreover, in the cells treated with heme (n=6) and heme+SnMP (n=3), PGE_2 levels were 44,620±1,656 and 42,370±2,627 pg/ml, respectively, whereas 6-keto-$PGF_{1\alpha}$ levels were 2,799±402 and 2,222±131 pg/ml in heme- and heme+SnMP-treated cells, respectively.

Table 1. PGE_2 and 6-keto-$PGF_{1\alpha}$ levels in rabbit microvessel endothelial cells. Cells were treated with or without heme (10 μM) or $CoCl_2$ (150 μM) for 24 h. The levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 in the culture media were measured by EIA. The results are expressed in pg of prostaglandins in 1 ml culture medium and are the mean±SE (n=3; *$p<0.05$ compared to the corresponding control).

Treatments	PGE_2 (pg/ml)	6-keto-$PGF1_\alpha$ (pg/ml)
Control	31,007±2,600	1,806±290
Heme	*47,166±4,125	*2,905±482
$CoCl_2$	*9,054±700	*612±137

As shown in Figure 1, HO activity was increased by two- and four-fold, respectively, in endothelial cells treated with heme or Ang II. HO activity in cells treated with Ang II increased 1.91-fold ($p<0.05$) when compared to control and was decreased by concurrent treatment with ZnDPP (data not shown). Opposite results were obtained when Ang II-treated endothelial cells were concurrently treated with heme.

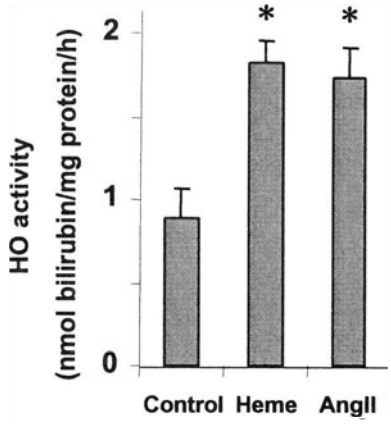

Figure 1. HO activity in endothelial cells treated with Ang II and different inducers or inhibitors of HO. Cell lysate HO activity was assayed by bilirubin using the difference in absorbency from λ 460 to λ 530 nm with an absorption coefficient of 40 mM^{-1} and cm^{-1}. HO activity (nmol bilirubin/mg protein) is expressed as the mean±SE of 4 different flasks, respectively. Statistical analyses were performed by t-test. * $p<0.05$ compared to control.

Effect of HO induction and inhibition on prostaglandin levels in human microvessel endothelial cells

Under basal conditions, PGE_2 levels in the culture medium of human endothelial cells were approximately 20 times higher than those of 6-keto-$PGF_{1\alpha}$. This is in agreement with previous reports [11]. The addition of heme to the culture medium increased the levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 35% and 98%, respectively, which is indicative of enhanced prostaglandin synthesis (Figure 2). The addition of $SnCl_2$ decreased PGE_2 and 6-keto-$PGF_{1\alpha}$ productions, respectively. Treatment with ZnDPP alone did not significantly affect prostaglandin levels in the culture medium. However, treatment of cells concurrently with ZnDPP and Ang II, further increased PGE_2 from 38±3.5 to 68.6±10.6 ng/ml ($p<0.05$) and 6-keto-$PGF1_{\alpha}$ from 3.9±0.8 to 4.98±0.79 ng/ml ($p<0.05$).

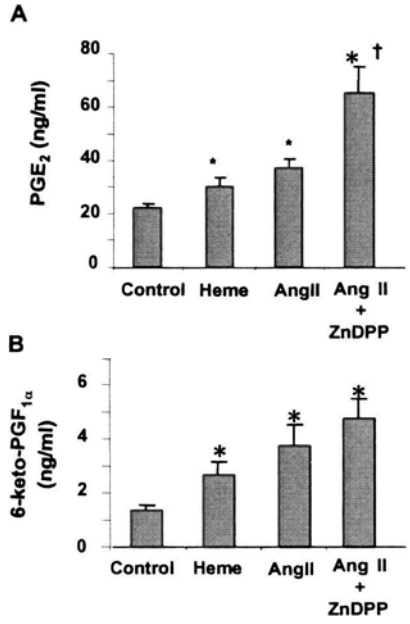

Figure 2. PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) levels in endothelial cells. Cells were treated with heme (10 μM), $SnCl_2$ (10 μM), ZnDPP (10 μM) and Ang II for 24 h. The levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 in the culture medium were measured by EIA. The results are expressed in ng of prostaglandins in 1 ml of culture medium (1.2 x 10^4 cell/well) and are the mean±SE (n = 3; *$p < 0.05$ compared with the corresponding control).

Effect of HO-1 expression on COX-2 activity

In complementary studies, the synthesis of 6-keto-$PGF1_{\alpha}$ by lysates of control untreated endothelial cells (0.15±0.006 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=6) was exceeded ($p<0.05$) by that of endothelial cells treated with heme (0.71±0.047 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=6) or Ang II (0.66±0.02 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=6). Interestingly, the synthesis of 6-keto-$PGF_{1\alpha}$ by lysates of heme-treated cells was greatly reduced ($p<0.05$) by inclusion in the reaction mixture of NS-398 to inhibit COX-2 (0.16±0.06 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=5) or of indomethacin to inhibit both COX-1 and -2 (0.1±0.017 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=3). Likewise, 6-keto-$PGF_{1\alpha}$ synthesis by lysates of endothelial cells treated with Ang II was greatly diminished ($p<0.05$) by NS-398 (0.08±0.055 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=5) and indomethacin (0.06±0.06 ng x mg of protein^{-1} x ml^{-1} x min^{-1}; n=3)

Effect of heme on prostaglandin levels and COX expression in primary cultures of endothelial cells

High-passage cultured endothelial cells may differ functionally from first-passage cultured endothelial cells, which are less likely to de-differentiate. Therefore, additional experiments using primary cultures of bovine artery endothelial cells were performed to assess the effect of the heme-HO system on prostaglandin levels and COX expression. Incubation of cells, with heme for 24 h increased 6-keto-$PGF_{1\alpha}$ and PGE_2 by two- and four-fold, respectively. Western blot analysis showed that incubation of primary cultures with heme resulted in upregulation of HO-1 with no significant effect on either HO-2, COX-1 or COX-2 protein levels (data not shown). The lack of effect of heme on COX protein may be due to the presence of heme levels in primary cells as compared with cell lines.

Effect of human HO-1 gene transfer on rabbit endothelial cells

Rabbit endothelial cells transfected with the human HO-1 gene expressed human HO-1 mRNA, but there was no increase in human HO-1 gene expression following treatment with $CoCl_2$ or heme due to the absence of promoter in the transduced gene (data not shown). The increase in HO expression brought about by HO-1 gene transfer was accompanied by augmentation in HO activity. Importantly, cellular heme content in rabbit endothelial cells transduced with human HO-1 was 56% lower than that in nontransduced rabbit cells (0.21±0.02 versus 0.48±0.03 nmol heme/mg protein), indicating that chronic augmentation in HO activity in cells expressing human HO-1 cDNA brings about depletion of cellular heme levels. As shown in Table 2, HO activity in rabbit cells was greatly increased by human HO-1 gene transfer.

Table 2. PGE_2 and 6-keto-$PGF_{1\alpha}$ levels in bovine artery endothelial cells treated with and without heme (10 μM). Results are the mean±SE (n=3; *$p<0.05$).

Treatments	PGE_2 (pg/ml)	6-keto-$PGF1_{\alpha}$ (pg/ml)
Control	189±67	2,158±153
Heme	*803±149	*4,426±209

Prostaglandin levels and COX expression in rabbit cells expressing human HO-1

The basal levels of 6-keto $PGF_{1\alpha}$ and PGE_2 in culture medium were decreased by 85% in cells expressing the human HO-1 transduced cells compared to nontransduced rabbit cells (Table 3). Rabbit cells transduced

with the empty expression vector expressed a level of PGE_2 and 6-keto-$PGF_{1\alpha}$ similar to that seen in nontransduced cells (data not shown). The decrease in PGE_2 and 6-keto-$PGF_{1\alpha}$ in cells overexpressing human HO-1 was associated with elevation in HO activity.

Table 3. Effect of human HO-1 on PGE_2 and 6-keto-$PGF_{1\alpha}$ levels. The levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 in the culture media of rabbit endothelial cells and rabbit HHO-1 cells were measured by EIA. The results are expressed in pg of prostaglandins in 1 ml culture medium and are the mean±SE (n=3; *$p<0.05$ compared to the corresponding control).

Treatments	HO activity (pmol bilirubin/mg/hr)	PGE_2 (pg/ml)	6-keto-$PGF_{1\alpha}$ (pg/ml)
Control cells	0.98±0.13	27,138±3,168	1,875±106
Cells transduced with human HO-1 gene	*6.71±0.23	*5,613±348	*406±68

Discussion

This study demonstrates, for the first time, that the heme-HO system of endothelial cells participates in the regulation of prostaglandin production by these cells, presumably by influencing the availability of heme for the manufacture of catalytically active COX. We found that the production of COX products, PGE_2 and 6-keto-$PGF_{1\alpha}$, the nonenzymatic derivative of PGI_2, was greatly diminished in rabbit endothelial cells treated with $CoCl_2$ or transfected with the human HO-1 gene. In agreement with previous reports, treatment with $CoCl_2$ increased the expression of HO-1 in rabbit endothelial cells, enhancing HO activity and presumably decreasing cellular heme [12]. Transfection of rabbit endothelial cells with the human HO-1 gene resulted in augmentation of HO-1 expression, elevation of HO activity and reduction of cellular heme levels. Hence, the production of COX products by vascular endothelial cells appears to be downregulated in experimental settings in which HO-1 is overexpressed and cellular heme is reduced. In addition, Ang II-induced stimulation of prostaglandin production is magnified in endothelial cells treated with ZnDPP to prevent HO mediated metabolism of endogenous heme; it is also magnified in cells treated with exogenous heme to compensate for reductions in cellular heme levels due to HO-1 overexpression. We also found, Ang II-induced stimulation of prostaglandin production is attenuated in endothelial cells treated with $SnCl_2$ to further upregulate HO-1 and reduce cellular heme. Collectively, these observations suggest that the stimulation of prostaglandin production elicited by heme and Ang II in endothelial cells is limited by the accompanying overexpression of HO-1, leading to lowering of cellular heme to a level below that which is required for optimal expression of Ang II-induced prostaglandin production.

To assess the role of HO-1 on COX activity, we used NS-398 and indomethacin, a putative specific inhibitor of COX-2 and a nonselective inhibitor of COX-1 and -2, respectively, on COX activity, both of these drugs were equally effective in suppressing prostaglandin synthesis during incubation of arachidonic acid with lysates prepared from endothelial cells treated with Ang II or heme. This implies that COX-2 rather than COX-1 is responsible for prostaglandin synthesis in the human femoral endothelial cells used in the study.

In summary, the present study documents a regulatory action of the heme-HO system, in endothelial cells, on prostaglandin production. Upregulation of HO-1 leading to reduction in cellular heme brings about a decrease in prostaglandin synthesis. We take this finding as indicative that variations in cellular heme levels impact prostaglandin production in endothelial cells by influencing the amount of catalytically active COX, presumably COX-2.

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Chapter #13

CYTOCHROME P450 ARACHIDONIC ACID METABOLITES MODULATE RENAL TUBULAR FUNCTION

Mairead A Carroll, Nicholas R Ferreri, Bruno A Escalante, Adebayo O. Oyekan, and John C. McGiff

Introduction

Nephron function is segmented. Each segment has characteristic transport mechanisms and individual eicosanoid profiles [1,2]. Mapping the distribution of AA metabolizing enzymes within the nephron is essential to understanding the contribution of eicosanoids to excretory function. The study of Bonvalet et al addressed the localization of cyclooxygenase (COX) in the nephron and uncovered large segments that had negligible COX activity, particularly the proximal tubules (PT) and the thick ascending limb (TAL) [3], which later studies identified as segments heavily invested with cytochrome monooxygenase (CYP450) activity [4,5].
Our first step in characterizing these segments of the nephron was to isolate them in relative homogeneity. The medullary (m) TAL of the rabbit initially [5], and later the rat [6], were isolated and subject to biochemical and functional analyses. The most important finding involved identification of 20-hydroxyeicosatetraenoic acid (20-HETE) [5] and localization of its site of action in the mTAL [7]. The sites of activity of 20-HETE in the mTAL were the Na^+-K^+-$2Cl^-$ cotransporter and the 70 pS K^+ channel, each of which was inhibited by 20-HETE [8].

Results and Discussion

A series of studies followed that addressed the effects of Angiotensin II (ANG II) [9] and extracellular calcium (Ca^{2+}) [10] concentrations on activity of ω hydroxylase, the enzyme that synthesizes 20-HETE. Long-term exposure to ANG II (>3 h) induced COX-2 expression which displaced ω hydroxylase as the principal pathway of arachidonic acid (AA) metabolism in the mTAL [6]. The functional significance in the intact animal of this

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switching mechanism has not been examined, although it presumably contributes to the pathophysiological alterations of renal function in states associated with elevated ANG II levels intrarenally. This switching mechanism is dependent on activation of tumor necrosis factor alpha (TNFα) [6] and may represent a long-term adaptive mechanism mediated by prostaglandin (PGE_2).

We also investigated the role of ω hydroxylase in the PT in terms of 20-HETE acting as a second messenger for endothelin-1 (ET-1) [11]. This work was occasioned by our findings that ET-1, despite its renal vasoconstrictor action, produced diuresis-natriuresis associated with increased renal release of 20-HETE [12]. Our working hypothesis was that 20-HETE mediated the inhibitory action of ET-1 on PT ion transport in the rat. We addressed the effects of ET-1 on PT transport because of the heavy endowment of this tubular segment with ω hydroxylase [4]. Further, the absence of significant COX activity in the PT [3] eliminated a potentially complicating factor because of the avid metabolism of 20-HETE by COX [13]. We found that 20-HETE functioned as a second messenger, mediating the effects of ET-1 on ion transport in PTs [11].

We initially established that changes in ^{86}Rb uptake reflected changes in the activity of the two transporters most involved in transepithelial Na^+ flux; viz., the basolateral Na^+-K^+-ATPase (the Na^+ pump) and the apical Na^+/H^+ exchanger [11]. ET-1 produced a dose-dependent inhibition of ouabain-sensitive ^{86}Rb uptake in PT over a dose range of 0.1 to 100 ng. Dibromododec-11-enoic acid (DBDD), a selective inhibitor of ω hydroxylase activity which was without effect on basal ^{86}Rb uptake, abolished the inhibitory effect of 10 nM ET-1 on ^{86}Rb uptake, suggesting that the effects of ET-1 were mediated by a CYP450-AA metabolite, probably 20-HETE as ET-1 released 20-HETE from the PTs. Indeed, 20-HETE inhibited ^{86}Rb uptake by the PT as did 1μM AA; the effects of AA were also abolished by DBDD whereas those of 20-HETE were expectedly unaffected by DBDD. As NO exerts an inhibitory action on ω hydroxylase activity, we also examined the effect of disinhibiting ω hydroxylase with L-NAME treatment. This intervention increased AA conversion to 20-HETE and potentiated the inhibitory effects of AA on PT ion transport.

We conclude that, in the PTs, 20-HETE is a second messenger for ET-1 acting as a mediator of the inhibitory effects of ET-1 on transepithelial Na^+ movement in this tubular segment. The critical role of CYP450-derived AA products is further expanded and now involves a number of hormones that affect renal tubular function: ANG II, parathyroid hormone (PTH), dopamine and epidermal growth factor (EGF) [1].

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Chapter #14

THROMBOXANE–INDUCED ERK PHOSPHORYLATION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

Carole Gallet, Stéphanie Blaie, Sylviane Lévy-Toledano and Aïda Habib

GPCRs (G-protein coupled receptor) activation of extracellular signal-regulated kinases (ERK) has been extensively explored the last decade and showed a multitude of intracellular pathways involved including the activation of PKC, Ras, raf-1, etc [1]. Recently, it has been demonstrated that some GPCRs such as the lysophosphatidic acid, thrombin, bombesin and angiotensin receptor can activate ERK through the phosphorylation of the epidermal growth factor (EGF) receptor [2]. This was mainly done using selective inhibitors of the tyrosine kinase activity of the EGF receptor such as the tyrphostin AG1478. Also, direct demonstration of the phosphorylation of the receptor of EGF in response to these different ligands has been demonstrated [3].

Thromboxane (TX) A_2 receptors (TP) belong to the family of GPCRs [4]. TX activates ERK kinases in rat aortic smooth muscle cells [5] whereas activation of JNK kinase was predominant in porcine aortic smooth muscle cells [6]. However, little is known about the mechanisms involved in the activation of ERK in human primary vascular cells.

In this study, we evaluated the effect of IBOP, a stable analogue of TX, on ERK phosphorylation in human aortic smooth muscle cells in culture.

After 10 min treatment of cells with 100 nM of IBOP, there was a significant increase in ERK phosphorylation. This effect was inhibited when cells were pre-treated for 30 min with 250 nM of AG1478, a selective inhibitor of the tyrosine dependent phosphorylation of the EGF receptor. Under the same conditions, EGF-dependent activation of ERK was blocked by the same inhibitor while the PDGF (platelet derived growth factor) -BB and the fibroblast growth factor (FGF)-2- dependent activation of ERK was not modified confirming its selectivity among the growth factor receptors (Figure 1).

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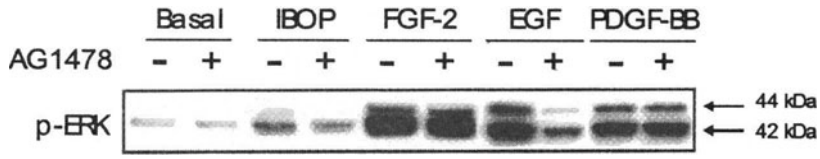

Figure 1. Effect of AG1478 on ERK phosphorylation in human aortic smooth muscle cells in culture. Cells were pre-treated in the absence or presence of 250 nM of AG1478 prior to the addition of 100 nM IBOP, 10 ng /ml of EGF, FGF-2 or PDGF-BB. After 10 min incubation, cells were harvested and lysates were subjected to SDS-Electrophoresis and immunoblot was performed using a polyclonal antibody specific of phosphorylated ERK (p-ERK). Detection was performed using ECL.

No modification of the phosphorylation of ERK by IBOP was examined when AG1296, a selective inhibitor of the phosphorylation of the PDGF receptor, was used. Strong inhibition in response to PDGF was strongly observed whereas AG1296 did not modify EGF-dependent phosphorylation of ERK (Figure 2).

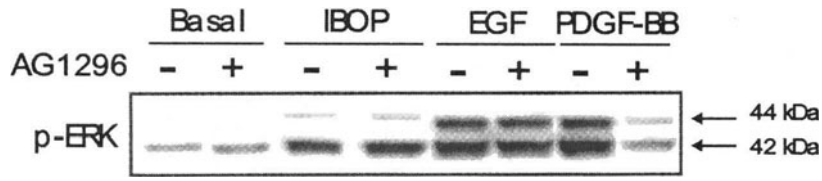

Figure 2. Effect of AG1296 on ERK phosphorylation. Human aortic smooth muscle cells were pre-treated in the absence or presence of 10 μM of AG1296 prior to the addition of IBOP, EGF or PDGF-BB. phospho-ERK (p-ERK) detection was done as described in the legend to figure 1.

We have characterized in human vascular smooth muscle cells the role of growth factor receptors in IBOP dependent phosphorylation of ERK. We showed that phosphorylation of ERK is dependent on EGF and not PDGF activation. Some studies have demonstrated the existence of a transactivation of the PDGF receptor by GPCRs, i.e. angiotensin [7], while others have demonstrated the only activation of the EGF receptor in response to the same ligand. [8]. The importance of the transactivation is still under investigation mainly in relation to the degree of participation of this pathway in cellular growth, migration and differentiation.

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Notes

* Carole Gallet and Stéphanie Blaie contributed equally to the work.

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Chapter #15

ARACHIDONIC ACID INHIBITS CYSTEINYL-LEUKOTRIENE RECEPTOR ACTIVATION IN HUMAN PULMONARY VESSELS

Laurence Walch, Xavier Norel, Jean-Pierre Gascard, and Charles Brink

Introduction

There are a few reports suggesting that arachidonic acid may have direct regulatory actions in cells or tissues which are independent of the released endogenous metabolites. Brotherton and co-workers [1] reported that endothelial cells in culture which released prostacyclin in the presence of arachidonic acid became refractory subsequent to a second stimulation, that is, the cells failed to release significant levels of prostacyclin when compared with the initial challenge. These observations were also supported by the work of Revtyak and co-workers [2] who demonstrated that both histamine- and A23187-induced prostacyclin release was inhibited in human endothelial cells subsequent to arachidonic acid exposure. These results suggested that arachidonic acid may play a direct regulatory role via interaction with cell surface receptors. The aim of this investigation was to examine the effects of arachidonic acid on human pulmonary arteries and veins by measuring the release of several metabolites subsequent to the arachidonic acid exposure and evaluate the effects of cysteinyl-leukotrienes on this release.

Material and Methods

Intrapulmonary arteries and veins were removed from the human lung and cut into rings which weighed 220 ± 23 mg (arteries) and 167 ± 54 mg (veins). In some experiments the endothelium was removed to evaluate the relative contribution of the endothelial layer to the mediators released.

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Preparations were placed in microtitre plates (24 wells) containing Tyrode's solution (1 ml) and allowed to equilibrate for 15 min in a humidified incubator (37°C, 5% CO_2/air). The Tyrode's solution composition (mM) was: NaCl: 149.2; KCl: 2.7; $NaHCO_3$: 11.9; $CaCl_2$: 1.8 $MgCl_2$: 0.5; NaH_2PO_4: 0.4 and glucose 5.5. At the end of the equilibration period (15 min) the medium was replaced with fresh Tyrode's solution containing arachidonic acid (10 μM) and the tissues were then maintained for 15 min (period I, basal production). Subsequently, the preparations were washed and then exposed to fresh Tyrode's solution with arachidonic acid (10 μM) for another 15 min (period II). At the end of the 1st and 2nd period the medium was collected and stored at -80°C until eicosanoid (6-keto-$PGF_{1\alpha}$, PGE_2, PGD_2 and LTE_4) and 8-iso-prostaglandin $F_{2\alpha}$ type III measurements were performed.

The effects of the leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4), were evaluated by exposing the preparations to these agonists at different concentrations (0.01, 0.1 and 1.0 μM) for the duration of period II. In another series of protocols a methanolic extraction of the tissues was performed to determine the quantities of 8-iso-prostaglandin $F_{2\alpha}$ type III present in the different types of vascular preparations. Human pulmonary arteries and veins were rinsed in Tyrode's solution (10 ml) and set up in unsealed tubes (Falcon, 50 ml) containing Tyrode's solution (10 ml) with arachidonic acid (10 μM) and allowed to equilibrate for 15 min in a humidified incubator (37°C, 5%CO_2/air). The preparations were removed and immersed in methanol, vortexed (3 min) and centrifuged at 4°C for 20 min at 3000 g. The supernatants were then collected and stored at -80 °C until assayed.

The following measurements (LTE_4, PGE_2, PGD_2 and 6-keto-$PGF_{1\alpha}$) were performed according to the instructions provided for each specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI. USA). The determinations of 8-iso-prostaglandin $F_{2\alpha}$ type III in the tissues was performed using GC-MS according to the methods reported by Bessard and co-workers [3].

All data are expressed as means ± S.E.M. The results of the mediator measurements were expressed as released substance in pg mg^{-1} of tissue wet weight or in the case of 8-iso-prostaglandin $F_{2\alpha}$ type III as pg ml^{-1}. Statistical evaluation was performed using a Student's *t*-test. A P-value of less than 0.5 was considered significant.

Results

Intact human isolated pulmonary arteries and veins challenged with arachidonic acid (10 μM; 15 min) exhibited a significant difference in the quantities of 6-keto-$PGF_{1\alpha}$ released (period II). These values were: arteries, 148 ± 21 pg mg^{-1} tissue and veins, 48 ± 9 pg mg^{-1} tissue and were similar to that observed at the end of period I. In preparations where the endothelium had been removed there was a marked reduction in the quantities of 6-keto-

$PGF_{1\alpha}$ released in both types of preparations. However, there was no significant difference between the amounts released in rubbed arteries (9 ± 5 pg mg $^{-1}$ tissue) versus rubbed veins (6 ± 2 pg mg^{-1} tissue). The other metabolites derived from human pulmonary arteries and veins exposed to the low concentration of arachidonic acid (10 μM) were either not modified or not detected (arteries: PGE_2, 4 ± 0.4 pg mg^{-1} tissue; LTE_4, 3 ± 0.7 pg mg^{-1} tissue). While the amounts of LTE_4 detected in arteries and veins were similar, removal of the endothelium caused a significant reduction in the quantities of LTE_4 released from the veins (intact: 6 ± 3 pg mg^{-1} tissue and rubbed: 0.3 ± 0.06 pg mg^{-1} tissues) but not from the arteries. The results obtained using GC-MS enabled the detection of very low levels of 8-iso-prostaglandin $F_{2\alpha}$ type III in the medium, which were not quantifiable (below 20 pg ml^{-1}). The values detected were obtained from intact arterial (1015 ± 20 mg tissue wet weight, n=3) and intact venous (740 ± 45 mg tissue wet weight, n=3). In arteries and veins devoid of an endothelium (one lung sample) 8-iso-prostaglandin $F_{2\alpha}$ type III was also below the threshold levels of detection (data not shown).

LTC_4 and LTD_4 failed to modified the release of 6-keto-$PGF_{1\alpha}$ in human pulmonary arterial and venous preparations which had been exposed to arachidonic acid (10 μM). A high concentration of LTC_4 (1 μM) also failed to modify prostaglandin E_2 release in intact preparations (arteries; 6 ± 3 pg mg^{-1} tissue wet weight, n=3 and veins; 15 ± 11 pg mg^{-1} tissue wet weight, n=3). Challenge of preparations with LTD_4 (1 μM) did not significantly alter the PGE_2 release in intact arteries (5 ± 2 pg mg^{-1} tissue wet weight; n=3) or veins (18 ± 11 pg mg^{-1} tissue wet weight; n=3). These results were not significantly different from the data obtained in intact preparations exposed to arachidonic acid (10 μM). Furthermore, the cysteinyl-leukotrienes did not modify the release of either 6-keto-$PGF_{1\alpha}$ or PGE_2 in rubbed preparations (data not shown).

Discussion

The data presented in this report demonstrate that human pulmonary arteries and veins release 6-keto-$PGF_{1\alpha}$ subsequent to stimulation with arachidonic acid (10 μM). The quantities were significantly greater in arteries than in veins and were not altered when the tissues were exposed to a second challenge. In contrast, rubbed preparations exhibited a marked reduction in the quantities of 6-keto-$PGF_{1\alpha}$ released and there was no difference between arteries and veins. These data suggest that the quantities detected were derived principally from the endothelium in both types of preparations. The data also show that 6-keto-$PGF_{1\alpha}$ was the major metabolite detected since the amounts measured for PGE_2 and LTE_4 were significantly lower whereas PGD_2 and 8-iso-prostaglandin $F_{2\alpha}$ type III were well below the threshhold levels of detection.

Previous studies [1, 4] had shown that endothelial cells in culture are refractory to a second challenge with arachidonic acid. The data (this report) demonstrate that human pulmonary vascular muscle preparations do not exhibit this refractoriness. Whether these differences are due to the concentrations of arachidonic acid [5], the techniques employed [4] or the vascular bed [6] from which the cells are derived remains to be explored.

While activation of a CysLT receptor [7, 8] has been reported to be associated with the release of 6-keto-$PGF_{1\alpha}$, the data (present report) show that subsequent to arachidonic acid challenge the cysLTs failed to induce release of 6-keto-$PGF_{1\alpha}$. These results are in agreement with the early reports [2]. Together these results suggest that arachidonic acid interfers with cell surface receptor activation associated with the cyclooxygenase pathway. One explanation for this observation is that arachidonic acid may cause a conformational change in the receptor since previous reports have shown that fatty acids are known to modulate receptor binding [9]. Another explanation for this interference may be that arachidonic acid plays a role as a second messenger by activating protein kinase C [10] a pathway also known to be activated by the cysLTs [11]. However, O'Flaherty and co-workers [12] recently reported that low concentrations of arachidonic acid directly regulate protein kinase C translation within cells and suggested that this function of arachidonic acid may be pivotal in the mechanism of action rather than direct activation of protein kinase C enzymes. Whether or not arachidonic acid directly activates protein kinase C or simply signals the movement of the enzymes (translocation from cytosol to membrane) remains to be elucidated.

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Chapter #16

CYTOSOLIC PHOSPHOLIPASE $A_2\alpha$ IN MOUSE PLATELETS
Clinical And Therapeutic Implications

Dennis A. Wong, Naonori Uozumi, Yoshihiro Kita and Takao Shimizu

Introduction

The role of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) in the release of arachidonic acid (AA) from lipid membranes has been intensely studied [1]. Its ubiquitous expression and precise regulation initially suggested a role in both the regulation of membrane phospholipids and its release, triggered by extracellular signaling, to be converted into potent intracellular and extracellular signaling molecules. The survival of $cPLA_2\alpha$ knockout mice, however, suggested that this enzyme has a somewhat redundant role in the upkeep of lipid membranes narrowing its role to its function of providing sharp spikes of intracellular AA to enzymes (cyclooxygenases (COX), lipoxygenases (LOX)), which then produce potent autocrine and/or paracrine lipid mediators upon activation [2]. The multitude of lipid mediators that can be derived from the release of AA makes the study of the precise role of $cPLA_2\alpha$ *in vivo* difficult to dissect. The results, however, of recent studies in knockout mice platelets point to a non-redundant role for $cPLA_2\alpha$ in the generation of thromboxane A_2 (TXA_2) at levels needed to perform a paracrine function, vasoconstriction [3]. These findings provide a basis to understand a number of previous studies in various disease models. Further the separation autocrine and paracrine pathways of TXA_2 generation provide a possible explanation for the therapeutic utility of low dose aspirin in the prevention of acute arterial vascular events and insight into the potential of inhibiting this enzyme as therapy for a number of vascular and inflammatory diseases.

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Platelet Aggregation

The role of $cPLA_2\alpha$ in platelets is tied primarily but not exclusively to the vital role of TXA_2 in platelet function. In humans and mice deficient in TXA_2 activity leads to a bleeding diathesis [4,5]. The absence of the enzyme responsible for conversion of AA to TXA_2, COX 1, or the lack of the TXA_2 receptor leads to delayed aggregation and increased bleeding times consistent with the effect of aspirin which irreversibility blocks COX activity. In contrast, *in vitro* and *in vivo* studies in platelets deficient in $cPLA_2\alpha$ show a minor defect in aggregation. Using $cPLA_2\alpha$ knockout mice along with mice lacking both, $cPLA_2\alpha$ and group II secretory phospholipase A_2 ($sPLA_2$ grp II), it was found that platelets have alternate phospholipase A_2(s) that can generate low levels of TXA_2 but not the two log-fold higher levels needed to produce the vasoconstriction typically associated with collagen activation. Aggregation of platelets through stimulation of the G-protein coupled receptors, ADP and TXA_2, directly was independent of the presence of either $cPLA_2\alpha$ or $sPLA_2$ group II. The production of TXA_2 was in fact equal or higher in both sets of deficient mice. Collagen stimulation of platelets showed a two log-fold decrease in TXA_2 production but there continued to be some TXA_2 production. Therefore collagen stimulation and G-protein activation can lead to the low level of AA release in platelets without $cPLA_2\alpha$ or $sPLA_2$ group II while only collagen stimulation appears to activate $cPLA_2\alpha$ in wild-type mice. These results point to the presence of other phospholipases associated with G-protein receptors in platelets which can maintain the autocrine role of TXA_2 in platelets because the inhibition of TXA_2 directly leads to a much more profound inhibition of aggregation. The loss of $cPLA_2\alpha$ leads mainly to inability of platelets to secrete the large amounts TXA_2 needed for the paracrine function of vasoconstriction. The possible clinical implication of this is that partial or complete inhibition of $cPLA_2\alpha$ would likely cause less disruption of platelet aggregation than aspirin which completely blocks COX activity and therefore TXA_2 production in platelets.

Vascular effects

One of the key roles of platelets in the vascular system is to prevent bleeding. Platelets are a vital part of the clotting cascade. To stop bleeding in the arterial system vasoconstriction or stopping the flow of blood is need in addition to clot formation. The TXA_2 secreted by platelets performs this function. This was observed in the differences in the evaluation of bleeding times between the $cPLA_2\alpha$ deficient mice and wild-type mice. When bleeding times were measured in the tails of the mice, wild type mice

typically would abruptly stop bleeding within 30-90 seconds, the result of vasoconstriction. Without $cPLA_2\alpha$ male mice with much large tail vessels just continued to bleed. This vasoconstrictive non-redundant role of $cPLA_2\alpha$ is further supported in results in collagen-induced thromboembolism. $cPLA_2\alpha$ deficient mice were more resistant to effects of pulmonary thromboembolism. A comparison of the lungs removed from the mice showed the presence of thrombi in both sets of mice but the difference was that in the wildtype mice the thrombi were consistently in the arteries verses the presence of larger thrombi in veins in the deficient mice. The induction of vasoconstriction leads to platelet aggregation in the arterial vessels, which is more lethal than venous clots. Another group has found a similar result in a hypoxic pulmonary model using mice deficient in both $cPLA_2\alpha$ and $sPLA_2$ group II. The hypoxic pulmonary vascular response was inhibited in deficient mice and restored by augmenting the vascular response [6]. These findings help explain several clinical observations and hint at the cardio-protective potential of a $cPLA_2\alpha$ inhibitor. Aspirin is particularly effective in inhibiting acute arterial vasoconstrictive events such as myocardial infarction and cerebrovascular attacks but appears to have little effect on venous thrombosis. Our studies suggest that the loss of TXA_2 induced vasoconstriction is behind this effect. Aspirin is protective in small intermittent doses while larger doses are not more effective and cause more bleeding. Aspirin irreversibly inhibits platelet COX for the life of a platelet, 7 days. Low intermittent dosing may allow small amounts of TXA_2 to be produced by newly generated platelets but not allow the production of the large amounts needed for arterial vasoconstriction. The implication is that a specific $cPLA_2\alpha$ inhibitor would be a more precise way to block this vasoconstriction. Also the lack of COX-1 inhibition would imply that current COX-2 drug would lack the cardiovascular protective effects of aspirin and other NSAIDS [7].

Inflammation

Platelets are also a key ingredient in the inflammatory process and studies on inflammatory models must now be viewed in light of these findings in platelets. A major component of the protective effect of $cPLA_2\alpha$ deficiency in anaphylaxis is likely do to the lack of TXA_2 induced vasoconstriction since TXA_2 receptor knockout mice are also resistant to anaphylaxis [2,5]. Macrophage-platelet and neutrophil-platelet complexes are known to be present and may play a important role in inflammatory diseases [8]. The release of AA in platelets appears to be important to the generation of leukotrienes in adjacent cells through intercellular sharing [9]. Adherent platelets on macrophage/monocyte and neutrophils would likely be unable to

provide AA for sharing in cPLA$_2\alpha$ knockout mice. The protective effect of cPLA$_2\alpha$ in lung allergen challenge [2], ARDS models [10] and pulmonary fibrosis models [11] of disease could be impacted by the lost of AA production from platelets. Inhibition of cPLA$_2\alpha$ even in one cell type may have potent anti-inflammatory effects.

Signaling

Activation of platelets through a number of different receptors leads to aggregation. The activation of cPLA$_2\alpha$ appears to be independent of G-proteins receptors for ADP and TXA$_2$. Both ADP and thromboxane analogue showed identical ATP secretion, aggregation curves and TXA$_2$ production patterns in knockout and wild-type mice. In contrast collagen stimulation leads to a small but significant delay in degranulation and a marked decrease in TXA$_2$ production. Further, studies on integrin $\alpha_2\beta_1$ deficient mice, one of the collagen receptors, imply that cPLA$_2\alpha$ is related to the other known collagen receptor protein IV [12,13]. Bleeding times were normal in the absence of the integrin suggestion the presence of cPLA$_2\alpha$ related vasoconstriction. Different PLA$_2$(s) therefore appear to be linked to specific signaling pathways. One can conclude that molecules upstream and downstream of cPLA$_2\alpha$ can be targeted to inhibit the cPLA$_2\alpha$ related function of platelets and perhaps in other cells types.

Future research

The search for a specific inhibitor for the various PLA$_2$(s) has started and has the potential to lead to a "better aspirin". The availability of specific inhibitors, back crossing various knockout mice and the generation of chimera mice (hemopoietic cell line cPLA$_2\alpha$ deficient in a wild type host) will allow one to dissect the exact role of cPLA$_2\alpha$ in platelets and other cells.

Conclusion

The role of cPLA$_2\alpha$ in platelets appears to be restricted to the production of large amounts of AA during marked activation with other PLA$_2$(s) available to produce smaller amounts sufficient for autocrine use. During *in vivo* platelet activation this AA production is needed to produce the large amounts of TXA$_2$ required to induce local vasoconstriction. The demonstration of independent pathways of AA release linked to specific PLA$_2$s in platelets suggests that in platelets and perhaps other cells specific pathways/PLA$_2$s can be targeted with preservation of phospholipid membrane and other autocrine functions of AA manipulation maintained by

other pathways. The specific targeting of $cPLA_2\alpha$ inhibition would likely have a small affect on platelet aggregation but a potent effect on platelet induced vasoconstriction as well as anti-inflammatory effects.

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Chapter #17

CELLULAR ARACHIDONATE-RELEASING FUNCTIONS OF VARIOUS PHOSPHOLIPASE A_2s

Makoto Murakami And Ichiro Kudo

Phospholipase A_2 (PLA_2), which hydrolyzes the *sn*-2 position of glycerophospholipid to liberate free fatty acid and lysophospholipid, occurs in multiple forms in mammals. To date, four major classes of PLA_2 enzymes have been identified, including secretory PLA_2s ($sPLA_2$s), cytosolic PLA_2s ($cPLA_2$s), Ca^{2+}-independent PLA_2s ($iPLA_2$s) and platelet-activating factor acetylhydrolases (PAF-AHs). In this paper, we summarize our current understanding of the regulation of cellular arachidonate (AA) metabolism by $sPLA_2$, $cPLA_2$ and $iPLA_2$ enzymes. More details are described in our recent reviews [1,2].

$sPLA_2$s

To date, 10 $sPLA_2$s (IB, IIA, IIC, IID, IIE, IIF, V, X, III, and XII) have been identified in mammals. $sPLA_2$s belonging to the group I/II/V/X collection are closely related, 14-19 kDa secreted enzymes with a highly conserved Ca^{2+}-binding loop and a catalytic site. In addition, there are 6 absolutely conserved disulfide bonds and up to 2 additional unique disulfide bonds, which contributes to the high degree of stability of these enzymes. The genes for $sPLA_2$-IIA, -IIC, -IID, -IIE, -IIF and -V are clustered on the same chromosome locus and often referred to as the group II subfamily $sPLA_2$s. $sPLA_2$-III and $sPLA_2$-XII share homology with the I/II/V/X collection of $sPLA_2$s only in the Ca^{2+}-binding loop and catalytic site, thereby representing distinct group III and XII collections. $sPLA_2$s exert their enzymatic activity in the presence of mM concentrations of Ca^{2+} with no strict fatty acid selectivity. In general, group II subfamily $sPLA_2$s act on anionic phospholipids in marked preference to charge-neutral phosphatidylcholine (PC), $sPLA_2$-V and -X hydrolyze both anionic phospholipids and PC efficiently, and $sPLA_2$-IB is intermediate. Some of the group II subfamily of $sPLA_2$s are highly cationic and bind tightly to anionic heparanoids such as heparin and heparan sulfate. The phospholipid head

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group specificity and heparan sulfate proteoglycan (HSPG)-binding property affect the cellular functions of each $sPLA_2$ [3-8].

$sPLA_2$-IB, a pancreatic PLA_2, has a unique 5 amino acid extension termed the pancreatic loop in the middle part of the molecule and a group I-specific disulfide Cys^{11}-Cys^{77}. An N-terminal heptapeptide of the inactive zymogen is cleaved by trypsin or other proteases to yield an active enzyme. The digestion of dietary phospholipids appears to be a primary function of pancreatic $sPLA_2$-IB. This enzyme is a high affinity ligand for the M-type $sPLA_2$ receptor, which in turn transduces signals leading to activation of $cPLA_2\alpha$ or promotes the internalization and lysosomal degradation of $sPLA_2$.

$sPLA_2$-IIA, a synovial PLA_2, has a group II-specific disulfide linking Cys^{50} with Cys at the C-terminus and a C-terminal extension with 7 amino acid length. The expression of $sPLA_2$-IIA is up-regulated by inflammatory stimuli, and its levels in sera or exudating fluids are well correlated with the severity of inflammatory diseases. Although $sPLA_2$-IIA can act poorly on the surface of quiescent cells because of its weak binding capacity to the PC-rich external leaflet of the plasma membrane, it can be sorted into caveolin-rich vesicular and perinuclear compartments through association with glypican, a glycosylphosohatidylinositol-anchored form of HSPG, in activated cells [5,7]. After proper sorting, $sPLA_2$-IIA releases AA from membrane microdomains, in which cell activation-directed membrane rearrangements may occur. This route, called *the HSPG-shuttling pathway*, allows this enzyme to accumulate into perinuclear membrane compartments, where the downstream AA-metabolizing enzymes, such as cyclooxygenases (COXs) and 5-lipoxygenase (5-LO), exist.

$sPLA_2$-IIC has an additional disulfide Cys^{87}-Cys^{93} in an extended loop region and is expressed in rodent testis, whereas it is a pseudogene in the human. $sPLA_2$-IID is constitutively expressed in the immune and digestive organs and is upregulated by proinflammatory stimuli in some restricted tissues. It augments stimulus-induced cellular AA release through the HSPG-shuttling pathway, as does $sPLA_2$-IIA. [7]. $sPLA_2$-IIE is expressed in several tissues at low levels and is also upregulated by proinflammatory stimuli. Although the affinity of $sPLA_2$-IIE for HSPG is weaker than that of $sPLA_2$-IIA and -IID, it is still capable of augmenting stimulus-induced cellular AA release through the HSPG-shuttling mechanism. $sPLA_2$-IIF has a unique 30 amino acid C-terminal extension that contains an additional Cys residue. Its expression is limited to the testis of adult mice, whereas it is detected in various human tissues at low levels. High level of its expression is found in mouse embryo, implying that its expression is developmentally regulated. Although $sPLA_2$-IIF does not bind HSPG, it has the ability to increase cellular AA release probably through acting on the perturbed microdomain on the plasma membrane [8]. The unique C-terminal extension, which plays a role in duration of the interaction between the enzyme and the plasma membrane, is essential for cellular AA releasing function of $sPLA_2$-IIF.

$sPLA_2$-V does not have the group I- and group II-specific disulfides and the group II-specific C-terminal extension, although it shows a higher level of identity with group II $sPLA_2$s. Its expression is also markedly induced by proinflammatory stimuli. $sPLA_2$-V shows high affinity for both HSPG and PC, and releases cellular AA in two distinct mechanisms [3,7]. As in the case of $sPLA_2$-IIA, high HSPG affinity allows $sPLA_2$-V to utilize the HSPG-shuttling pathway. $sPLA_2$-V is also capable acting on the plasma membrane surface independently of HSPG. The latter action, called *the external plasma membrane pathway*, depends primarily on its ability to bind PC in the outer plasma membrane. Since this pathway does not require membrane rearrangements, $sPLA_2$-V as well as $sPLA_2$-X (see below) can release AA from various types of unstimulated cells far more efficiently than other group II subfamily $sPLA_2$s.

$sPLA_2$-X has both the group I- and II-specific disulfides, the group II-specific C-terminal extension, and the group I-specific propeptide. Like $sPLA_2$-IB, $sPLA_2$-X is synthesized as a zymogen, and removal of the N-terminal propeptide produces an active mature enzyme. The mature enzyme interacts with the $sPLA_2$ receptor with high affinity. $sPLA_2$-X is expressed in the immune and digestive organs and testis. Due to its high activity toward PC and no affinity for HSPG, $sPLA_2$-X releases cellular AA predominantly through the external plasma membrane pathway [6]. The AA released by $sPLA_2$-V or -X from the external cell surface can diffuse across the cytosol, reaching the perinuclear COX and 5-LO enzymes for conversion to eicosanoids.

$sPLA_2$-III is an unusually large protein (55 kDa) amongst the $sPLA_2$ family and consists of three domains, in which a central $sPLA_2$ domain that displays all of the features of group III bee venom $sPLA_2$s, including 10 cysteines and the key residues of the Ca^{2+} loop and catalytic site, is flanked by large and unique N- and C-terminal regions. It is expressed in the kidney, heart, liver and skeletal muscle. The $sPLA_2$ domain alone is sufficient for eliciting AA release from the plasma membrane, whereas either the N- or C-terminal domain mediates the HSPG dependent cellular action of this enzyme.

$sPLA_2$-XII is a 19 kDa enzyme containing a central catalytic domain with a His/Asp catalytic dyad, yet the location of cysteines outside the catalytic domain is distinct from that of other $sPLA_2$s. Strong expression of $sPLA_2$-XII is found in the heart, skeletal muscle, kidney and pancreas and weaker expression in various tissues in the human. Cellular function of this enzyme remains unresolved.

$cPLA_2$s

$cPLA_2$s consist of 3 enzymes, $cPLA_2\alpha$, $cPLA_2\beta$ and $cPLA_2\gamma$, (group IVA, IVB and IVC, respectively). The lipase consensus sequence, GXSGS, as well as other residues required for catalysis is conserved in these enzymes.

$cPLA_2\alpha$ and $cPLA_2\beta$ have an N-terminal C2 domain, which is critical for Ca^{2+}-dependent binding to PC-rich phospholipid membranes. $cPLA_2\alpha$ is ubiquitously and constitutively expressed in most cells and tissues. $cPLA_2\alpha$ shows a remarkable selectivity toward phospholipids bearing AA at the *sn*-2 position, whereas $cPLA_2\beta$ and $cPLA_2\gamma$ do not show fatty acid selectivity.

The C2 domain-promoted, Ca^{2+}-dependent translocation of $cPLA_2\alpha$ from the cytosol to perinuclear membranes is an essential step for the initiation of stimulus-coupled AA release [9]. Duration of $[Ca^{2+}]_i$ stabilizes the association of $cPLA_2\alpha$ with the perinuclear membrane. This spatiotemporal localization permits efficient functional coupling between $cPLA_2\alpha$ and downstream COXs and 5-LO. The maximal activation of $cPLA_2\alpha$ requires sustained phosphorylation of Ser^{505} and Ser^{727} by mitogen-activated protein kianses (MAPKs) and by MAPK-activated protein kinases, respectively [10]. Membrane hydrolysis by $cPLA_2\alpha$ is facilitated by PIP_2, where several cationic residues in the C2 and catalytic domains are crucial for binding to PIP_2. During apoptosis, $cPLA_2\alpha$ is cleaved by caspase-3 at Asp^{522} and is inactivated [11].

Macrophages and mast cells obtained from $cPLA_2\alpha$ knockout mice produce minimal PGs, LTs and PAF in response to stimuli. The airway anaphylactic response, adult respiratory distress syndrome, and ischemic brain injury are markedly reduced in $cPLA_2\alpha$ knockout mice compared with those in wild-type mice. Female $cPLA_2\alpha$ knockout mice have defects in reproduction, particularly in implantation and parturition. Aggregation of platelets derived from $cPLA_2\alpha$-knockout mice is partially impaired. $cPLA_2\alpha$ knockout in Apc^{min} mice markedly decreases the size of the intestinal polyps, implying its role in the COX-2-dependent colon cancer development. Intestinal epithelium in $cPLA_2\alpha$-knockout mice has numerous small ulcerative lesions, indicating its role in the production of cytoprotective PGE_2 in the gastrointestinal tracts.

Although $cPLA_2\gamma$ lacks the C2 domain, it contains an isoprenylation site at the C-terminus and is membrane-bound. This enzyme is capable of augmenting spontaneous and stimulus-induced AA release. The C-terminal isoprenylation appears to be essential for stable association of this enzyme with the perinuclear membrane and attendant cellular AA-releasing function.

$iPLA_2$s

The classical $iPLA_2$, $iPLA_2$-VIA ($iPLA_2\beta$), occurs in several splice variants. $iPLA_2$-VIA-1 is an 85 kDa protein that contains 8 ankyrin repeats in the N-terminal region, followed by a catalytic domain containing a consensus lipase motif GXSXG, where Ser^{465} acts as a catalytic center. $iPLA_2$-VIA-2, an 88 kDa isoform, has a primary structure essentially identical to VIA-1, except that the eighth ankyrin repeat is interrupted by an additional 54 amino acids. The ankyrin repeats may allow this enzyme to oligomerize. Other splice variants lack the C-terminal catalytic domain and may act as dominant-negative inhibitors for $iPLA_2$-VIA-1 and IVA-2.

$iPLA_2$-VIB ($iPLA_2\gamma$) contains a consensus lipase motif, a glycine-rich nucleotide-binding motif, and unique a C-terminal peroxisome localization signal. Sequences conserved between $iPLA_2$-VIA and -VIB are clustered in the C-terminal half, whereas the N-terminal region of $iPLA_2$-VIB lacks the ankyrin repeats. Both $iPLA_2$-VIA and VIB are ubiquitously expressed in various tissues. They are fully active in the absence of Ca^{2+}, and at least $iPLA_2$-VIA shows no strict *sn*-2 fatty acid and head group specificity of the substrate phospholipids.

$iPLA_2$-VIA plays a pivotal role in phospholipid remodeling through constitutive deacylation of phospholipids [3,4]. Beyond the housekeeping role of $iPLA_2$-VIA; *i.e.* maintenance of phospholipid homeostasis, several lines of evience suggest that this enzyme is also involved in stimulus-coupled AA release [3,4]. Even though $iPLA_2$-VIA does not require Ca^{2+} for activity *in vitro*, it may be regulated by Ca^{2+} or Ca^{2+}-dependent factors in cells. In apoptotic cells, $iPLA_2$-VIA is cleaved by caspase-3-like proteases at Asp^{183}, which results in enzyme activation and attendant increase in fatty acid release [11]. This event may be responsible for membranous changes during apoptosis. Although the function of $iPLA_2$-VIB is poorly understood, our preliminary study has revealed that it can promote spontaneous fatty acid release, suggesting its contribution to membrane remodeling, as does $iPLA_2$-VIA.

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Chapter #18

BIOLOGICAL FUNCTIONS OF GROUP X SECRETORY PLA_2

Kohji Hanasaki and Hitoshi Arita

Secretory phospholipase A_2s ($sPLA_2$s) are a diverse family of low molecular mass enzymes that hydrolyze the *sn*-2 fatty acid ester bond of glycerophospholipids to produce free fatty acids and lysophospholipids. In addition to the classical types of group IB and IIA ($sPLA_2$-IB and IIA), recent advances in molecular biology have led to the identification of novel types of $sPLA_2$s, including group IID and IIE that we have cloned [1]. Among the nine types of human $sPLA_2$s, group X $sPLA_2$ ($sPLA_2$-X) has a structural similarity with the classical type of $sPLA_2$-IB and IIA [2]. We have shown that $sPLA_2$-X has a potent hydrolyzing activity toward phosphatidylcholine (PC) and elicits more potent production of unsaturated fatty acids and lysophosphatidylcholine (lysoPC) in various inflammatory cells and colon cancer cells compared with $sPLA_2$-IB and IIA [3,4]. The released arachidonic acid by $sPLA_2$-X was efficiently converted to prostaglandins (PGs) by the action of endogenous cyclooxygenase (COX). The expression of $sPLA_2$-X was detected in human lung epithelial cells and mouse splenic macrophages, and greatly augmented in human colon adenocarcinoma tissues in concert with enhanced expression of COX-2 [4]. These findings suggest that $sPLA_2$-X plays a critical role in the lipid mediator productions under various inflammatory states and in the COX-2-dependent PGE_2 biosynthesis during colon tumorigenesis. Recently, we have shown that $sPLA_2$-X is a high-affinity ligand for mouse PLA_2 receptor (PLA_2R) and identified a soluble PLA_2R in mouse plasma [5]. Using PLA_2 receptor-deficient mice, we have shown that the membrane and soluble form of the receptor are involved in the endogenous inhibitory mechanisms against the strong PC-hydrolizing activity of $sPLA_2$-X in mice [5].

Recent studies have shown that $sPLA_2$-IIA is expressed in the atherosclerotic arterial intima and associated with extracellular matrix structures and lipid droplets. In addition, $sPLA_2$-IIA was shown to induce the lipolysis of LDL leading to enhanced retention of LDL to human aortic

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proteoglycans [6], suggesting a potential role of $sPLA_2$-IIA in the accumulation of LDL in the proteoglycan matrix on the subendothelial layer of the arterial intima. However, $sPLA_2$-IIA can induce only weak modification of LDL, as this enzyme preferably hydrolyzes anionic phospholipids and has very low enzymatic activity toward PC, a major phospholipid component of LDL. We have shown that $sPLA_2$-X is one of the enzymes with a potent hydrolyzing activity toward PC. During the analysis of $sPLA_2$-X functions, we unexpectedly found that $sPLA_2$-X elicits potent release of fatty acids from culture medium containing fetal calf serum in cell-free systems. These observations prompted us to examine its potential role in the lipolysis of human plasma lipoproteins.

We have found that $sPLA_2$-X can elicit marked release of various types of unsaturated fatty acids from human low density lipoprotein (LDL) in the order of linoleic acid>arachidonic acid>oleic acid ≈ docosahexaenoic acid [7]. $sPLA_2$-X induced significant release at 5 nM, while $sPLA_2$-IB evoked slight but significant release at 500 nM and there was little, if any, release with $sPLA_2$-IIA treatment even at 500 nM. As shown in Figure 1, the PC contents in human LDL time-dependently decreased after treatment with $sPLA_2$-X and oxidation (1 mg/ml LDL plus 50 nM $sPLA_2$s or 20 μM $CuSO_4$).

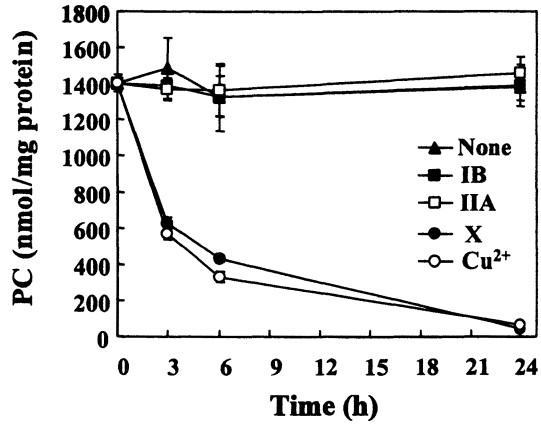

Figure 1. Effects of human $sPLA_2$s on PC contents in LDL.

Over half of the PC was diminished in LDL by $sPLA_2$-X within 3 h, and PC was completely degraded after 24-h treatment. Corresponding to the reduction of PC contents, the amounts of lysoPC in LDL increased up to 24 h after $sPLA_2$-X treatment. Incubation with $sPLA_2$–specific inhibitor Indoxam or anti-$sPLA_2$-X Ab resulted in significant suppression of $sPLA_2$-X-induced lipolysis of LDL. In contrast, treatment with $sPLA_2$-IB or IIA caused little change in either PC or lysoPC contents in LDL. Treatment with three types of $sPLA_2$ did not alter the oxidative parameters. These findings

demonstrate that $sPLA_2$-X can induce potent PC hydrolysis in LDL leading to the production of large amounts of lysoPC and unsaturated fatty acids without any oxidative modification.

Treatment with $sPLA_2$-X caused an increase in the negative charge of LDL with little modification of apoB in contrast to the excessive aggregation and fragmentation of apoB in oxidized LDL. The $sPLA_2$-X-modified LDL was efficiently incorporated into macrophages to induce the accumulation of cellular cholesterol ester [7]. The cellular lipid droplets were then stained with oil red O and analyzed by light microscopy. In contrast to little staining in the macrophages treated with native LDL, the formation of numerous intracellular lipid droplets was observed in macrophages incubated with $sPLA_2$-X-modified LDL, and the lipid droplets were obviously larger than those observed in macrophages treated with oxidized LDL. No lipid droplets could be detected after incubation with $sPLA_2$-IB- or $sPLA_2$-IIA-treated LDL. When Indoxam was added during the modification of LDL with $sPLA_2$-X followed by incubation with macrophages, the formation of lipid droplets was significantly prevented. In contrast, the COX inhibitor Indomethacin and lipoxygenase (LOX) inhibitor did not affect lipid droplet formation in the macrophages treated with $sPLA_2$-X-modified LDL, suggesting that $sPLA_2$-X-induced formation of intracellular lipid droplets is completely dependent on the modification of LDL and is not related to the action of eicosanoid metabolites.

In the atherosclerotic lesions *in vivo*, three types of intracellular lipid deposits have been reported in macrophage foam cells: non-membrane-bound lipid droplets, lysosomal lipid bodies and cholesterol crystals. Electron microscopic analysis has confirmed the presence of numerous non-membrane-bound lipid droplets and cholesterol crystals in the cytoplasm of macrophages incubated with acetylated LDL, whereas numerous multilamellar structures were found in the cytoplasm of the cells treated with oxidized LDL. The formation of non-membrane-bound lipid droplets was also detected in the macrophages incubated with $sPLA_2$-X-treated LDL, suggesting a degradation pathway similar to that of acetylated LDL [7]. Large lipid droplets having more than 0.4 μm profile diameters have been observed in the fatty streak regions in human arteries. In addition, non-membrane-bound lipid droplets have been demonstrated to be the main form of lipid accumulation at the early stage of the lesions. In this context, $sPLA_2$-X might be one of the endogenous molecules involved in the initiation of atherogenesis, as acetylated LDL is an artificial product. Immunohistochemical analysis revealed marked expression of $sPLA_2$-X in foam cell lesions in the arterial intima of high-fat-fed apolipoprotein E-deficient mice [7]. These findings suggest that modification of LDL by $sPLA_2$-X in the arterial vessels is one of the mechanisms responsible for the generation of atherogenic lipoprotein particles as well as the production of various lipid mediators including unsaturated fatty acids and lysoPC. As $sPLA_2$ inhibitors, but not COX or LOX inhibitors, suppressed the $sPLA_2$-X-

induced lipolysis of LDL, the availability of sPLA$_2$ inhibitors as antiatherogenic drugs should be evaluated in future studies.

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Chapter #19

PROTECTION OF CELL MEMBRANE FROM EXOGENOUS PLA_2 AND RELATED INFLAMMATORY STIMULI BY MEMBRANE-ANCHORED LIPID CONJUGATES

Saul Yedgar, Miron Krimsky and Ouri Schwob

Phospholipase A_2 (PLA_2) is a super-family of enzymes that hydrolyze cell membrane phospholipids (PL) to produce lysophospholipids (LysoPL) and free fatty acids [for review of the PLA_2 types see Ref. 1]. The PLA_2 family consists of two main kinds of enzymes: the secreted ($sPLA_2$) and the intracellular ones, which include the cytosolic (cPLA2) and the Ca^{++}-independent ($iPLA_2$) enzymes. While $cPLA_2$ is specific to arachidonic acid (AA)-carrying PL, the others do not exhibit preference for a fatty acyl chain in the PL [2].

The activity of the secretory PLA_2s is prominent in pathological processes, as the enzymes responsible for this activity are secreted by inflammatory cells upon their activation and by damaged tissues, in pancreatitis and other inflammation-related diseases.

By hydrolyzing cell membrane PL to produce LysoPL and free fatty acids, particularly AA, PLA_2 initiates the production of numerous metabolites that mediate diverse pathological states, particularly those related to inflammatory processes.

AA is metabolized via the cyclooxygenase (COX) and lipoxygenase (LOX) pathways to produce large families of eicosanoids; many of them are involved in the development of numerous pathological conditions, especially in inflammation-related processes [3].

LysoPL induce white cell activation and extravasation [4,5], activate mast cells (by lyso-phosphatidylserine in particular) [6], induce tissue damage, such as gastric ulceration [7], and act as growth factors (especially lyso-phosphatidic acid), to induce proliferation of cancer cells [8] and tumor metastasis [9]. Furthermore, LysoPL are also the precursors of platelet

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activating factor (PAF), possibly the most potent mediator of inflammatory processes [10]. In the above processes the secretory PLA_2s play a major role, since in activated states the majority of PL hydrolysis is carried out by $sPLA_2$s. Furthermore, the activation of cellular $sPLA_2$ is also involved in the action of inflammatory agents, such as endotoxins [11] and interferon [12,13].

In addition to producing lipid mediators, PLA_2 secreted into the extracellular fluids act synergistically with other inflammatory mediators, such as reactive oxygen species, in the tissue damaging that occurs in inflammatory states [14], in which the actual membrane lysis is executed by $sPLA_2$. Altogether, $sPLA_2$ activity is involved directly and indirectly in the pathophysiology of inflammatory conditions, and its control is thus a desired therapeutic target [for review see ref. 15].

Important players in controlling the action of exogenous PLA_2 on cell membrane are the cell surface glycosaminoglycans (GAG). A number of studies have shown that the action of $sPLA_2$ on the cell membrane is inhibited by cell surface GAG, such as heparin [16], chondroitin sulfate [17] and hyaluronic acid [18]. In accord with this, in a study of the synergistic action of hydrogen peroxide (HPO) and $sPLA_2$ on cultured epithelial cells [19], we have observed that exogenous $sPLA_2$ applied to cultured epithelial cells does not hydrolyze the cell membrane PL, unless the cell is treated with HPO prior to application of $sPLA_2$. It was found that HPO degrades the cell surface GAG and thus renders the cell membrane accessible to lysis by the exogenous $sPLA_2$. Cell surface GAG also play a key role in protecting cells from bacterial and viral infections, and their stripping exposes the cell to interaction and subsequent internalization of the bacteria [20]. Although the relationship of these processes to production of lipid mediators is not unequivocally clear, it has been proposed that enrichment of cell surface GAG would assist in protection of the cell from bacterial infection, and inflammatory processes in general.

This hypothesis was tested by using membrane-anchored lipid conjugates designed and synthesized in our laboratory. These compounds (previously denoted ExPLIs as they were originally designed to be extracellular PLA_2 inhibitors) consist of PLA_2-inhibiting molecules, such as N-derivatized phosphatidyl-ethanolamine (PE), linked to polymeric carriers, including natural macromolecules, such as hyaluronic acid, heparin, and chondroitin sulfates, or polymers used in drug administration and clinical treatment (e.g., carboxy-methylcellulose, dextrans, hydroxy-ethylstarch or polygeline). Due to their structure, the lipid conjugates fulfil a multiple function in cell protection; the lipid moiety, which incorporates into the cell membrane, suppresses the activation of endogenous $sPLA_2$ [21-23] and the polymeric carrier mimics the cell surface GAG in protecting membrane lipids from inflammatory stimuli [19,21,24].

These multifunctional anti-inflammatory drugs (MFAID) have been examined in a number of in-vitro and in-vivo systems, and have been found effective in amelioration of diverse inflammatory processes: The MFAIDs

inhibit the hydrolysis of lipid membranes by diverse types of exogenous $sPLA_2$s [25], as well as the activation of PLA_2 in cultured macrophages stimulated by LPS [22], and protect epithelial cells from PLA2 activation and lysis induced by the synergistic action of ROS, hemolysins and proteases [21].

Concordant with reports that PLA_2 activity is involved in cellular response to interferon [12,13], we have found that the MFAIDs can block the IFN-induced expression of MHC class I and II, and of mixed lymphocyte reaction (MLR)-induced T cell proliferation and secretion of IL-2 and IFNγ [26].

Several studies have demonstrated that cellular stimulation by endotoxin (LPS and LTA) involves the activation of cellular $sPLA_2$, and possibly also $cPLA_2$ [11,22]. Accordingly, we have found, in cultured human lung microvascular endothelial cells, that the MFAIDs are effective in blocking the endotoxin-induced production of chemokines and adhesion molecules, as well as the activation of NFκ-B [27]. As this transcription factor is involved in the activation of $cPLA_2$, its inhibition by the MFAIDs, which are cell-impermeable PLA_2 inhibitors, may suggest that activation of $sPLA_2$ is required for the LPS-induced activation of $cPLA_2$. In a subsequent in-vivo study, we found the MFAIDs efficient in amelioration of endotoxin-induced sepsis in rats [28]. This was expressed by reduced mortality rate, suppression of blood level of cytokines (TNFα, IL-6), and expression of $sPLA_2$, iNOS, IL-1, IL-6, TNFα, and ICAM-1 in the liver and kidney of the septic rats.

In another recent study we have found that the MFAIDs protected HeLa cells from infection by *chlamydia*, and reduced the level of inflammatory mediators in *chlamydia-infected* mice (Darville et al., unpublished]. Last, the MFAID were found effective in suppression of capillary formation (angiogenesis) in fibrin gel by human endothelial cells stimulated by different growth factors [29]. It should be emphasized that in all these studies the membrane-anchored lipid conjugates were impressively effective, while their corresponding polymers alone (without the lipid moiety) were ineffective.

The experimental results and considerations summarized above demonstrate the therapeutic advantage of protecting cells from inflammatory stimuli by using multifunctional inhibitors like the membrane-anchored lipid conjugates. The combined action of the MFAIDs, being cell-impermeable PLA_2 inhibitors, and at the same time enriching the cell surface GAG, seems to provide a wide range protection from diverse inflammatory stimuli and related injurious processes.

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Chapter #20

REGULATORY FUNCTIONS OF PROSTAGLANDIN E_2 SYNTHASES

Ichiro Kudo and Makoto Murakami

Introduction

Prostaglandin (PG) E_2, the most common prostanoid with a variety of bioactivities, has been implicated in various pathophysiologies. PGE_2 is produced via three sequential enzymatic reactions: release of arachidonic acid (AA) from membrane glycerophospholipids by phospholipase A_2 (PLA_2), conversion of AA to PGH_2 by cyclooxygenase (COX), and isomerization of PGH_2 to PGE_2 by PGE synthase (PGES). During last few years, multiple PGES enzymes have been identified molecularly. In this report, we will overview the structures, enzymatic properties, expression, cellular functions, and possible *in vivo* functions of two of these enzymes, membrane-bound PGES (mPGES)-1 and cytosolic PGES (cPGES).

mPGES-1

mPGES-1 shows significant homology with other proteins belonging to the MAPEG (for membrane-associated proteins involved in eicosanoid and glutathione (GSH) metabolism) superfamily, including microsomal GSH-*S*-transferase (MGST)-1, -2 and -3, 5-lipoxygenase-activating protein (FLAP) and leukotriene C_4 synthase (LTCS), with the highest homology being found with MGST-1 (~40%) [1-3]. All MAPEG proteins have similar molecular masses of 14-18 kDa and appear to have similar three dimensional and membrane-spanning topographic features. Arg^{110} in mPGES-1, which is the residue strictly conserved in all MAPEG proteins, is essential for the catalytic function of mPGES-1 [2]. mPGES-1 requires GSH as an essential cofactor for activity. mPGES-1 activity is inhibited by NS-398, a COX-2 inhibitor, by MK-886, a FLAP inhibitor, and by LTC_4 [3]. The region of

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FLAP that is essential for binding to MK-886 (and thereby binding to AA) is highly conserved in LTCS and mPGES-1.

The expression of mPGES-1 is up-regulated in response to various stimuli [1-3]. Coordinate induction of COX-2 and mPGES-1 is accompanied by production of PGE_2, which is reversed by glucocorticoids [2,3]. A recent study using mPGES-1 knockout mice have provided unequivocal evidence that LPS-stimulated production of PGE_2 by macrophages largely depends on mPGES-1 [4]. The gene for human *mPGES-1* maps to chromosome 9q34.3, spanning over 15 kb and being divided into three exons [5]. The promoter region is TATA-less and contains several potential transcription factor-binding sites, including two GC-boxes, C/EBP, AP-1 and glucocorticoid-responsive elements (GRE) [5]. Binding of the transcription factor Egr-1 to the proximal GC box is critical for stimulus-evoked induction of mPGES-1, whereas the GRE may exert a negative regulatory effect [5]. Cotransfection of mPGES-1 with either of the COX isozymes demonstrates that mPGES-1 is functionally coupled with COX-2 in marked preference to COX-1 [2]. COX-2 and mPGES-1 are colocalized in the perinuclear membrane [2]. Conceivably, colocalization of these two enzymes in the same subcellular compartment may allow efficient transfer of the unstable substrate PGH_2 between them.

Induction of mPGES-1 by proinflammatory stimuli argues that this enzyme is an essential component for COX-2-dependent PGE_2 production during inflammation. Indeed, mPGES-1 expression is increased in many tissues of LPS-treated rats [2,3], hind paws of rats with adjuvant arthritis [3] and synoviocytes from patients with rheumatoid arthritis [6]. mPGES-1 may also be involved in pain hypersensitivity, in which COX-2 and PGE_2 play a critical role. LPS increases the expression of COX-2 and mPGES-1 in brain vascular endothelial cells [7], PGE_2 produced by which can pass across the blood-brain barrier into the central nervous system, thereby evoking the febrile response.

COX-2-derived PGE_2 has also been implicated in other diseases, such as tumorigenesis and osteoporosis. The involvement of mPGES-1 in tumorigenesis has been suggested by the observation that transfection of mPGES in combination with COX-2, but not with COX-1, into cells leads to cellular transformation [2]. Furthermore, the expression of COX-2 and mPGES-1 is markedly elevated in several human cancers [8]. mPGES-1 is highly inducible in osteoblasts [2] and osteoclast formation and resultant bone resorption are markedly impaired in mPGES-1 knockout mice (unpublished results). Thus, mPGES-1 may participate in bone disease such as peritodontitis and osteomyelitis, which are associated with bacterially induced bone loss. Induction of mPGES-1 in β-amyloid-treated astrocytes suggests its role in Alzheimer's disease [9].

Physiologically, mPGES-1 is induced in granulosa cell layer of follicles after treatment with gonadotropins [10] providing support for the role of mPGES-1 in ovulation, a process during which COX-2-dependent PGE_2 synthesis is obligatory. mPGES-1 is also highly expressed in male genital

organs [11] although its role in reproduction remains elusive. mPGES-1 is constitutively expressed in the kidney, particularly in epithelia of distal tubules and medullary collecting ducts and in transitional epithelial cells of bladder and ureter [12] suggesting a role of mPGES-1 in physiological supply of PGE_2 in the urogenital tract.

cPGES

cPGES is a 23 kDa cytosolic protein that is identical to the Hsp90-associated protein p23 [13]. cPGES requires GSH as an essential cofactor for its activity. Although the homology between cPGES and other known cytosolic GSTs is low (~20%), near its N-terminus cPGES has a tyrosine residue (Tyr^9) that is conserved in several other cytosolic GSTs. Mutation of this tyrosine abrogates the activity of cPGES [13] suggesting that this residue serves as a GSH acceptor, as it does in other cytosolic GSTs. cPGES is expressed ubiquitously and in abundance in a variety of tissues and cells. In most cases, its expression is constitutive and is not affected by proinflammatory stimuli. cPGES is capable of converting COX-1-, but not COX-2-, derived PGH_2 to PGE_2 in cells during the immediate PGE_2-biosynthetic response elicited by Ca^{2+}-evoked stimuli [13]. Thus, in line with the roles of COX-1 *in vivo*, cPGES may contribute physiologically to the production of PGE_2 required for the maintenance of tissue homeostasis.

Activation of cPGES is spatiotemporally controlled by several post-transcriptional and -translational events (unpublished results). After cell activation, cPGES appears to translocate from the cytosol to the endoplasmic reticulum, where COX-1 resides. Stimulus-induced temporal association of cPGES with Hsp90 is crucial for cPGES activation in cells. Glucocorticoids facilitate dissociation of the Hsp90-cPGES complex, thereby attenuating cPGES-directed PGE_2 production. Furthermore, cPGES also binds to and phosphorylated by casein kinase II, a Hsp90-associating kinase, which leads to increased cPGES activity.

Conclusion

Although COX-2 inhibitors have reduced gastrointestinal toxicity as compared with traditional COX-nonselective NSAIDs, there are also some adverse effects associated with this new group of drugs probably due to inhibition of vascular PGI_2 production. More selective modulation of the PGE_2 pathway would be desirable in certain circumstances. From this standpoint, PGESs represent attractive novel targets for prophylactic and therapeutic intervention for patients with various inflammatory diseases, osteoporosis, and cancer.

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Chapter #21

A NOVEL TYPE OF MEMBRANE-ASSOCIATED PROSTAGLANDIN E SYNTHASE

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Introduction

Prostaglandin (PG) E_2 is widely distributed in various organs, and exhibits various biologically important activities such as smooth muscle dilatation/contraction, body temperature regulation, induction of pain, stimulation of bone resorption, and inhibition of immune responses. PGE synthase catalyzes the conversion of PGH_2 to PGE_2. About 25 years ago, Ogino *et al* [1] reported that glutathione (GSH) was required for the PGE synthase activity, and laid the groundwork for the study of membrane-associated PGE synthase (mPGE synthase). Tanaka *et al* [2] characterized PGE synthase in sheep vesicular gland microsomes by use of a monoclonal antibody. In 1999, using a clone of microsomal GSH *S*-transferase 1-like 1, Jakobsson *et al* [3] expressed human GSH-specific mPGE synthase (mPGE synthase-1) in *E. coli*. The mPGE synthase-1 had high GSH-dependent PGE synthase activity, and the protein expression was induced by IL-1β. Moreover, Ogorochi *et al* [4] and Meyer *et al* [5] independently purified the PGE synthase from the cytosol fraction of human brain and *Ascaridia galli*, respectively, and reported that the enzyme was GSH *S*-transferase (GST). Recently Tanioka *et al* [6] isolated the PGE synthase from the cytosol fraction of rat brain, and reported that the enzyme belonged to GST family.

In 1997, we examined the distribution of the mPGE synthase in various rat organs, and reported two types of mPGE synthase, GSH-specific and -nonspecific types [7]. We purified the GSH-nonspecific mPGE synthase (mPGE synthase-2) to apparent homogeneity from bovine heart [8], and we recently clarified that the enzyme belonged to thioredoxin family [9].

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Results and Discussion

We found mPGE synthase-2, GSH-nonspecific type, in rat heart, which was different from mPGE synthase-1, GSH-specific type. We tried to solubilize and to purify the mPGE synthase-2 from bovine heart micorosomes [8]. The mPGE synthase-2 activity of bovine heart microsomes as the starting materials was about 1 μmol, and bovine heart microsomes were a rich source for its purification. The enzyme was solubilized by sodium deoxycholate, and was purified using DEAE-Toyopearl, first Superdex 200, Mono Q, and second Superdex 200 column chromatographies. Through 5 steps, the enzyme was purified about 800-fold with an overall yield of 6%. The specific activity of the purified PGE synthase was about 830 nmol/min.mg of protein. The PGE synthase activity was eluted as the main protein from the second Superdex 200 column chromatography, and was purified to apparent homogeneity on SDS-PAGE. The molecular weight of the purified enzyme was about 31-kDa on SDS-PAGE, and was about 60-kDa on Superdex 200 column chromatography. The molecular weight is assumed to be 60-kDa consisting of a homo-dimer of 30-kDa subunits. However, it is possible that the value by gel filtration is overestimated due to the enzyme-detergent complex.

We determined the N-terminal 22-amino acid sequence of the purified enzyme. The sequence was identical to that of the 88^{th}-109^{th} amino acids deduced from the monkey (AB046026) or human (AK024100) cDNA, that encodes a hypothetical protein with unknown function. The amino acid sequences derived from the human and monkey cDNAs showed 97% identity with each other. The sequence had 87 amino-acid residues N-terminal to the sequence of PGE synthase purified from the microsomal fraction of bovine heart. The amino acid sequences derived from human and monkey cDNAs did not belong to any GST family, but the amino acid sequence from ^{104}Leu to ^{120}Leu was the consensus region of glutaredoxin and of thioredoxin.

We constructed the expression plasmid using polyhistidine-tagged peptide fused vector (pTrc-HisA) and the monkey cDNA coding a 290-amino acid polypeptide, and expressed the proteins in *E.coli.* Moreover, we purified the expressed protein to homogeneity. The approximately 33-kDa protein was recognized by anti-recombinant mPGE synthase-2 antibody. The expressed protein had PGE synthase activity in the presence of SH reagent, GSH, dithiothreitol (DTT), or 2-mercaptoethanol (β-Mer), as same as the enzyme purified from bovine heart. The enzyme activity was most stimulated, about 5-fold, by DTT, compared with that in the absence of SH reagent (Figure 1). These results suggest that the enzyme shows broad specificity for thiol requirement.

Molecular weight of this protein was about 33-kDa. The specific activity was about 3 μmol/min.mg of protein, and the Km value was 28 μM, indicating that kcat/Km value was about 65 x 10^3 $M^{-1}s^{-1}$. This enzyme did not show GST activity.

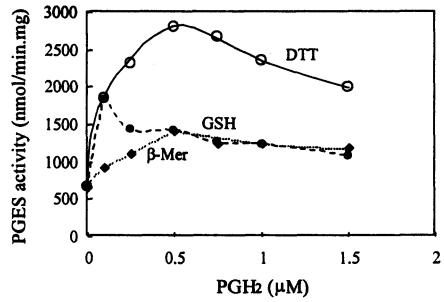

Figure 1. Effects of SH reagents on the PGE synthase-2 activity.

^{110}Cys – x ~ x – ^{113}Cys in the consensus region were the active site of glutaredoxin and thioredoxin, and were conserved among these proteins, including human mPGE synthase-2 (Figure 2). Although the PGE synthase activity of the mutant at C113S was almost the same as that of the wild type, the enzyme activities of the mutant at C110S and of the double mutant at C110, 113S were lost. These results indicate that ^{110}Cys was important for the catalytic activity.

	104	110 113	125
hPGES-2	LYQYKT	**C**PF**C** SKVRA F	LDFH AL
hGrx1*	VF I KPT	**C**PY**C** RRAQE I	LS QL P I
hGrx2*	I FS KT S	**C**SY**C** TMAKKLF	HDMNV
rTrx**	DFSATW	**C**KP**C** KMI KP F	FHAL S E

Figure 2. Comparison of amino acid sequences among human mPGE synthase-2, glutaredoxine, and thioredoxine.
*J. Biol. Chem. 276, 30374-30380, 2001 **J. Biol. Chem. 263, 9589-9597, 1988

Using the human cDNA as a probe, Northern blot analysis revealed that the probe was hybridized to a 2-kilobase mRNA in skeletal muscle, heart, kidney, and brain. In the brain, the probe hybridized to a 2-kilobase mRNA present in the cerebral cortex, occipital pole, frontal lobe, temporal lobe, putamen, and cerebellum (Figure 3). The result that the mPGE synthase-2 was detected in heart, but not in vesicular gland, coincided with that for the distribution of PGE synthase activity in the absence of GSH in rats [7] and sheep [8]. The mPGE synthase-2 is a novel type of mPGE synthase based on molecular properties as well as on enzymatic properties.

Moreover, the gene of mPGE synthase-2 was localized to chromosome 9q33-q34, and those genes of cyclooxygenase-1, mPGE synthase-1, and lipocalin-type PGD synthase were located close together on chromosome 9, at 9q32-q33.3, 9q34.3, and 9q34.2-q34.3, respectively. These results indicate

that the region of q32-34 in chromosome 9 may be related to the metabolism of PGs.

Recently, Hu *et al* [10] reported that the expression of the protein having the same amino acid sequence as mPGE synthse-2, was induced by interferon-γ treatment.

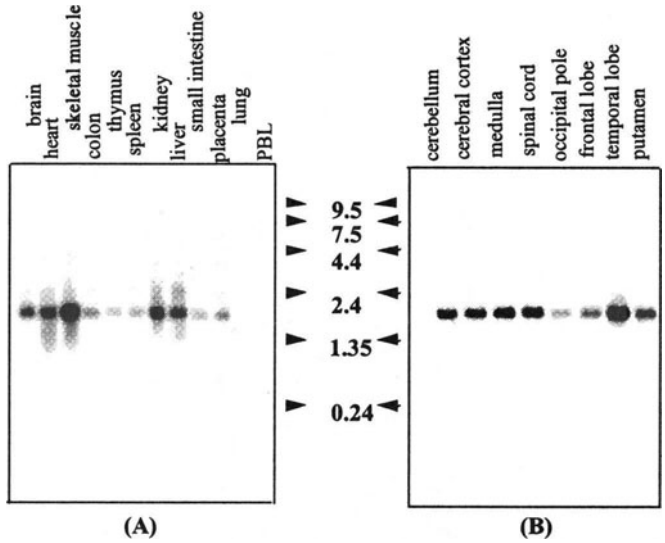

Figure 3. Northern blot analyses of various human tissues (A) and of various regions of the human brain (B).

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Chapter #22

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN MICROSOMAL PGE_2 SYNTHASE-1

Marc Ouellet, Ally Pen, Po-Hien Ear, Jean-Pierre Falgueyret, Tammy G. LeRiche, Joseph A. Mancini, Denis Riendeau, and M. David Percival.

Introduction

Prostaglandin E synthases (PGES) comprise a number of structurally different enzymes that convert the product of the cyclooxygenase enzymes, PGH_2, into the physiologically important PGE_2. An inducible microsomal PGES (mPGES-1) couples preferentially with Cox-2 [1], whereas a constitutively and widely expressed cytosolic PGES (cPGES or p23) is believed to couple with Cox-1 [2]. Both of these enzyme activities are dependent on GSH. Most recently, a second, membrane-associated PGES (mPGES-2) that has a wide tissue distribution and is activated by a number of thiol-containing reagents was identified and purified [3]. Two cytosolic glutathione transferases (μ2 and μ3) also have some PGES activity. As the expression of mPGES-1 is strongly induced by pro-inflammatory stimuli and is down-regulated by glucocorticoids, mPGES-1 is a potential drug target for anti-inflammatory therapy. A solubilization and partial purification of a microsomal PGES activity from bovine vesicular glands has been reported [4]. It appears that this enzyme is the same as mPGES-1. Here we report the baculovirus over-expression of recombinant human mPGES-1 in Sf9 insect cells, its purification to apparent homogeneity and the characterization of its enzymatic properties.

Results and Discussion

The cDNA fragment encoding the full length mPGES-1 (GenBank: AF027740) was subcloned into the pFastBac plasmid and the bacmid preparation and isolation were performed in bacteria. Sf9 insect cells were

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transfected with bacmid DNA containing the mPGES-1 cDNA insert and viral stocks were prepared and amplified. Immunoblot analysis of Sf9 membrane fractions revealed mPGES-1 expression by 48 h with optimal expression at 96 to 120 h [5]. Diheptanoylphosphatidylcholine (DHPC) was used to solubilize mPGES-1 from Sf9 membranes. Solubilized mPGES-1 was applied to a hydroxyapatite column (in the presence of 1 % octyl glucoside) and eluted with a linear potassium phosphate gradient (10 to 600 mM). Active fractions were pooled, concentrated (Centriplus 50, Amicon) and applied to a Superdex 200 HR gel filtration column (GFC) and eluted with 20 mM Tris-HCl pH 7.0, 1 mM GSH, 0.1 mM EDTA, 200 mM NaCl and 1 % octyl glucoside. Figure 1 shows a silver stained SDS-PAGE gel of the various steps in mPGES-1 purification. LC-MS analysis of GFC purified mPGES-1 revealed no apparent post-translational modification (Figure 2). The two main peaks (MW 16980 and 17110 Da) correspond to the intact predicted mass without and with the initial methionine respectively.

Purified mPGES-1 was desalted by GFC in buffer without GSH and the enzyme activity was measured. In the absence of added GSH no mPGES-1 activity was detected. No activity was also detected in the presence of 2.5 mM dithiothreitol, N-acetylcysteine, cysteine, lipoic acid, β-mercaptoethanol or S-nitrosoglutathione. Enzyme activity was only observed in the presence of GSH [4,5]. GSH was also shown to increase the stability of mPGES-1 [4,5]. A preparation of mPGES-1 was tested for glutathione-S-transferase (GST) activity using a sensitive radio HPLC assay employing [^{3}H]-GSH. No detectable GST activity was found with mPGES-1 with 1-chloro or 1-fluoro-2,4-dinitrobenzene and various other substrates while activity was observed with CDNB and PGD_2 synthase and microsomal GST-1 [5].

The specific activity of mPGES-1 improved only 7-fold with the purification (1 vs 7 μmol/min/mg; microsomal mPGES-1 vs GFC purified mPGES-1). Figure 1 shows that the protein enrichment as determined by gel electrophoresis was more than 7-fold. The closest homolog of mPGES-1 is microsomal GST-1 and its activity is known to be stimulated by the presence of detergents such as Triton X-100 [6]. The activity of mPGES-1 was activated 3-7 fold by the addition of detergents (DHPC, octyl glucoside, dodecylmaltoside, Triton X-100 or captoprylsulfobetaine) at concentrations above their critical micelle concentrations [5]. The kinetic parameters of purified mPGES-1 were determined in the presence of 0.3 mM dodecylmaltoside. In the absence of detergent the K_m values for PGH_2 and GSH were 14 μM and 0.75 mM respectively while in the presence of detergent they were 10 μM and 0.6 mM. The V_{max} value was increased from 5.5 to approximately 40 μmol/min/mg with 0.3 mM dodecylmaltoside [5]. This indicates that the detergent activation of mPGES-1 results in an increase in V_{max}, but no change in the K_m of PGH_2 and GSH. Based on the highest specific activity of mPGES-1, the purification scheme described here yielded at least a 40 fold purification.

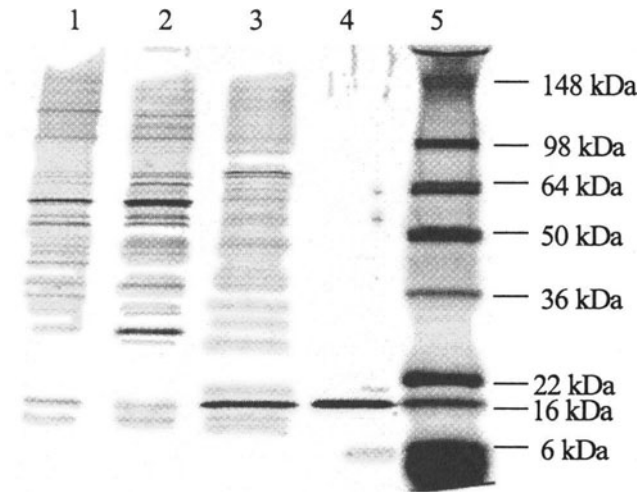

Figure 1. SDS-PAGE analysis of the various fractions generated during mPGES-1 purification. 500 ng of total protein was loaded per lane. Lane 1:microsomal fraction, lane 2: microsomal fraction solubilized with 1% DHPC, lane 3: pooled fractions from hydroxyapatite column, lane 4: pooled fractions after GFC, lane 5: MW markers.

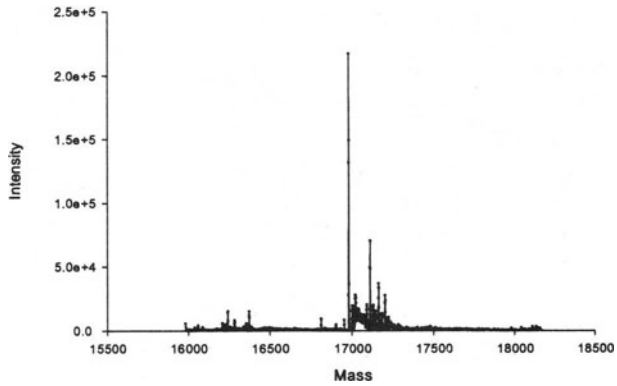

Figure 2. Deconvoluted LC-MS spectrum of purified recombinant mPGES-1. A Q-TOF Ultima instrument was used to collect the spectrum.

The present study shows that mPGES-1 is activated by detergents and that GSH is essential for mPGES-1. The methods for the expression and

purification described here and previously [5] should provide sufficient material for mechanistic and biophysical characterization of mPGES-1.

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Chapter #23

FORMATION OF PROSTAGLANDIN E_2, LEUKOTRIENE B_4 AND 8-ISOPROSTANE IN ALVEOLAR MACROPHAGES BY ULTRAFINE PARTICLES OF ELEMENTAL CARBON

Ingrid Beck-Speier, Niru Dayal, Erwin Karg, Konrad L. Maier, Holger Schulz, Gabriele Schumann, Axel Ziesenis, and Joachim Heyder

Introduction

Acute exposure to inhaled ambient particles is found to be associated with adverse health effects worldwide. Because of their very small diameter (< 0.1 μm) and their large specific surface area, ultrafine particles (UFP) represent a special fraction of the ambient aerosol. They are considered as a major factor contributing to adverse health effects including pulmonary and cardiovascular diseases [1].

Animal studies using instillation of UFP into the lungs of rats indicate, that the toxicity of the particles is associated with their large surface area and with the induction of inflammatory reactions in the lungs [2]. However, the molecular mechanisms underlying these in-vivo responses are still unknown.

We have shown recently that agglomerates of ultrafine particles of elemental carbon and of TiO_2 activate in alveolar macrophages the release of arachidonic acid, and the synthesis of various products of the cyclooxygenase (COX)-dependent pathway such as prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2) and 12-hydroxyheptadecatrienoic acid, and of the 5-lipoxygenase (5-LO)-dependent pathway such as leukotriene B_4 (LTB_4) and 5-hydroxy-eicosatetraenoic acid [3]. The comparison of particle-induced biologic effects with their physical properties, for example the specific particle surface area and particle mass concentration, revealed that the synthesis of PGE_2/TXB_2 is related to their particle surface area rather than to their mass concentration.

Using a cell-free in-vitro system we have obtained evidence that the large surface area of UFP show an oxidative potential [4]. This study was designed to evaluate the significance of the oxidative potential of UFP to

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induce oxidative stress in cellular systems and to release lipid mediators. Agglomerates of ultrafine particles of elemental carbon (AUFP-EC) were used as model for UFP. Alveolar macrophages (AMs), being part of the primary pulmonary defence system, were chosen as target cells.

Materials and Methods

Ultrafine particles of elemental carbon (AUFP-EC) were generated by spark discharge [5]. The oxidative potential of these particles was determined in an in-vitro system consisting of AUFP-EC and methionine (500 μM) in phosphate buffer, pH 7, measuring oxidation of methionine to the sulfoxide by HPLC.

Canine AMs were obtained by bronchoalveolar lavage of healthy beagles [3]. After labelling with ^{14}C-arachidonic acid, AMs (1 x 10^6 cells/ml) were incubated with AUFP-EC for 1 h. The ^{14}C-labelled metabolites were extracted and separated by thinlayer chromatography [3]. Intracellular concentration of 8-isoprostane was determined by an 8-isoprostane-specific enzyme immunoassay after deproteinization of particle-treated AMs by 80 % methanol containing 0.5 mM EDTA and 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl.

Results and Discussion

The oxidative potential of AUFP-EC was determined by their ability a) to oxidize methionine to methionine sulfoxide in a cell-free in-vitro system, and b) to induce oxidative stress in a cellular system with AMs by formation of 8-isoprostane indicating lipid peroxidation. As shown in Figure 1A, AUFP-EC with increasing mass concentrations or corresponding specific surface areas caused oxidation of methionine to methionine sulfoxide. Figure 1B illustrates the formation of 8-isoprostane by the particles in AMs. Concomitant with an increase in oxidation of methionine AUFP-EC enhanced the production of 8-isoprostane in AMs. These data give evidence for the oxidative potential of AUFP-EC.

To evaluate the influence of the oxidative potential of AUFP-EC on formation of lipid mediators in AMs, the generation of PGE_2/TXB_2 and LTB_4 was determined using particles with high and low oxidative potential. As shown in Table 1, freshly suspended AUFP-EC exhibited a high oxidative potential as determined by formation of methionine sulfoxide. At the same mass concentration, the high innate oxidative potential of AUFP-EC was reduced drastically by about 80 % during a 24 h incubation in PBS. In addition Table 1 shows that AUFP-EC with high oxidative potential induced generation of 8-isoprostane, PGE_2/TXB_2 and LTB_4 in AMs, whereas particles with low oxidative potential did not increase formation of 8-isoprostane and LTB_4, but still enhanced production of PGE_2/TXB_2.

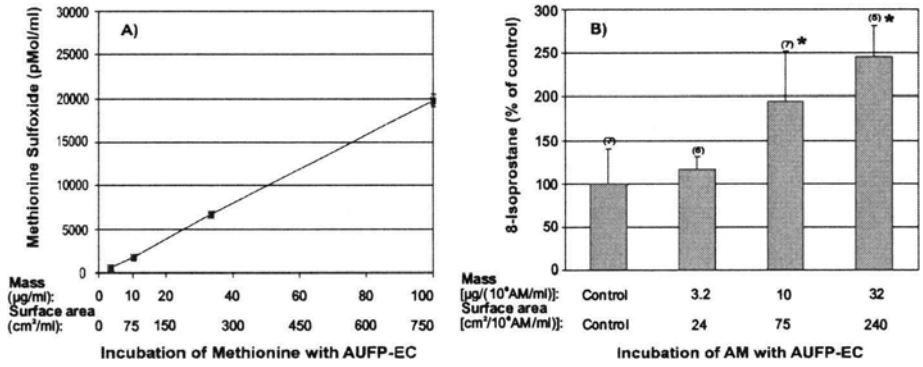

Figure 1. Effect of oxidative potential of AUFP-EC in (A) cell-free in-vitro system with oxidation of methionine to methionine sulfoxide, and in (B) cellular system with formation of 8-isoprostane in AUFP-EC-treated AMs.

Our results indicate that the oxidative potential of AUFP-EC is crucial for the biologic effects induced by the particles. AUFP-EC with varying oxidative potential activate different pathways in AMs. Particles with high oxidative potential stimulate the 5-LO pathway with formation of pro-inflammatory LTB_4, the COX pathway with synthesis of anti-inflammatory PGE_2, and in addition the nonenzymatic formation of 8-isoprostane as marker for oxidative stress. Interestingly, particles with low oxidative potential still induce the COX-pathway with production of anti-inflammatory PGE_2 but not the 5-LO pathway nor formation of lipid peroxides. We conclude that the oxidative potential of particles is able to induce inflammatory reactions and oxidative stress.

Table 1. Effect of oxidative potential of AUFP-EC on oxidation of methionine, and alveolar macrophage-derived synthesis of 8-isoprostane, PGE_2/TXB_2 and LTB_4. AUFP-EC was used freshly (0 h) and incubated for 24 h in PBS (24 h). AUFP-EC (100 µg/ml) were tested for formation of methionine sulfoxide (n=3). AUFP-EC (32 µg/ml) were added to AMs and analyzed for arachidonic acid metabolites. Control values of 8-isoprostane, normalized to 100 %, correspond to 27 ± 12 pg 8-isoprostan/ml (n=4). Control values for arachidonic acid metabolites, normalized to 100 %, represent percentages of radioactivity of single metabolites in relation to total cell radioactivity, and are 0.61 ± 0.21 for PGE_2/TXB_2 and 0.41 ± 0.18 for LTB_4 (n=6). Asterisks indicate significant difference between metabolite production in control cells and particle-affected cells ($p < 0.05$).

Parameter	AUFP-EC 0 h in PBS	AUFP-EC 24 h in PBS
Methionine sulfoxide [pMol/ml]	14144 ± 397	3247 ± 350
8-Isoprostane [% of control]	329 ± 79*	183 ± 70
PGE_2/TXB_2 [%of control]	240 ± 21*	209 ± 40*
LTB_4 [%of control]	183 ± 24*	112 ± 11

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Chapter #24

EFFECTS OF $PGF_{2\alpha}$ ANALOGUES IN EXPERIMENTAL MORPHINE –INDUCED PHARMACODEPENDENCE

Mihai Nechifor, Dan Chelãrescu, Elena Teslariu, Florian Cocu, and Adriana Negru

Background

Prostaglandines and other eicosanoids are involved in a multitude of normal and pathological processes at CNS level.

Synthesis of PGE_2, PGD_2 $PGF_{2\alpha}$ and PGI_2, TxA_2 and LTC_4, LTB_4, 12-HETE and other eicosanoids in the brain was well demonstrated [1].PGD_2 and $PGF_{2\alpha}$ are synthesized in the greatest quantity into the mammalian brain In some pathological conditions, eicosanoids synthesis and concentration into the brain are modified.

One of the important and frequent pathological manifestations is pharmacodependence. This is a chronic, relapsing illness, characterized by compulsive drug seeking and use.

Aims

We tested the influence of 2 $PGF_{2\alpha}$ synthetic analogues: optically active cloprostenol (ClPGOA) and $PGF_{2\alpha}$ isopropyl ester (IPEF) (Figure 1) on experimental morphine–induced pharmacodependence in rat.

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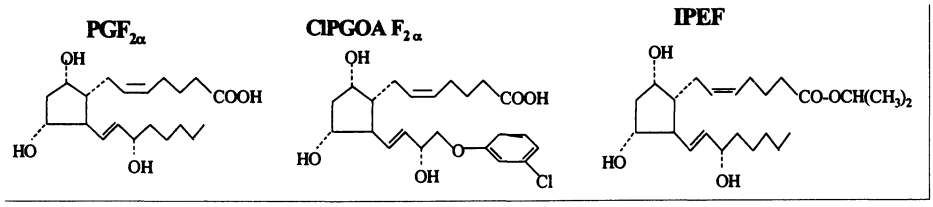

Figure 1. Chemical structures of $PGF_{2\alpha}$, ClPGOA and IPEF

Material and Method

We worked on 4 groups of 20 Wistar, adult, male rats each. **Group I** got morphine (M) in daily administration (15 days) increasing progressive the doses (from 7mg/kg/day i.p to 45mg/kg/day i.p). **Group II** got M the same as group I + ClPGOA 25 μg/kg i.p. with 1 h before morphine . **Group III** got M the same as group I + IPEF 25 μg/kg i.p. with 1 h before morphine. **Group IV** received saline daily i.p. (Control group).

On the 16^{th} day, administration of morphine and $PGF_{2\alpha}$ analogues was ceased and all animals received naloxone (2 mg/kg i.p.), 2 hours after the last morphine administration. Immediately after naloxone administration, animals were placed individually in test chambers consisting of round boxes (30 cm diameter x 35 cm height) and withdrawal signs were evaluated during a 25 min period. Intensity of with-drawal was tested by measuring several classical signs of morphine abstinence. The number of bouts of teeth chattering, grooming, aggressive positions and jumping were counted. Diarrhea was evaluated over 5 min periods with one point being given for the presence of each sign during each period. The number of periods showing the sign were then counted (max. score: 5). Locomotor activity (explorations- open field test) was also evaluated over 5 min periods giving a value between 0 and 2 for each period. The values were added for the whole 25 min period (max. score 10). Weight loss was also quantified. Results were statistically interpreted with ANOVA one-way test.

Results

The results shown that ClPGOA and IPEF administrated during morphine-induced pharmacodependence influenced moderately but significantly statistic some of the symptoms of withdrawal syndrome. Aggressive postures decreases with 19% in IPEF group vs morphine only group ($p<0.05$). Grooming was decreased with 24% ($p<0.05$) in ClPGOA group vs. control group (Fig 2, 3). Explorations decreased with 34% in ClPGOA group vs. control group ($p<0.01$). There were not influenced significantly weight loss, diarrhea, jumpings.

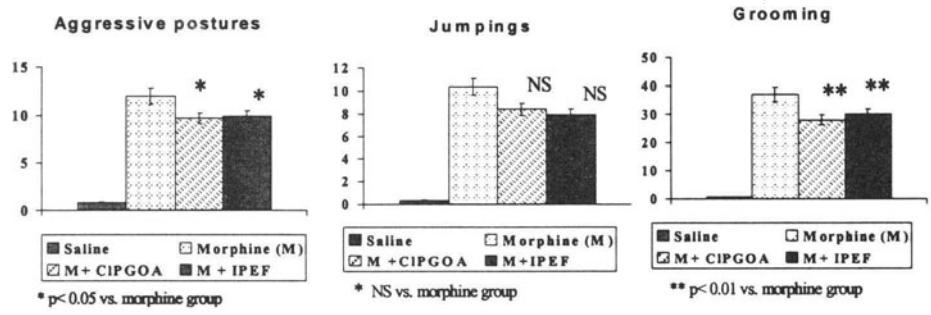

Figure 2. ClPGOA and IPEF influence on aggressive posture, jumping and grooming (number of bouts evaluated over 25 minutes).

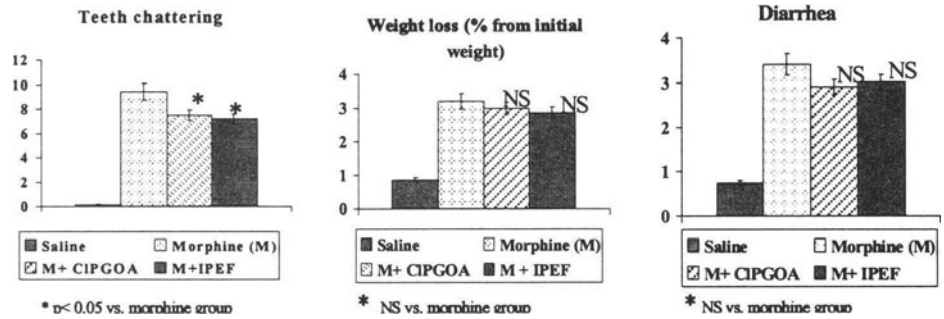

Figure 3. ClPGOA and IPEF influence on teeth chattering (number of bouts evaluated over 25 minutes), weight loss (% vs. initial) and diarrhea (evaluated over 5 min periods with one point being given for the presence of sign during each period).

Discussion

ClPGOA and IPEF are stabile analogues of $PGF_{2\alpha}$ without vaso-constrictive but with luteolytic actions. Our results are in accordance with Nielsen and Sparber [2] that observed $PGF_{2\alpha}$ (acting on dopaminergic neurons) in i.c.v. administration in rat alleviates apomorphine-induced dependence. Nakagawa et al, 1995 [3] have shown that some prostaglandin analogues acting on EP_3 receptors attenuated withdrawal jumping in morphine dependent mice but consider that i.c.v. $PGF_{2\alpha}$ administration had no effect in this situation. According to this we didn't observe a positive influence on jumpings but we observed that these 2 analogues of $PGF_{2\alpha}$ tested [ClPGOA and IPEF] significantly modify other withdrawal signs in morphine-induced dependence. In our previous studies about cloprostenol (raceme) [4] we observed also a decrease of some symptoms in rats with morphine withdrawal syndrome A possible involvement of eicosanoids into

the the pharmacodependence mechanism is suggested by some factors: a) Discovery of anandamides in 1991 (N-arachidonyl ethanolamine) and 2-arachidonyl-glycerol, substances considered as elements of "endogenous cannabinoid regulatory system"[5]; b) Involvement of prostaglandines and other eicosanoids in presynaptic release of neuromediators (dopamine and serotonine), considered as milestones in molecular mechanism of dependence; c) The eicosanoids influence on behavior (both prostaglandines, leukotriens and other).

Conclusions

Some $PGF_{2\alpha}$ synthetic analogues modify several symptoms from withdrawal syndrome in rats with morphine-induced pharmacodependence.

Not all symptoms were equally influenced. We consider that cerebral $PGF_{2\alpha}$ is involved in morphine-induced pharmacodependence.

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Chapter #25

INHIBITION OF PROSTAGLANDIN H_2 SYNTHASES BY SALICYLATE IS DEPENDENT ON THE OXIDATIVE STATE OF THE ENZYMES

David M. Aronoff, Olivier Boutaud, Lawrence J. Marnett, and John A. Oates

Introduction

Salicylic acid (SA) is an effective antipyretic and analgesic agent, yet at therapeutic concentrations exerting these effects SA lacks antiinflammatory action, distinguishing it from non-steroidal antiinflammatory drugs (NSAIDs) and selective inhibitors of prostaglandin H synthase-2 (PGHS-2). Only at concentrations much greater than are required for antipyresis does SA demonstrate antiinflammatory effects. In contrast to acetylsalicylic acid (aspirin), which irreversibly inhibits platelet PGHS-1 by acetylating a serine in the cyclooxygenase active site, SA is a weak inhibitor of platelet PGHS. The distinctive pharmacologic behavior of SA suggests that the molecular basis for its action differs from that of NSAIDs and PGHS-2 inhibitors.

Although numerous investigations have demonstrated only weak inhibitory effects of SA on purified PGH-synthase preparations [1-6], Mitchell et al. reported that SA inhibited PGE_2 production by A459 cells with an IC_{50} of approximately 30 μM in the absence of exogenous arachidonic acid (AA) or with low concentrations of exogenous substrate [3]. However, increasing the concentration of exogenous AA abrogated the inhibition by SA, an effect considered to be consistent with SA acting as a competitive inhibitor of PGHS-2 in these cells. This inverse relationship between substrate concentration and the inhibitory potency of SA was confirmed in vivo [6].

Of interest is the analogy between the pharmacology of SA and that of acetaminophen, which also exerts antipyretic and analgesic effects at doses/concentrations having little or no antiplatelet or antiinflammatory action. We recently demonstrated that the cellular selectivity of

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acetaminophen as a PGHS inhibitor is dependent on hydroperoxide concentrations within the cell [7]. Acetaminophen acts as a co-substrate of PGHS-peroxidase, reducing the higher oxidative state of the heme prosthetic group, preventing the generation of the catalytic tyrosyl radical within the cyclooxygenase site, thereby blocking AA oxygenation [7]. Inhibition of PGH synthases by acetaminophen is inversely proportional to the AA concentration, a finding explained by the enhanced production of the lipid hydroperoxide cyclooxygenase product PGG_2 [7].

In light of the evidence that increasing the concentration of AA similarly suppresses the inhibitory effect of SA, we considered whether peroxides could suppress the action of SA. We have sought to address the hypothesis that inhibition of PGH-synthases by SA is contingent on a low concentration of the lipid peroxides that drive the enzyme to its catalytically active, higher oxidative state.

Results and Discussion

Salicylate inhibition of PGHS-1 and -2 requires the phenolic structure: To assess the structural requirement of the hydroxyl group on the inhibition of PGH synthases by SA, we incubated equipotent amounts of both isoforms in the presence or absence of analogues of SA differing only at the *ortho* position. We found that SA demonstrated a similar inhibitory potency against purified preparations of PGHS-1 5.4 nM and PGHS-2 10 nM: IC_{50} 648 +/- 130 μM for PGHS-1 and 763 +/- 257 μM for PGHS-2; mean +/- SD. Both *o*-anisic acid and benzoic acid were much less inhibitory than salicylate (IC_{50} > 2 mM for both compounds against either PGHS isoform).

Salicylate inhibition of PGHS-1 and -2 is reversed by lipid hydroperoxides: The inhibitory potency of many phenolic PGHS inhibitors is inversely related to ambient peroxide concentrations [7-10]. We therefore assessed the effect of the platelet-derived lipid hydroperoxide (and PGHS-peroxidase substrate) 12-HPETE on the PGHS-1 inhibition by SA. We found that 12-HPETE (0.3 μM) fully reversed PGHS-1 inhibition by SA at a concentration below that of substrate AA (0.5 μM). 12-HPETE (0.2 μM) also reversed PGHS-2 inhibition by SA. This effect was not seen with 12-HETE.

Enhanced generation of PGG_2 prevents salicylate inhibition of PGHS-2: The primary substrate of PGHS-peroxidase is PGG_2, the hydroperoxide product of AA oxygenation by PGHS-cyclooxygenase. Because increased peroxidase activity is capable of reversing SA inhibition, it follows that greater PGG_2 production should diminish the inhibitory effect of SA. We therefore assessed the effect of increasing either AA or PGHS-2 concentration on SA inhibition. For these studies the SA level was kept constant at 2 mM, as this amount showed greater than 75% inhibition of

PGHS-2 under these conditions. Increasing either AA or PGHS-2 concentrations significantly reversed the inhibition by 2 mM salicylate.

SA is not an effective reducing co-substrate of PGHS –peroxidase: In contrast to other phenolic PGHS inhibitors it has been reported that SA does not serve as reducing co-substrates for the PGHS-peroxidase active site [11]. We examined whether SA has important redox interactions with PGHS-peroxidase cycling under our *in vitro* conditions. Reduction of the PGHS-peroxidase substrate PPHP to the alcohol PPA can be followed by HPLC [11]. Utilizing the same reaction conditions employed for testing SA against PGHS-1, we substituted PPHP as the substrate and confirmed that salicylate fails to serve as a reducing co-substrate for the peroxidase activity. This contrasts with acetaminophen, an effective reducing co-substrate for the peroxidase [11]. That SA does not act to reduce the PGHS-peroxidase implicates the PGHS-cyclooxygenase site as the primary target for the drug.

The effect of lipid peroxides on SA inhibition is mediated through electron transfer between PGHS-peroxidase and -cycloocygenase subunits: To assess whether the effect of 12-HPETE on SA inhibition observed with Fe-PGHS-1 is mediated through an electron transfer between the peroxidase ferriprotoporphyrin radical and the catalytic tyrosine of the cyclooxygenase site (Tyr385 in PGHS-1), we repeated the 12-HPETE experiments using Mn-PGHS-1, which has minimal peroxidase activity [12,13]. We found that salicylate was slightly more potent as an inhibitor of Mn-PGHS-1 than Fe-PGHS-1 (IC_{50} 437 +/- 34 μM for Mn-PGHS-1 compared with 648 +/- 130 μM for Fe-PGHS-1; mean +/- SD), and that 12-HPETE did not appreciably reverse salicylate inhibition of Mn-PGHS-1.

Conclusion

In conclusion, the finding that inhibition of PGH synthases by SA is antagonized by lipid hydroperoxides provides a basis for considering that cellular levels of hydroperoxides determine the selective action of the drug *in vivo*.

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Chapter #26

5-LIPOXYGENASE ACTIVATION BY MAPKAPK-2 and ERKs

Oliver Werz, Eva Bürkert, Lutz Fischer, Dagmar Szellas, David Dishart, Bengt Samuelsson, Olof Rådmark, and Dieter Steinhilber

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of proinflammatory leukotrienes from arachidonic acid (AA) [1]. Ca^{2+}, phosphatidylcholine, ATP, and hydroperoxides stimulate the enzymatic activity of 5-LO *in vitro* [1]. However, the mechanisms involved in the agonist-induced 5-LO activation in intact cells are less clear. Stimuli that cause an elevation of the intracellular Ca^{2+} levels activate 5-LO. Ca^{2+} binds 5-LO *in vitro* at the enzyme's C2 domain [2], which is a prerequisite for association with membranes [3].

On the other hand, phosphorylation has a significant impact on cellular 5-LO activity [4-6]. p38 MAPK-regulated MK-2 and -3 phosphorylate 5-LO *in vitro*, that is potently promoted by unsaturated fatty acids (e.g. AA), and a strong correlation between such phosphorylation events and 5-LO activity in intact cells was found [4,5,7]. Intriguingly, activation of 5-LO in polymorphonuclear leukocytes (PMNL) by cell stress, involving p38 MAPK and MKs, is Ca^{2+}-independent [7].

5-LO was dose-dependently phosphorylated by active ERK2 *in vitro* (Figure 1A). In analogy to MK-2, 5-LO was only a poor substrate for ERK2 and AA increased 5-LO phosphorylation by ERK2 in a dose-dependent fashion up to 25-fold (Figure 1B). Also oleic acid and linoleic acid increased ERK2 activity towards 5-LO, whereas saturated fatty acids failed to enhance 5-LO phosphorylation rates. Therefore, upon cell stimulation, efficient phosphorylation may occur only when free AA is provided.

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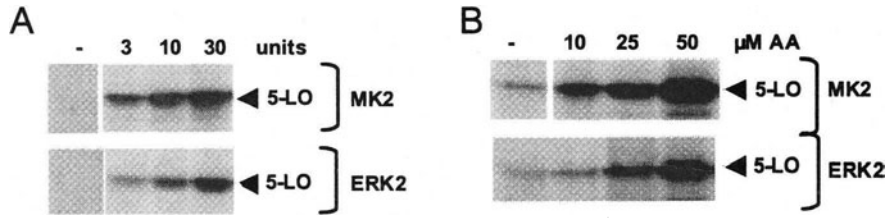

Figure 1. 5-LO is phosphorylated by MK-2 and ERK2; effects of AA. Protein phosphorylation was determined by in vitro kinase assay. (A) Purified recombinant 5-LO (3 µg) was incubated with the indicated amounts of kinases for 30 min at 30°C. (B) 5-LO was incubated with 10 mU kinase together with the indicated amounts of AA. Proteins were separated by SDS-PAGE and phosphorylated 5-LO (arrow) was detected by autoradiography of the dried gel.

Addition of exogenous AA to PMNL caused a dose-dependent induction of 5-LO activity that was accompanied by activation (phosphorylation) of ERK2 and p38 MAPK (Figure 2A). This AA-induced 5-LO activity was partially reduced by U0126, an ERK pathway inhibitor, but also by the p38 MAPK inhibitor SB203580. Combination of both inhibitors gave additive effects, indicating that ERK and p38 MAPK pathways act in conjunction to stimulate 5-LO. Importantly, 5-LO activity induced by AA plus Ca^{2+}-ionophore (leading to high levels of intracellular Ca^{2+} that directly activates 5-LO) was hardly affected by U0126 and SB203580 (not shown).

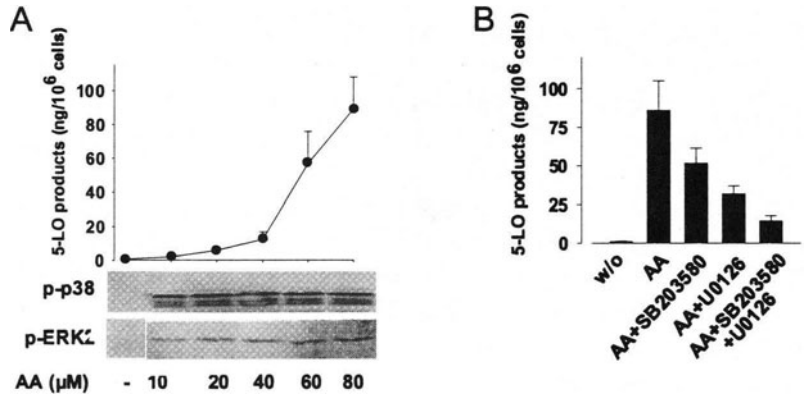

Figure 2. Activation of p38 MAPK and ERKs correlates with 5-LO activity. Human isolated PMNL were incubated with the indicated amounts of AA at 37°C (A) or preincubated with SB203580 (10 µM) and/or U0126 (3 µM) and then stimulated with 60µM AA (B). 5-LO products were analyzed by HPLC 10 min after addition of AA. ERK and p38 MAPK activation was determined 2.5 min after addition of AA by Western blotting using phospho-specific antibodies against the kinases.

The 5-LO sequence reveals putative phosphorylation motifs for MK-2 (LERQLS; 266-271) as well as for ERK2 (YLSP; 661-664), with Ser271 and Ser663 as potential phosphorylation sites. Mutation of these residues to alanine leads to 5-LO mutant proteins that were no longer substrates for the respective kinases. HeLa cells were transfected with wt or the 5-LO mutants and cellular 5-LO activity was determined. Almost identical amounts of 5-LO products were formed from wt and mutated 5-LO enzymes, when cells had been treated with AA plus ionophore, whereas significantly less 5-LO products as compared to wt-5-LO were formed, when cells had been challenged with AA alone (Figure 3). Finally, deletion of both phosphorylation sites resulted in a even more pronounced reduction of AA-induced 5-LO activity. Together it appears that phosphorylation of 5-LO at Ser271 and Ser663 is of importance for 5-LO activation at low Ca^{2+} levels. In this scenario AA may play a pivotal regulatory role on several levels in as much as it (1) serves as substrate for 5-LO, (2) activates 5-LO kinases, and (3) may promote the phosphorylation of 5-LO by ERKs and MKs.

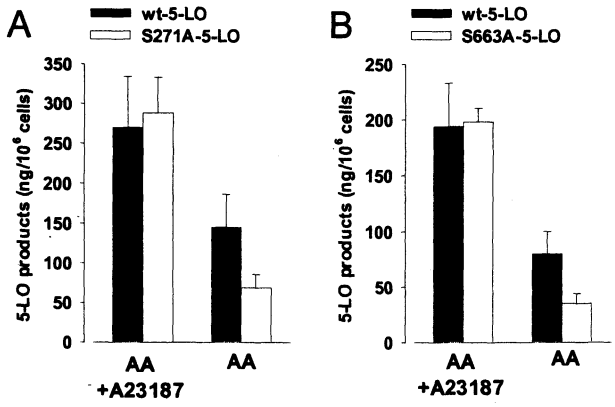

Figure 3. Effects of deletion of 5-LO phosphorylation sites. HeLa cells were transiently transfected with wt-5-LO, S271A-5-LO (A), or S663A-5-LO (B), stimulated with 40 μM AA with or without 10 μM ionophore A23187 and after 10 min at 37°C, 5-LO products were analyzed by HPLC.

Acknowledgements

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Chapter #27

RENAL EFFECTS OF SELECTIVE CYCLOOXYGENASE INHIBITION IN EXPERIMENTAL LIVER DISEASE

Marta López-Parra, Joan Clària, Anna Planagumà, Esther Titos, Jaime L. Masferrer, B. Mark Woerner, Alane T. Koki, Wladimiro Jiménez, Vicente Arroyo, Francisca Rivera, and Joan Rodés

Introduction

Renal synthesis of vasodilator prostaglandins (PGs) plays a key role in the maintenance of renal function in decompensated cirrhosis [1,2]. In fact, acute PG inhibition with nonsteroidal anti-inflammatory drugs (NSAIDs) in patients with cirrhosis and ascites is associated with a significant impairment in renal hemodynamics, sodium excretion, free water clearance and the renal response to furosemide and spironolactone [1-3]. Thus, in clinical practice, patients with decompensated cirrhosis cannot be treated with NSAIDs on a long-term basis because of the high risk of developing renal failure and refractory ascites.

Cyclooxygenase (COX) is the key enzyme in the formation of PGs from arachidonate and is the major therapeutic target for NSAIDs [4]. Two isoforms of COX, designated COX-1 and COX-2, have been identified [5-7]. Conventional NSAIDs inhibit both COX-1 and COX-2 and this feature accounts not only for their therapeutic actions but also for their unwanted side effects. The discovery of two isoforms of COX has led to the development of novel compounds that unlike conventional NSAIDs selectively inhibit either COX-1 or COX-2.

The present study was designed to characterize which COX isoform is involved in the maintenance of renal function in experimental liver disease. To this end, we first analyzed COX-1 and COX-2 mRNA and protein expression in kidneys from rats with carbon tetrachloride (CCl_4)-induced cirrhosis, an experimental model that closely reproduces the systemic and renal abnormalities seen in human cirrhosis [8]. In these animals, we also examined the effects on renal function of the following COX inhibitors: 6-MNA -the active metabolite of nabumetone- and ketorolac (mixed COX inhibitors), SC-560 (selective COX-1 inhibitor) and celecoxib and SC-236 (selective COX-2 inhibitors).

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Material and Methods

The study was performed in adult male Wistar rats. In these animals, cirrhosis was induced by CCl_4 inhalation following a method described elsewhere [8]. Renal COX-1 and COX-2 mRNA expression was assessed by RT-PCR [9]. COX-1 and COX-2 protein levels were determined by Western Blot using specific murine antisera for either COX-1 or COX-2 [10].

Urinary sodium excretion ($U_{Na}V$) and glomerular filtration rate (GFR) were measured before and 60 minutes after the i.v. administration of 6-MNA (34 mg/kg), ketorolac (3 mg/kg), SC-560 (20 mg/kg), celecoxib (20 mg/kg) and SC-236 (3 mg/kg) to cirrhotic rats.

Results and Discussion

Constitutive COX-1 and COX-2 mRNA (Figure 1A) and protein (Figure 1B) expression were detected by RT-PCR and Western blot analysis, respectively, in kidneys from both control and cirrhotic rats. However, whereas COX-1 was found to be unchanged, as compared to controls, COX-2 protein expression was consistently up-regulated in kidneys from cirrhotic rats (Figure 1B).

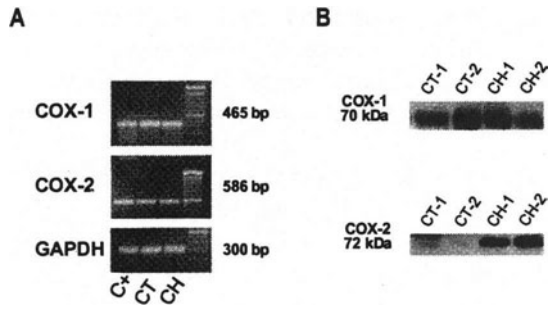

Figure 1. COX-1 and COX-2 mRNA and protein expression in renal tissue isolated from control (CT) and cirrhotic rats with ascites (CH). (A) Total RNA was subjected to RT-PCR using specific oligonucleotides for COX-1, COX-2, and GAPDH. RNA isolated from Raw 264.7 macrophages was used as a positive control (C+). (B) Representative Western blot analysis of COX-1 and COX-2. Renal protein extracts were electrophoresed and probed with specific anti-COX-1 and anti-COX-2 antibodies. (Adapted from references 9 and 10 with permission from Elsevier Science and Nature Publishing Group, respectively).

These results are consistent with the notion that COX-2, but not COX-1, is regulated in a cell-specific fashion in response to altered volume status

[11,12]. However and although previous studies suggest that renal COX-2 up-regulation is likely to be a consequence of changes in volume status [13], the exact mechanism for this phenomenon in cirrhosis is, at present, not well understood.

Despite having increased renal COX-2 protein levels, renal function in cirrhotic rats appears to be mainly dependent on PGs derived from COX-1. As shown in Figure 2, the impairment in $U_{Na}V$ and especially in GFR induced by SC-560 was similar to that induced by mixed COX inhibitors such as ketorolac and 6-MNA. In contrast, selective inhibition of COX-2 with celecoxib or with its related compound SC-236 did not seriously compromise renal function in cirrhotic animals. The physiological significance of PGs derived from COX-2 in the kidneys of cirrhotic rats is at present unknown.

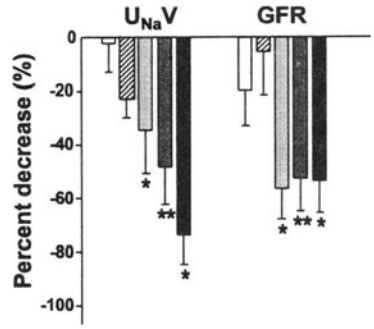

Figure 2. Effects of COX inhibition on renal function in rats with cirrhosis and ascites. Urinary sodium excretion ($U_{Na}V$) and glomerular filtration rate (GFR) were measured before and 60 minutes after the administration of SC-236 (□), celecoxib (▨), 6-MNA (▩), ketorolac(■) and SC-560 (■) to cirrhotic rats. Results represent the mean±s.e.m and are expressed as the percent decrease as compared to basal conditions. *, $p<0.05$ and **, $p<0.01$ vs. baseline conditions.

Taken together, our findings in the CCl_4 experimental model of cirrhosis encourage further studies in humans investigating the feasibility of selective COX-2 inhibitors possibly being the anti-inflammatory option of choice not only in patients with chronic liver disease but also in those circumstances that are more susceptible to NSAID-induced renal failure, such as congestive heart failure and nephrotic syndrome.

Acknowledgements

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Chapter #28

VARIABLE ANTIPYRETIC EFFECT OF SC-58236, A SELECTIVE CYCLOOXYGENASE (COX)-2 INHIBITOR, IN RATS

Eyup S. Akarsu, Soner Mamuk and Sibel Arat

Introduction

Fever is the most common body temperature (T_b) change accompanied to inflammation [1]. It has been reported that increased prostaglandin (PG) formation is generally essential for the genesis of fever [2]. The COX enzyme is required for PG synthesis. There are two isoforms of the COX enzyme as COX-1 and COX-2 [3]. Recent studies have suggested that COX-2 isoenzyme is critical for the mediation of lipopolysaccharide (LPS)-induced fever in rodents [4,5]. But, our data did not support this suggestion [6]. Briefly, selective inhibitors of COX-2 and COX-1, either alone or in combination, did not inhibit the initiation of the fever component of LPS-induced dual T_b response in rats. It may be proposed that the model we used could be the source of this variance since we observed an initial hypothermia and subsequent fever by LPS at 50 μg/kg dose. The mechanisms of fever may have been determined by the pattern of the response implying that the fever as a component of dual response may have different characteristics than the fever, which develops without hypothermia. In this connection, a lower dose of LPS such as 2 μg/kg produces only fever in our experimental conditions [7]. Another source of variability might be related with LPS itself. We have reported that LPS causes serotype-specific T_b changes in rats [7]. Thus, it may be proposed that diverse signalling pathways might be activated depending on the dose or the serotype of LPS. In order to evaluate this proposal, the effects of COX-1 or COX-2 selective inhibitors (valeryl salicylate [VS] and SC-58236; respectively) on fever induced by low dose of two different serotype of LPSs were investigated in rats.

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Materials and Methods

Adult male Wistar albino rats were used. The T_b was monitored with a biotelemety (Mini-Mitter, Bend, OR) at 24-26 °C ambient temperature. LPS of *E.coli* serotype O111:B4 (lot 3922-25-0, Difco, Detroit, MI) or *E. coli* O55:B5 (lot 56H4096, Sigma) was injected intraperitoneally (ip) at 2 μg/kg dose. VS (Cayman Chem, Ann Arbor, MI) and SC-58236 (Searle, Skokie, IL) were suspended in Tween 20 (0.025 %) and injected subcutaneously at 20 mg/kg and 10 mg/kg doses; respectively. Drug and LPS injections were made simultaneously.

The data were evaluated as T_b changes from baseline value (ΔT). ΔT curves expressed as mean±SEM were analysed by one-way ANOVA with Newman-Keuls *post-hoc* test. Significance was noted when $p<0.05$.

Results and Discussion

E. Coli O111:B4 LPS caused fever at 2 μg/kg, ip. The T_b began to rise about 5 h after LPS injection and remained elevated for about 3 h (Figure 1). Before that, there was an upward change on T_b which did not attain statistical significance. SC-58236 treatment completely abolished the late

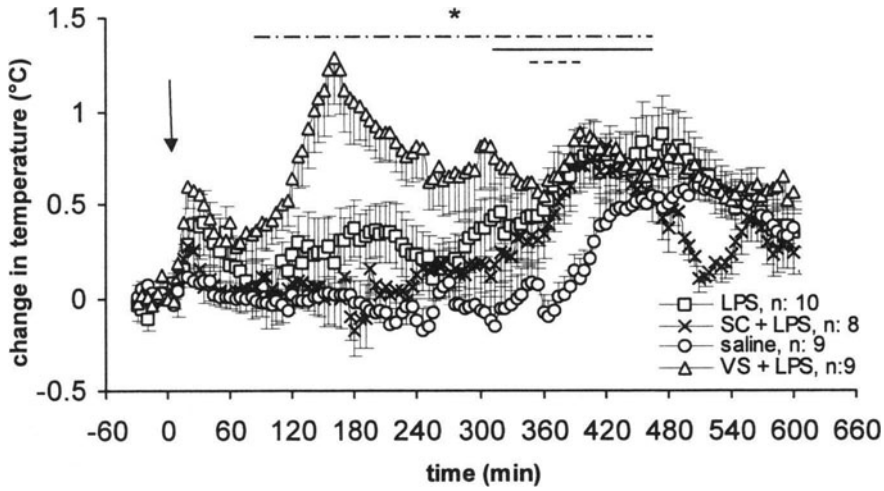

Figure 1. Effects of VS or SC-58236 treatment on *E. coli* O111:B4 serotype LPS-induced fever. Arrow indicates the injection time for drug and/or LPS. * $p<0.05$ compared to saline. The statistically significant points of each curve are indicated as: (—) LPS; (– –) SC + LPS; (– · –) VS + LPS.

phase of fever without any inhibition on the initiation phase. Meanwhile, VS treatment facilitated the fever as the T_b rised about 2 h after LPS injection. The maximal ΔT value was not significantly different. VS had no effect on the fever observed 5 h after LPS challenge.

These data indicate that in terms of sensitivity to VS and SC-58236, *E.coli* O111:B4 LPS-induced fever at 2 ug/kg, ip dose has similar characteristics with the fever obtained by the same LPS at 50 μg/kg, ip dose [6]. The presence of hypothermia is presumably not changed the mechanisms, which are responsible for the generation of fever. Thus, we may conclude that COX-1 and COX-2 isoenzymes may not be involved in the initiation of *E. coli* O111:B4 LPS-induced fever in rats.

On the other hand, *E. coli* O55:B5 LPS also produced fever when injected 2 μg/kg, ip. A small but significant elevation on T_b observed 2 h after LPS injection. The T_b remained to be high for 3 h. (Figure 2). But in this case, SC-58236 treatment completely inhibited the fever. VS treatment prolonged the febrile response because an additional late fever peak occurred about 5 h after injection. VS had no effect on the first fever peak.

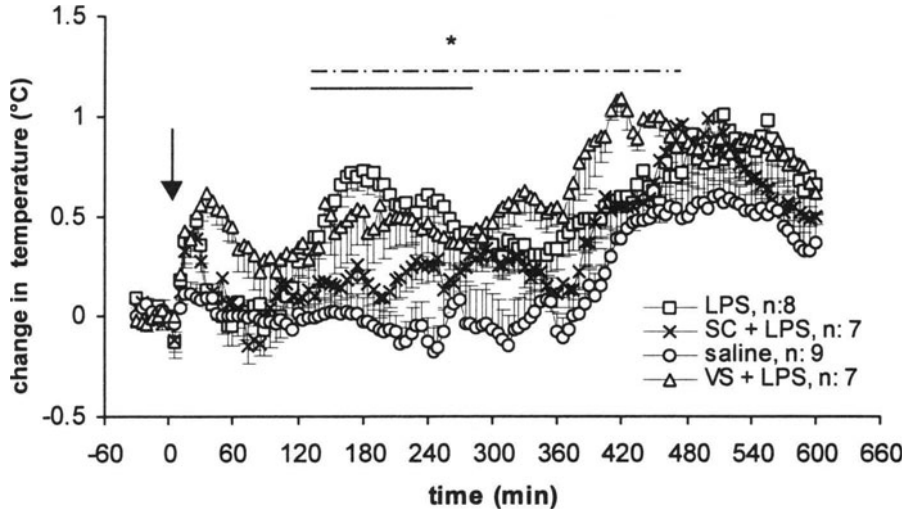

Figure 2. Effects of VS or SC-58236 treatment on *E. coli* O55:B5 serotype LPS-induced fever. Arrow indicates the injection time for drug and/or LPS. * $p<0.05$ compared to saline. The statistically different points of each curve are indicated as: (—) LPS; (– · –) VS + LPS.

Taken together, these data suggest that LPSs activate alternative pathways, which may presumably be responsible for the initiation of fever in rats. The structure of the LPS should be critical for the determination of these pathways. Our recent data have indicated the importance of the polysaccharide (PS) component of exogenous pyrogens (including LPS) for the genesis of fever in rats [8]. Thus, different PSs may activate different signalling molecules, which may account for the heterogenous nature of fever. The role of COX-2 in fever seems to be variable depending on the LPS itself. COX-1 does not seem to be involved in fever. However, it appears that COX-1 may have a predominant role for the development of LPS-induced hypothermia in rats.

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Chapter #29

INDUCTION OF CYCLOOXYGENASE-2 EXPRESSION BY FLUID SHEAR STRESS IN VASCULAR ENDOTHELIAL CELLS

Hiroyasu Inoue, Yoji Taba, Yoshikazu Miwa, Chiaki Yokota, Megumi Miyagi, and Toshiyuki Sasaguri

Introduction

Vascular endothelial cells are exposed to a wide variety of biomechanical stimuli including fluid shear stress caused by blood flow. Shear stress modulates several endothelial functions, such as control of vascular tone, maintenance of antithrombotic surfaces, regulation of inflammation, protection against oxidative stresses, and regulation of endothelial cell proliferation and apoptosis.

Cyclooxygenase (COX), a rate-limiting enzyme for prostaglandin (PG) biosynthesis, comprises two isozymes, COX-1 and COX-2. Laminar shear stress upregulates COX-2 gene expression [1-3]. We found that COX-2 is involved in lipopolysaccharide (LPS)-stimulated production of prostacyclin (PGI_2) in endothelial cells [4]. And that, shear stress promotes the production of PGD_2 in endothelial cells by stimulating the expression of lipocalin-type PGD_2 synthase (L-PGDS), whereas PGI_2 synthase is constitutively expressed in the presence or absence of the shear stress [5]. Therefore, it is necessary to evaluate the roles of COX-2 and L-PGDS expression by shear stress, which will be distinctly involved in the production of PGI_2 and PGD_2 in endothelial cells.

Three cis-acting elements, namely the NF-κB binding site, NF-IL6 binding site, and cyclic AMP response element, reside in the region between base pairs -327 and +59 (-327/+59) in the human COX-2 gene promoter. Their involvement in COX-2 gene transcription varies among cell species [6]. Recently, the COX-2 gene has been reported to be post-transcriptionally regulated through its 3'-untranslated region (3'-UTR) containing 17 copies

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of the "AUUUA" motif, which is assumed to promote mRNA degradation. However, the mechanism underlying shear stress-induced COX-2 gene expression remains to be elucidated.

We investigated the molecular mechanism for the shear stress-induced expression of COX-2 in vascular endothelial cells. The gene expression of COX-2 was more sensitive to shear strength than that of L-PGDS. We found that shear stress induces COX-2 expression not only at the transcriptional level but also at the post-transcriptional level through the 3'-UTR, which would make it possible to rapidly and persistently induce COX-2 expression in response to shear stress [7].

Materials And Methods

Materials and experimental procedures have been described in detail elsewhere [4-7].

Results and Discussions

Exposure of human umbilical vein endothelial cells (HUVECs) to laminar shear stress in the physiological range (1 to 30 dyne/cm^2) upregulated the expression of cyclooxygenase (COX) -2 but not COX-1. The expression of COX-2 mRNA began to increase within 0.5 hours after the loading of the shear stress and reached a maximal level at 4 hours, which was more sensitive to shear strength than that of L-PGDS. (Table 1)

Table 1.Comparison of induction of COX-2 and L-PGDS mRNAs by fluid shear stress

Shear stress	Induction of COX-2[7]	Induction of L-PGDS[5]
Lower shear stress (1 dyne/cm^2)	Yes	No
Higher shear stress (15 dyne/cm^2)	Yes Rapid (within 30 min) Maximal level at 4 hours	Yes Time-lag (6 hours) Maximal level at 18 hours

Roles of the promoter region and the 3'-UTR in the human COX-2 gene were evaluated by the transient transfection of luciferase (Luc) reporter vectors into bovine arterial endothelial cells. Shear stress elevated Luc activity via the region (-327/+59 bp) in the COX-2 promoter. Mutation analysis indicated that cAMP responsive element (-59/-53 bp) was mainly involved in the shear stress-induced COX-2 expression. Moreover, shear stress selectively stabilized COX-2 mRNA in HUVECs. When a 3'-UTR containing 17 copies of the AUUUA mRNA instability motif was inserted into downstream of the Luc coding region, shear stress elevated the Luc expression. These results suggested that transcriptional activation and post-

transcriptional mRNA stabilization both contribute to the rapid and sustained expression of COX-2 in response to shear stress. Taken these findings together, COX-2 but not COX-1 will be mainly involved in PGI_2 formation in blood vessels loaded with laminar shear stress in the physiological range. This assumption is consistent with recent reports as follows.

1) Selective COX-2 inhibitors suppress the systemic biosynthesis of PGI_2 in healthy humans [8].

2) Glucocorticoids do not depress excretion of urinary PGI_2 metabolite [9].

3) COX-2 expression is involved in PGI_2 formation, but is not suppressed by dexamethasone in vascular endothelial cells [4].

4) COX-2 preferentially cooperates with PGI_2 synthase more than COX-1 [10].

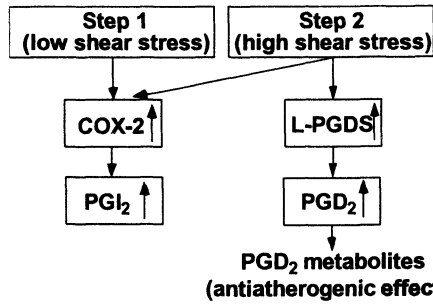

Figure 1. Possible roles of COX-2 and L-PGDS in endothelial cells

Concerning distinct regulation of COX-2 and L-PGDS expression by fluid shear stress, we hypothesize that there are two steps in COX-2-mediated arachidonate metabolism in endothelial cells (Figure 1). In this context, PGD_2 may also play a role in preventing the formation of atherosclerotic lesions by being converted to 15-deoxy-$\Delta^{12,14}$-PGJ_2, which has been reported to display several antiatherogenic effects on cultured vascular cells [11].

Macrophages were reported to express augmented levels of COX-2 in atherosclerotic lesions. This abnormally elevated COX-2 expression in macrophages may be related with inflammation in the lesions. Since the PGs produced in macrophages are different from those produced in endothelial cells, the regulation of COX-2 and downstream enzymes should be different between endothelial cells and macrophages. In this context, we have reported that COX-2 expression is negatively regulated by nuclear receptor PPARγ and its ligand candidate 15-deoxy-$\Delta^{12,14}$-PGJ_2 in macrophages but not in endothelial cells [6]. It should be examined whether changes in the pattern of blood flow, such as turbulence, influence the PG species produced in the cardiovascular system.

Acknowledgements

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Chapter #30

INDUCTION OF COX-2 EXPRESSION BY THE ENDOCANNABINOID DERIVATIVE R(+)-METHANANDAMIDE

Burkhard Hinz, Robert Ramer and Kay Brune

Introduction

Cannabinoids produce a broad array of potential therapeutical effects, including the reduction of nausea in cancer and AIDS patients undergoing chemotherapy [1]. In recent years, two G_i protein-coupled cannabinoid receptors, CB_1 and CB_2, have been identified and cloned. Whereas the CB_1 receptor is preferentially expressed in the central nervous system [2], the CB_2 receptor has been described as the predominant form expressed in peripheral immune cells [3]. The discovery of specific cannabinoid receptors was followed by the identification of two endogenous arachidonic acid derivatives, anandamide (N-arachidonylethanolamide) and 2-arachidonyl-glycerol, which exhibit specific binding affinity to cannabinoid receptors [4, 5]. However, emerging evidence suggests that various cannabinoid actions are mediated via cannabinoid receptor-independent pathways, comprising activation of mitogen-activated protein kinases (MAPKs) [6, 7], alteration of intracellular calcium [8] and cAMP levels [9, 10], inhibition of cytokine expression [11] and induction of histamine release [12].

Modulation of prostaglandin (PG) formation by cannabinoids has been widely established over the past years. In spite of this, the mechanism of action responsible for enhanced PG production attributed to cannabinoid compounds has been elusive. Previous studies suggest that cannabinoids may stimulate arachidonic acid release from cultured astrocytes [13], cortical slices [14] and neuroblastoma cells [15]. Additionally, Δ^9-THC has been shown to increase the amount of PGE_2 in the brain [16, 17]. A possible interference of cannabinoids with the expression of inducible enzymes involved in arachidonic acid metabolism has been recently reported by Chan et al. [18] who showed that the PG-dependent hippocampal neuronal toxicity of Δ^9-THC requires de novo synthesis of mRNA and protein. However, at

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present, no definitive studies support the notion that expression of cyclooxygenase-2 (COX-2) is required for cannabinoid-mediated PG formation.

The objective of the present study was to determine the effect of the potent and selective CB_1 agonist R(+)-methanandamide (R-(+)-arachidonyl-1'-hydroxy-2'-propylamide), a metabolically stable analogue of the endocannabinoid anandamide [19], on the expression of COX-2 by H4 human neuroglioma cells.

Materials and Methods

H4 human neuroglioma cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2. All incubations were performed in serum-free medium. For quantitative RT-PCR analysis, H4 neuroglioma cells were grown to confluence in 24-well plates. Following incubation for the indicated times total RNA was isolated using the RNeasy total RNA Kit (Qiagen, Hilden, Germany). ß-Actin- (internal standard) and COX-2 mRNA was determined by quantitative real-time RT-PCR as decribed previously [20, 21]. For Western blot analysis, cells grown to confluence in 10-cm dishes were used. The expression of different proteins was assessed using specific antibodies raised to COX-2 (BD Biosciences, Heidelberg, Germany), phospho-p38 MAPK and phospho-p42/44 MAPK (New England BioLabs GmbH, Frankfurt, Germany). Quantification was achieved by scanning the films with an optical scanner (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA, USA) and then analyzing band intensities of the images with the Multi-Analyst program, version 1.1 (Bio-Rad Laboratories, Hercules, CA, USA). PGE_2 concentrations were determined in supernatants of cell cultures grown to confluence in 24-well plates using a commercially available enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA).

Results

As shown by quantitative real-time RT-PCR analysis and Western blot analysis, R(+)-methanandamide induced a concentration-dependent increase of COX-2 mRNA and protein expression up to a 3.3- and 3-fold of the respective vehicle control (Figure 1A-C). Moreover, incubation of H4 cells with R(+)-methanandamide for 24 h concentration-dependently increased PGE_2 levels in cell culture supernatants up to 4.4-fold of control (Figure 1D). R(+)-methanandamide-induced PGE_2 synthesis was totally abolished in the presence of NS-398, a selective inhibitor of COX-2 activity (Figure 1D).

Induction of COX-2 expression by H4 cells was not unique for R(+)-methanandamide but shared by the structurally unrelated plant-derived cannabinoid Δ^9-THC (Table 1).

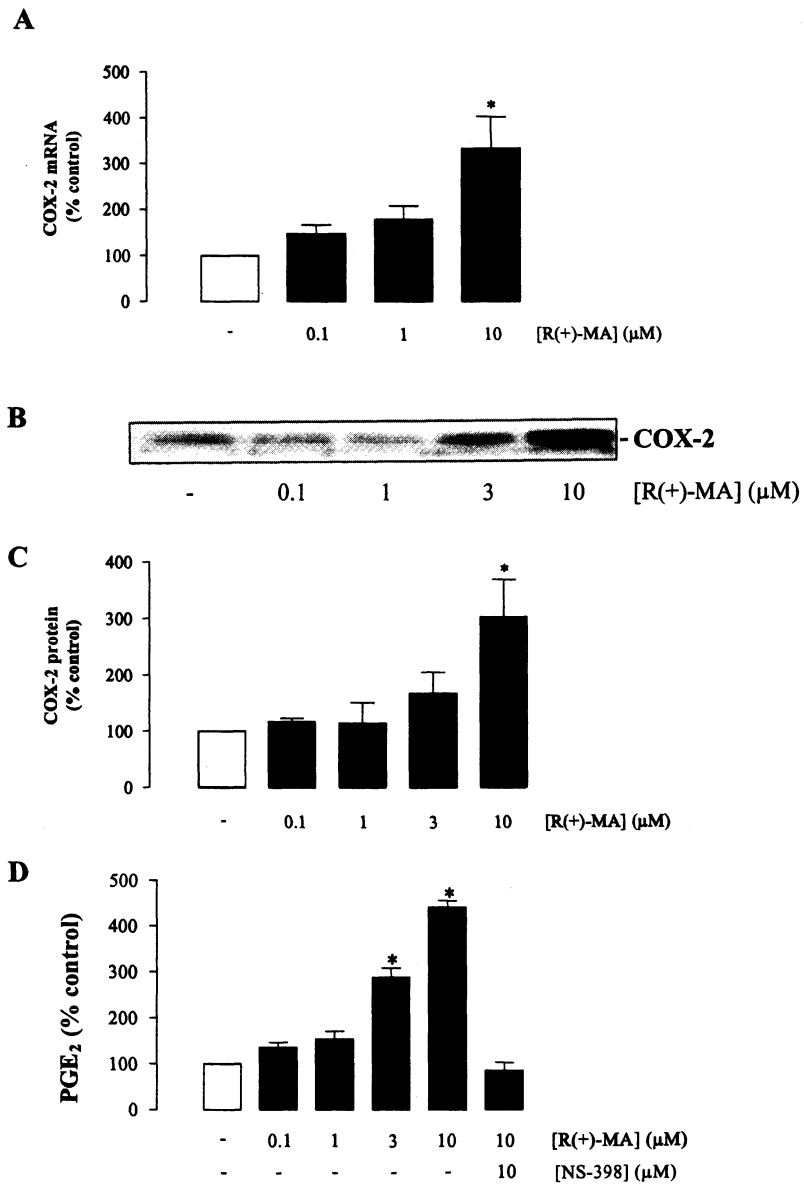

Figure 1. Effect of R(+)-methanandamide (R(+)-MA) on the expression of COX-2 mRNA (A) and COX-2 protein (B and C) and on PGE_2 (D) synthesis by H4 human neuroglioma cells. Cells were incubated for 4 h (COX-2 mRNA) or 24 h (COX-2 protein, PGE_2) with R(+)-MA or its vehicle. NS-398 was present throughout the 24-h incubation period. COX-2 protein expression is shown by a representative Western blot (B) and a densitometric analysis (C). Values (A,C,D) are means ± S.E.M. of n = 3 observations. * $P < 0.05$ vs. corresponding vehicle control (open column), Student's t-test.

Further experiments were performed to examine whether the stimulatory effect of R(+)-methanandamide on COX-2 expression and PGE_2 synthesis might be the result of a CB_1 receptor-mediated pathway. However, R(+)-methanandamide-induced COX-2 expression and PGE_2 synthesis remained unaltered in the presence of the selective CB_1 receptor antagonist AM-251 and the $G_{i/o}$ protein inactivator pertussis toxin (Table 1).

Next we performed experiments to address the issue whether MAPK activation is involved in R(+)-methanandamide-induced COX-2 expression. According to Fig. 2, an increase in the phosphorylated form of p38 MAPK was detected 10 min following R(+)-methanandamide treatment. Phosphorylation of p38 MAPK reached a maximum by 15 min that was still evident at 45 min post-stimulation (Figure 2). Phosphorylation of p42/44 MAPK began to increase within 5 min after the addition of R(+)-methanandamide, peaked after 15 min and declined thereafter (Figure 2). To confirm a causal link between activation of p38 and p42/44 MAPKs and induction of COX-2 expression by R(+)-methanandamide, the impact of specific inhibitors of p38 MAPK (SB203580) and p42/44 MAPK activation (PD98059) on COX-2 expression was assessed in further experiments. R(+)-methanandamide-induced COX-2 mRNA expression and PGE_2 synthesis were totally abolished in the presence of both kinase inhibitors (Table 1).

Table 1. Effect of Δ^9-THC on COX-2 mRNA expression and PGE_2 formation by H4 human neuroglioma cells and influence of AM-251, pertussis toxin (PTX), SB203580 and PD98059 on R(+)-methanandamide (R(+)-MA; 10 μM)-induced COX-2 mRNA expression and PGE_2 formation by H4 human neuroglioma cells.

	COX-2 mRNA (% control)	PGE_2 (% control)
Vehicle	100	100
Δ^9-THC (10 μM)	265 ± 20*	342 ± 76*
Vehicle	100	100
R(+)-MA	575 ± 158*	326 ± 18*
R(+)-MA + AM-251 (1 μM)	538 ± 133*	264 ± 31*
R(+)-MA + PTX (100 ng/ml)	585 ± 200*	308 ± 33*
Vehicle	100	100
R(+)-MA	273 ± 8*	440 ± 14*
R(+)-MA + SB203580 (30 μM)	88 ± 33	72 ± 16
R(+)-MA + PD98059 (30 μM)	57 ± 10	88 ± 24

Note. Cells were incubated with Δ^9-THC, R(+)-MA or its vehicle for 4 h (COX-2 mRNA) or 24 h (PGE_2 levels). AM-251 and PTX were added to cell cultures 1 h prior to R(+)-MA. SB203580 and PD98059 were added concomitantly with R(+)-MA to cells. Values are means ± S.E.M. of n = 3 experiments. * $P < 0.05$ vs. corresponding vehicle control, Student's t-test.

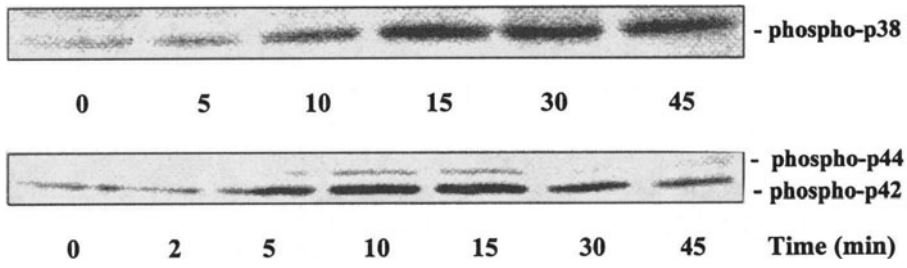

Figure 2. Time-course of p38 MAPK and p42/44 MAPK phosphorylation in R(+)-methanandamide-treated H4 human neuroglioma cells. Cells were incubated with R(+)-methanandamide (R(+)-MA; 10 μM) for the indicated times. Phosphorylation of p38 and p42/44 MAPKs was analyzed by Western blotting. Results are representative of three experiments with similar results.

Discussion

The present study demonstrates a stimulatory effect of the endocannabinoid derivative R(+)-methanandamide on the expression of the COX-2 enzyme in human neuroglioma cells. Enhanced expression of COX-2 was accompanied by increased levels of PGE_2 in cell culture supernatants. The involvement of COX-2 in the PG-elevating effect of R(+)-methanandamide was confirmed by experiments demonstrating that the selective inhibitor of COX-2 activity, NS-398, totally abolished R(+)-methanandamide-induced PGE_2 production. Our results further show that induction of COX-2 expression is not unique for R(+)-methanandamide but is shared by the structurally unrelated cannabinoid Δ^9-THC.

As cannabinoids are known to act via CB_1 receptors in the central nervous system, experiments were performed to check a possible involvement of this receptor subtype in the inductive action of R(+)-methanandamide on COX-2 expression. However, the selective CB_1 receptor antagonist, AM-251 as well as the $G_{i/o}$ protein inactivator pertussis toxin did not affect COX-2 induction by R(+)-methanandamide, suggesting that neither pertussis toxin-sensitive CB_1 receptors nor recently postulated pertussis toxin-sensitive non-CB_1/non-CB_2 G-protein-coupled cannabinoid receptor subtypes [10] confer R(+)-methanandamide-induced COX-2 expression.

To provide evidence for possible targets within the non-cannabinoid receptor-mediated pathway of COX-2 induction, our further interest has focused on the MAPK signaling cascade that has previously been linked to induction of COX-2 expression. Western blot analysis using antibodies specific for phospho-p38 MAPK and phospho-p42/44 MAPK revealed an activation of both kinases by R(+)-methanandamide. Consistent with the

activation of both kinases, R(+)-methanandamide-induced COX-2 expression and subsequent PGE_2 synthesis were completely abolished by SB203580, a selective p38 MAPK inhibitor, and PD98059, a specific inhibitor of p42/44 MAPK activation. On the basis of these data we conclude that both p38 and p42/44 MAPKs may play a crucial role in mediating up-regulation of COX-2 expression by R(+)-methanandamide.

A relationship between cannabinoids and PGs has been established by several lines of evidence. Accordingly, various actions of cannabinoids within the central nervous system, including the dilation of cerebral arterioles [22], psychoactive and behavioural effects [23-25] as well as the reduction of intra-ocular pressure [26, 27] have been associated with an increased production of PGs. However, the precise role of a cannabinoid-induced expression of the COX-2 enzyme that has been implicated in both pathophysiological and physiological functions [28] is presently unknown and has to be addressed in future studies. As COX-2 has been implicated in inhibition of apoptosis [29], it cannot be ruled out that cannabinoids may diminish their pro-apoptotic action [30] by virtue of their capacity to induce COX-2 expression. Moreover, COX-2 has been recently shown to possess a regulatory function in endocannabinoid signaling. In fact, both anandamide and 2-arachidonylglycerol are substrates for COX-2 and become inactivated upon binding to the latter [31, 32]. In the light of these findings, induction of COX-2 expression by anandamide could constitute part of a negative feedback mechanism by which anandamide facilitates its own metabolism and inactivation.

Overall, the present study demonstrates a non-cannabinoid receptor-mediated induction of COX-2 expression by the endocannabinoid derivative R(+)-methanandamide. Our results indicate that this action is mediated, at least in part, through a MAPK-dependent pathway. In conclusion, induction of COX-2 expression may represent a novel mechanism by which cannabinoids mediate their diverse actions within the central nervous system.

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Chapter #31

SPECIFIC GENE EXPRESSION OF ISOFORMS IN CYCLOOXYGENASE PATHWAY TO GENERATE PGJ_2 DERIVATIVES DURING THE DIFFERENTIATION OF 3T3-L1 ADIPOCYTES

Shan Lu, Masa-atsu Ohya, Kohji Nishimura, Mitsuo Jisaka, Tsutomu Nagaya, and Kazushige Yokota

Introduction

Obesity is a risk factor to cause complicated diseases such as diabetes and others. Obesity accompanies an increase in the number or size of adipocytes, which should involve the changes in the life cycle of fat cells. During these processes, peroxisome proliferator-activated receptor (PPAR)γ, which is a member of nuclear receptors, plays an important role in the differentiation and the progression of insulin resistance in adipocytes [4]. PPARγ was originally recognized by the fact of the activation by antidiabetic drugs. Later, fatty acids and eicosanoids were found to activate PPARγ as endogenous ligands [1,3].

Compared to the biological action of eicosanoids, much less is known about the mechanism of the activation of arachidonate cascade in adipogenesis and adipocyte hypertrophy. Hence, we sought to determine the specific gene expression of the isoforms of the cyclooxygenase (COX) pathway to provide prostanoids as endogenous ligands for nuclear receptors using mouse adipogenic cell line, 3T3-L1 cells.

Materials And Methods

Preadipogenic mouse 3T3-L1 cells were cultured to induce the differentiation of mature adipocytes as reported earlier [2]. Total RNA was extracted from cultured cells and subjected to the analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) as described before [6].

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The amplification of the target cDNA was done with the upstream primer 5'-GTCTTCACCACCATGGAGAA-3' versus the downstream primer 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (677 bp), 5'-GGGACCAAAGGGAAGAAACA-3' versus 5'-AACCCCATAGTCCACCAGCAT-3' for COX-1 (768 bp), 5'-GAGATGATCTACCCTCCTCA-3' versus 5'-CCCCTTCACATTATT-GCAGAT-3' for COX-2 (909 bp), 5'-GACAAGTTCCTG-GGGCGCTG-3' versus 5'-CTACTGTAGAGGGTGGCCAT-3' for lipocalin-type PGD synthase (L-PGDS) (344 bp), and 5'-AATATGAGGGGAAGAGCCGA-3' versus 5'-CAGTAGAAGTCTGCCCAGGT-3' for hematopoietic type of PGDS (H-PGDS) (431 bp).

Results And Discussion

By the RT-PCR method, we found significant increase in the level of the transcript of PPARγ after 3 h of the maturation phase, and thereafter, the increased levels were maintained over 7 days. This result clearly indicates that the PPARγ expression is inducible during the adipogenesis.

As known well, the PGD_2 dehydration product, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) is reported to be the most potent endogenous ligand for PPARγ [1, 3]. Therefore, attempts were made to determine the expression of the transcripts coding for the biosynthetic enzymes in the cyclooxygenase (COX) pathway which leads to the formation of PGD_2 and PGJ_2 derivatives from arachidonic acid. The rate-limiting step for prostanoids is known to be the COX reaction catalyzing the conversion of arachidonic acid to the intermediate PGH_2, which serves a common substrate for several prostanoids.

Next, the expression of COX isoforms was determined by RT-PCR (Figure 1). The expression of mRNA levels of both COX isoforms was found during the process of adipocyte differentiation. The analysis revealed that COX-1 was relatively expressed at constant levels. In sharp contrast, transient and biphasic increases of COX-2 were observed within several hours during the induction of the differentiation and maturation processes.

The additional step leading to the formation of PGJ_2 derivatives includes the action of PGDS catalysing the conversion of PGH_2 to PGD_2. This enzyme comprises two types of isoforms, namely H-PGDS and L-PGDS. However, until now, little is known about the expression of PGD synthase isoforms in adipocytes and related cells. The expression of L-PGDS mRNA was only detectable, which was the highest around 3 h after replacing with maturation medium within 24 h (Figure 2), whereas there was no significant expression of H-PGDS. Thus, the expression of PPARγ, COX-2, and L-PGDS seems to undergo the cooperative regulation during adipocyte differentiation.

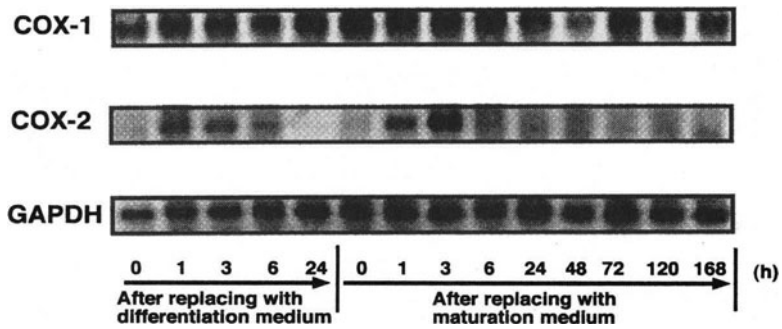

Figure 1. RT-PCR analysis of mRNA levels of COX isoforms in cultured mouse 3T3-L1 adipocytes during the process of adipogenesis.

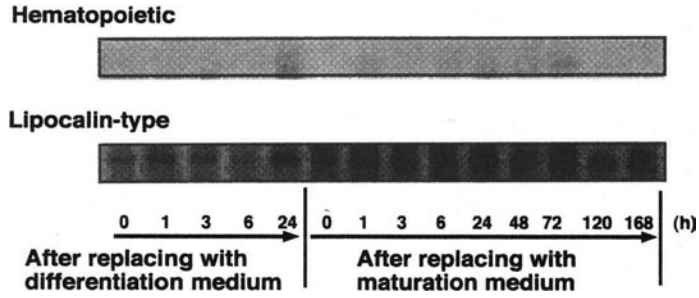

Figure 2. RT-PCR analysis of mRNA levels of PGDS isoforms in cultured mouse 3T3-L1 adipocytes during the process of adipogenesis.

PGD_2 has been shown to be sequentially converted to PGJ_2 and 15d-PGJ_2 in an albumin-independent manner, whereas serum albumin was reported to be involved in the formation of Δ^{12}-PGJ_2 from PGJ_2 [5]. Thus, further studies will be required to determine the levels of this PGJ_2 derivative during the adipogenesis under physiological conditions.

Conclusion

In cultured 3T3-L1 cells, PPARγ mRNA level increased significantly within several hours and was maintained during the maturation phase. The transcription of COX-2 isoform gene was also enhanced transiently during the induction of differentiation or the maturation process, whereas the COX-1 gene was constitutively expressed. Moreover, The expression of L-PGDS

gene was only detectable and the mRNA level increased gradually following the progression of mature adipogenesis. In contrast, there was no expression of H-PGDS. These results suggest that cultured 3T3-L1 cells have capacity to induce the specific isoforms of COX-2 and L-PGDS to form PGD_2 which would be converted to 15d-PGJ_2 serving as an endogenous ligand for PPARγ.

Acknowledgements

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Chapter #32

TRISTETRAPOLIN BINDS TO THE COX-2 mRNA 3' UNTRANSLATED REGION IN CANCER CELLS

Olivier Boutaud, Dan A. Dixon, John A. Oates, and Hitoshi Sawaoka

Introduction

Consistent evidence of several types indicates that the inducible cyclooxygenase (COX-2) can promote the multi-step sequence of events that lead to colon cancer [1,2]. Levels of COX-2 are increased in 85-90% of human colorectal adencarcinoma [3]. It also is expressed in cancers of the stomach [4], esophagus [5], pancreas [6], prostate [7], lung [8], and breast [9]. Whereas it is clear that COX-2 plays an important role in the initiation of colon cancer, the mechanisms that lead to its over-expression have not been fully elucidated. COX-2 expression is regulated at the transcriptional level in response to cytokines [10] and oncogenic signaling pathways [11]. Also, post-transcriptional mechanisms have been shown to play a role in the regulation of COX-2 expression during carcinogenesis [12], and to increase [13] or decrease [14] COX-2 mRNA stability. The 3' UTR of COX-2 mRNA contains 22 copies of a conserved AU-rich sequence element (ARE), the AUUUA pentamer. This pentamer, frequently located in or near a U rich region, has been associated with the regulation of the stability of a number of mRNAs, including those of proto-oncogenes and cytokines. A number of ARE-binding proteins have been identified, including HuR, AUF-1/hnRNPD, TIA-1, and tristetraprolin, that can exert either positive or negative effects on stability, translation and subcellular localization of the mRNA [15-17].

Tristetraprolin (TTP) [18] binds to AREs from mRNA coding for early response genes such as c-fos, interleukin-3, and TNF-α [19-21], and confers instability to the messages. Because the 3'UTR of COX-2 mRNA is homologous to TNFα, the possibility of TTP destabilizing the cyclooxygenase message is appealing.

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HCA-7 is one of the human colorectal adenocarcinoma cell lines that has the highest COX-2 activity. We have demonstrated the presence of two major transcripts of COX-2 in these cells. The larger was found to be the full length mRNA, 4465 nt in length. The smaller is 2577 nt in size and represents a polyadenylation variant that lacks the distal 2578-4465 segment of the 3' untranslated region. The consequences of deletion of the distal 1888 nt of the 3' UTR may be considered in the context that the deleted sequence between bases 2578 and 4465 contains 15 AREs. We found that these two mRNAs are differentially regulated, and that the amount of 4465 nt mRNA relative to the 2577 nt polyadenylation variant is inversely correlated with the expression of TTP, consistent with a destabilization of the 4465 but not the 2577 nt mRNA by TTP. We then demonstrated that TTP from HCA-7 cells binds to the 3125 to 3432 nt region of the COX-2 mRNA.

Results and Discussion

COX-2 transcripts are differentially regulated in HCA-7: One of the characteristics of cancer cells is the loss of contact inhibition. The HCA-7 shares that characteristic and cells continue to grow for several days after reaching confluence. We observed that before confluence, the full length COX-2 transcript was the main species but, after confluence, the ratio of 2577 to 4465 mRNA increased. This change in the ratio resulted from both a decrease in the abundance of 4465 and an increase in the abundance of 2577 nt mRNA. Treatment of HCA-7 with LPS leads to the induction of COX-2 transcription. As observed with confluence, the transcripts are differentially regulated: although 4.5 kb is not significantly increased, 2577 nt variant is strongly induced with the maximal effect at 4h. Importantly, the cyclooxygenase activity was unchanged during the time of the experiment, indicating that both transcripts are translationally competent. This suggests that confluence is associated with the synthesis of a transactivating regulatory protein that signals the degradation of COX-2 mRNA by binding to the 2578-4465 region of the 3' UTR.

TTP is expressed in HCA-7 cells: We demonstrated that TTP is expressed in HCA-7 cells and that its transcription is increased with the confluence stage of the cells. TTP expression is correlated with the decrease of the full length COX-2 transcript. We also found that LPS induced TTP transcription with a maximal effect at 2 hours. After four hours, the 2577 nt COX-2 RNA was increased whereas there was not a corresponding increase in the full length COX-2 RNA. This finding is consistent with the hypothesis that transactivating regulatory proteins such as TTP bind to the distal portion of COX-2 3'UTR conferring instability to the message.

TTP binds to COX-2 3'UTR between nucleotides 3125 and 3432: Because COX-2 full length RNA was down-regulated in a reciprocal way with TTP expression, we assessed whether TTP expressed in HCA-7 cells

50% confluent and 3 days overconfluent was binding to the COX-2 3'UTR. Protein extracts prepared from HCA-7 at these two conditions of confluence were incubated with a series of 8 radioactive sense RNA probes that span the 3'UTR distal to nucleotide 1941. After UV crosslinking between RNA and protein, TTP was immunoprecipitated and separated on SDS-PAGE [15]. The results demonstrated unambiguously that TTP binds to COX-2 3'UTR between nucleotides 3125 and 3432 and that more TTP was bound when the experiment was performed with the cytosolic extract obtained from overconfluent cells.

Conclusion

TTP binds the 3'UTR of COX-2 in a region that is missing from the 2577 nt polyadenylation variant found in the human colon cancer cell line, HCA-7. These findings provide the basis for a hypothesis that TTP binds to and destabilizes the full length COX-2 mRNA thereby restraining its expression, whereas the absence of TTP binding site in the short transcript permits it to escape the destabilizing effect of TTP, thus enhancing its expression in this cancer cell line.

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Chapter #33

PLATELET ACTIVATING FACTOR RECEPTORS

A Marilise Marrache, Fernand Gobeil Jr, Sylvie G Bernier, Jana Stankova, Marek Rola-Pleszczynski, Sanaa Choufani, Ghassan Bkaily, Annie Bourdeau, Martin G Sirois, Alejandro Vazquez-Tello, L Fan, Jean-Sebastien Joyal, Janos G Filep, Daya R Varma, Alfredo Ribeiro-da-Silva, and Sylvain Chemtob

Introduction

Platelet activating factor (PAF) is a potent pro-inflammatory lipid mediator. Its effects are mediated through cell surface G protein-coupled receptors (GPCRs) that are distributed on numerous cells notably on endothelium [1]. An intracrine mode of action for PAF is proposed based on evidence for intracellular PAF binding sites [2] and retention of newly generated PAF within its producing cells [3]. Separate functions for the intracellular and cell surface receptors are suggested using agents which putatively distinguish them [4, 5]; immediate effects are mediated by cell surface receptors whereas regulation of gene expression are dependent upon intracellular receptors consistent with presence of signaling effectors in nuclei including G proteins, Ca^{++}, kinases and NF-κB [6,7,8]; but intracellular PAF receptors; especially on nuclei, which may explain their presumed involvement in gene regulation [5] has never been explicitly demonstrated. We speculated that PAF receptors exist at the cell nucleus where they induce major pro-inflammatory gene cyclooxygenase-2 (COX-2) expression.

Detection of Functional Nuclear PAF Receptors and Signaling Cascade

Among the nuclear GPCRs, functionality for nuclear prostaglandin E_2 [6,7] and angiotensin II receptors [9] have been documented; however the latter peptide is not produced locally but translocates to the nucleus [10]. PAF on the other hand is produced locally [8] due to the presence of nuclear

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cytosolic phospholipase A_2 ($cPLA_2$) and acetyltransferase enzymes which play essential roles in the biosynthesis of PAF.

Similar binding kinetics [8] resulted when comparing plasma membrane and nuclear PAF receptors and migration profile of both receptors in a Western blot was similar implying that the same intact protein is present at the nuclear level [8]. The distribution of intracellular PAF receptors was visualized by immunofluorescent and immunogold labeling [8]. PAF receptors on plasma membrane and nuclear envelope were identified on brain endothelial cells from piglets and immunostaining possibly associated with euchromatin was also observed. PAF receptor distribution at the nuclear matrix may be related to gene regulation as decondensed chromatin represent active sites of transcription.

Mechanisms by which PAF can elicit gene expression are unknown but recently uncovered mechanisms of GPCR-mediated gene induction implicate β-arrestin-c-Src interaction leading to downstream activation of mitogen-activated protein kinases (MAPK), and metalloprotease-dependent transactivation of receptor tyrosine kinases involving *de novo* release of their ligand [11]. Alternatively, activation of nuclear PAF receptors could elicit gene expression [6,7]. Indeed, nuclear PAF receptors displayed functional relevance as they modulated COX-2 gene transcription (Figure 1). C-PAF-induced COX-2 expression was MAPK- and NF-κB-dependent [8], though it may seem surprising that nuclear stimulation can lead to NF-κB activation. NF-κB is complexed to its inhibitory unit IκB in cytoplasm and during activation, NF-κB is released to enter the nucleus. But like NF-κB, free IκB can also enter the nucleus [12] and re-associate with NF-κB. Shuttling of these subunits between the nucleus and cytoplasm results in activatable NF-κB complexes within the nucleus [12].

Signaling mechanisms seem to differ for the cell surface and nuclear PAF receptors. The former was associated with G_q protein and the latter was associated with G_i protein [8] implying distinct functions for PAF receptors depending on its localization [4,5]. Nuclear PAF receptors mobilized nuclear Ca^{++} pools [8] and presumably via the βγ-subunits of G_i protein, an independent event from inositol phosphate production as seen previously [7]. Our findings also indicate that inhibition of PAF-generating enzyme $cPLA_2$ in whole cells diminished C-PAF-evoked COX-2 expression [8]; this infers a role for auto-stimulated PAF generation in gene induction.

Potential Mechanims for Nuclear Targeting of PAF Receptors

The mechanism for nuclear localization of PAF receptors may involve putative nuclear localization signal ($KKFRKH^{298-303}$) found at the C-terminal tail of PAF receptors [13]. Endoplasmic reticulum retention sequences ($NSLK^{338-341}$) [14] may also participate in nuclear localization as to their implication in vesicular trafficking and translocation of receptors to the nucleus as suggested for angiotensin II [10]. However, deletion of these C-terminal segments did not alter PAF receptor localization. Also, interference

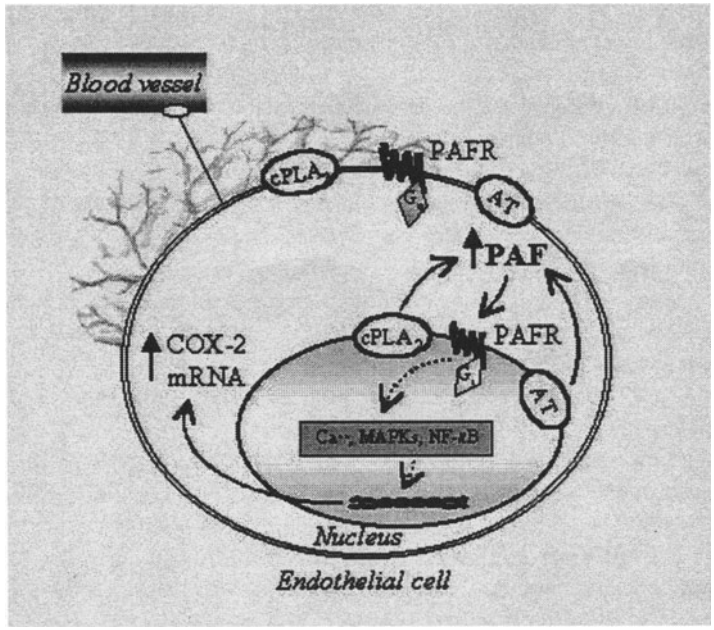

Figure 1. Schematic model of COX-2 induction by nuclear PAF receptors. AT and PAFR refer to acetyltransferase enzyme and PAF receptor respectively.

with agonist-induced receptor internalization did not affect C-PAF-induced COX-2 expression [8]. Hence, mechanisms responsible for nuclear localization of PAF receptors still remain to be elucidated.

The present data uncover the first clear evidence for nuclear functional PAF receptors. We not only described the nucleus as a putative organelle for PAF generation and site expressing its receptor but also provided a novel GPCR mechanism for MAPK activation and pro-inflammatory gene induction.

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Chapter #34

SPLICING MUTATION OF THE PROSTACYCLIN SYNTHASE GENE IN A FAMILY ASSOCIATED WITH HYPERTENSION

Tomohiro Nakayama, Masayoshi Soma, Yoshiyasu Watanabe, Buaijiaer Hasimu, Katsuo Kanmatsuse, Shinichiro Kokubun, Jason D. Morrow, and John A. Oates

Introduction

Prostacyclin inhibits platelet aggregation, and vasoconstriction [1]. Prostacyclin synthase (PGIS, CYP 8A1, EC 5.3.99.4), a catalyst of prostacyclin formation from prostaglandin H2, is widely distributed and predominantly found in vascular endothelial and smooth muscle cells [2]. The PGIS gene is localized to 20q13.11-13 and thought to be a candidate gene for cardiovascular disease. We reported the organization of this gene [3]. Furthermore, we identified a family with a nonsense mutation in exon 2 of the prostacyclin synthase gene, and this family displays a history of hypertension and cerebral infarction [4]. These findings suggested that abnormality of the prostacyclin synthase gene may lead to altered vasodilation and platelet aggregation.

In the present study, we searched for new mutations in the PGIS gene and assessed the relationship with essential hypertension (EH).

Materials and Methods

For polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of each exon and both acceptor and donor site of intron, oligonucleotide primers were designed from the genomic sequence of the prostacyclin synthase gene (Genbank/EMBL/DDBJ data bank accession numbers D84115-D84124). DNA was extracted from whole blood according to standard procedures. PCR-SSCP was used as described [4].

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Results and Discussion

Using SSCP analysis, we discovered a T to C transition at +2 the position in the splicing donor site of intron 9 in patients with EH (Figure 1a). In vitro expression analysis of an allelic minigene consisting of exons 8-10 showed that this nucleotide transition causes skipping of exon 9 (Figure 1a, b). This exon skipping alters the translational reading frame of exon 10 and creates a premature stop codon (TGA) 64 bp downstream from the boundary of exons 8-10. Therefore this transition is a splicing site mutation, which yields a truncated protein read with mismatch-codons from the codon 403.

This mutation was found in only one EH subject following the screening of 200 EH patients and 200 normotensive (NT) controls, and was heterozygous. As this patient (subject 4) was already deceased by the time we discovered the mutation, we analyzed the mutation in a number of other family members (Figure 2). Two (subject 1 and 2) of the three siblings (subject 1, 2, and 3) tested displayed the mutation. All subjects displaying this mutation had hypertension. Interestingly, serum creatinine levels in subjects with mutation were elevated (subjects 1, 2, 4 were 1.4, 1.6, 1.3) compared with those in subjects without mutation (subjects 3 and 5 were 1.0 and 0.7). These suggested that the mutation of PGIS causes hypertension complicated with renal dysfunction.

Urinary samples could only be taken from subjects 1, 2, and 5 (a daughter of subject 4) because subject 3 did not agree to sample collection. The data are listed in Figure 2. All results were corrected with urinary creatinine excretions. Prostacyclin metabolite 2,3-dinor-6-keto-PGF1 alpha in both samples 1 and 2 was reduced below the lower limit of the normal range (mean+/-SD, 0.179+/−0.015). Sample 1 was reduced below normal with a P value of 0.027 and sample 2 was reduced below the normal range with a P value of 0.037. By contrast, urinary 2,3-dinor-6-keto-PGF1 alpha excretion in sample 5 was slightly elevated (P=0.005). For the assay of 11-dehydro-thromboxane B2 (11-dehydro-TXB2), the upper limit of normal is 0.644 ng/mg creatinine).

PGIS belongs to a CYP8 family in the P450 superfamily. A three-dimensional model based on the crystal structure of P450 $_{BM-3}$ was constructed. The backbone structure of the PGIS model matched well with that of $P450_{BM-3}$ [5]. We could predict the PGIS model based on the $P450_{BM-3}$ crystal structure. It has been described that some residues in the C-terminal are involved in substrate access and ligand for the heme iron. The method of site-directed mutagenesis showed that mutation of Cys441 to Ser had resulted in a diminished enzyme activity (13%) [6]. Cys441 is in the heme binding region. The splice site mutation that we discovered produces a truncated protein with a deletion in the heme binding region. Thus, the PGIS activity of subjects having the mutation is decreased. It is thought that this mechanism may involve in the pathophysiology of their hypertension.

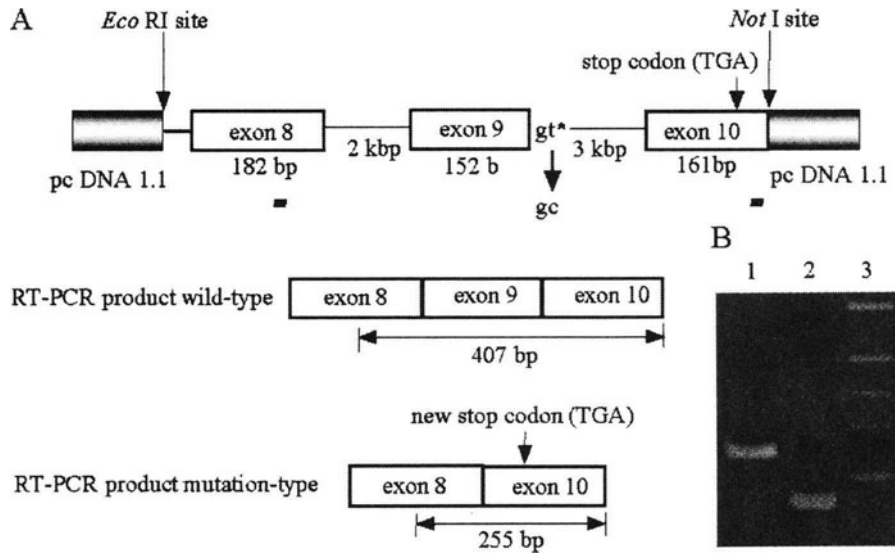
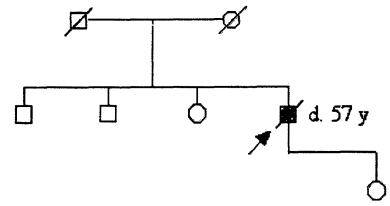


Figure 1. A: Allelic minigene construct consisting of exon 8-10 and RT-PCR products. B: Lane 1 is the wild-type product. Lane 2 is the mutation type. Lane 3 is a molecular marker



Subject number	1	2	3	4	5
Age (y.o.)	76	73	72		27
Diagnosis	HT	HT	NT	HT	NT
Mutation	+	+	-	+	-
Urinary 2,3-dinor 6-Keto F1α (ng/mg creatinine)	0.053*	0.077*			0.324**
Urinary 11 -dehydro TXB2 (ng/mg creatinine)	0.902**	0.482			0.247

□	Male	HT	Hypertension
○	Female	NT	Normotension
■	Patient firstly detected	*	decreased below normal ($p<0.05$)
↗⊠	Dead	**	increased above normal ($p<0.05$)

Figure 2. Genealogy of the patient with splicing site mutation of PGIS 11-dehydro-TXB2 in sample 1 was elevated above the upper limit of the normal range, while that in samples 2 and 5 was within normal limits.

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Chapter #35

DNA METHYLATION REGULATES 5-LIPOXYGENASE PROMOTER ACTIVITY

Johannes Uhl, Niko Klan, Matthias Rose, Karl-Dieter Entian, Oliver Werz and Dieter Steinhilber

5-Lipoxygenase (5-LO), the key enzyme in leukotriene biosynthesis, is expressed in a tissue- and cell-differentiation specific manner. The 5-LO core promoter required for basal promoter activity has an unique G + C-rich sequence which contains five tandem Sp1 consensus sequences [1]. Previously, we could show that the 5-lipoxygenase promoter is regulated by DNA methylation [2]. Treatment of the 5-LO negative cell lines U937 and HL-60TB with the demethylating agent 5-aza-2'-deoxycytidine (AdC) upregulated 5-LO activity and the expression of 5-LO primary transcripts and mature mRNA indicating that AdC stimulates 5-LO expression at the level of gene transcription. Analysis of the methylation status by methylation-sensitive sequencing of the 5-LO promoter revealed that the core promoter region was methylated in U937 and HL-60TB cells, whereas it was unmethylated in the 5-LO positive wild-type HL-60 cell line. In transient reporter gene assays, there was a dramatic reduction of 5-LO promoter activity when the reporter gene constructs were methylated in-vitro by Sss I methylase before transfection, indicating that 5-LO promoter activity is strongly inhibited when the promoter is methylated.

Usually, the methylation pattern of the DNA is inherited during cell division. Therefore, it was of interest to study the reversibility of the AdC effect on 5-LO expression. Interestingly, AdC-induced DNA demethylation was reversible in U937 cells, whereas no remethylation seems to occur in HL-60 cells (figure 1).

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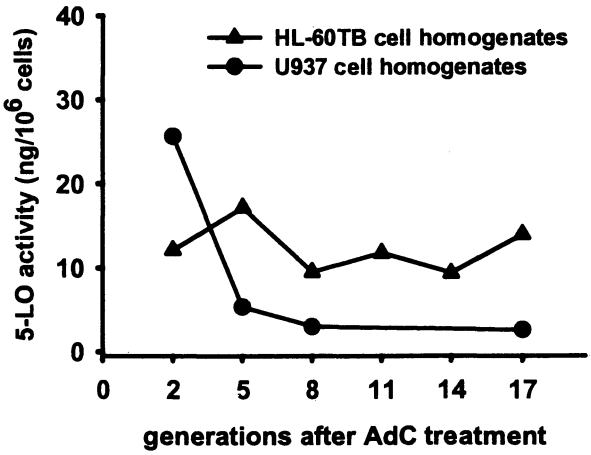

Figure 1. Reversibility of DNA demethylation by AdC. HL-60 cells were treated with AdC as described and cultured for the indicated generations in the absence of AdC before differentiation was induced by TGFβ and $1,25(OH)_2D_3$ [2]].

Thus, 5-LO activity of differentiated U937 cells is strongly reduced, when the cells are cultured in the absence of the demethylating agent AdC for five or more generations. In contrast, 5-LO activity of HL-60TB cells was constant even after cultivation for up to 17 generations in the absence of AdC.

Regulation of cellular 5-LO activity involves a second protein termed FLAP. Previously, we found that FLAP mRNA expression is high in both the 5-LO negative U937 and the 5-LO positive HL-60 cell line [3]]. In contrast to 5-LO, no significant effect of AdC was observed on FLAP mRNA expression in various cell lines (figure 2) indicating that the FLAP promoter is either demethylated in these cell lines or that DNA methylation does not affect FLAP promoter activity. Furthermore, AdC did not induce FLAP expression in the FLAP negative HeLa cell line, so that there is no evidence at present that DNA methylation is involved in the regulation of FLAP promoter activity.

The methylation status of DNA is regulated by DNA methyl transferases (DNMTs) and demethylases. Recently, a demethylase activity that depends on the promoter context was reported for MBD2 [5]]. However it has to be

mentioned that this demethylase activity could not be confirmed by other groups so that the significance of this finding is not clear at the moment. As can be seen from fig.3, there is no clear correlation between the methylation status of the 5-LO promoter and MBD2b expression in the cells. Thus, there is comparable MBD2b expression in HL-60 and HL-60TB cells and low expression of MBD2b in Mono Mac 6 and U937 cells. Recently, it has been shown that the DNA methyltransferases DNMT1 and DNMT3 participate in establishing and maintaining genomic methylation patterns [6]. Interestingly, we did not find significant differences in DNMT1 and 3a expression in the cell lines investigated here (data not shown) which suggests that methylation of the 5-LO promoter is subject to a more complex regulation than the simple overexpression of genes involved in DNA methylation and demethylation.

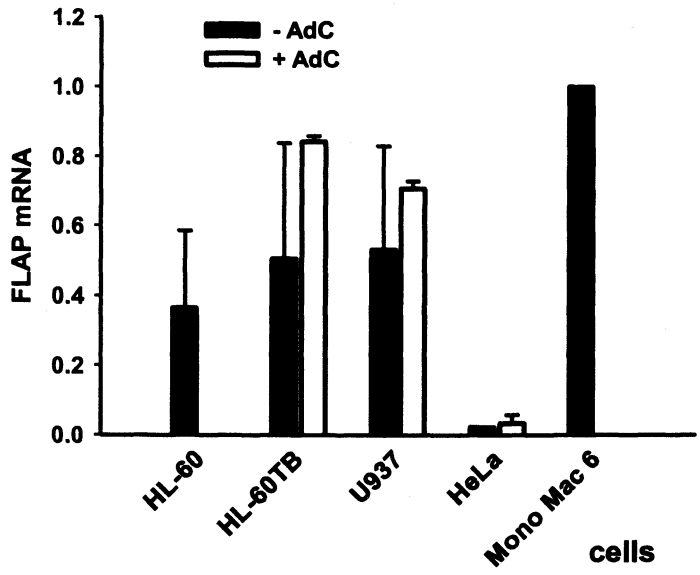

Figure 2. Effects of AdC on FLAP mRNA expression in different cell types. Cells were treated with AdC [2]] and analyzed for FLAP mRNA expression by RT-PCR as described [4]]. 24 PCR cycles were applied for β-actin and FLAP.

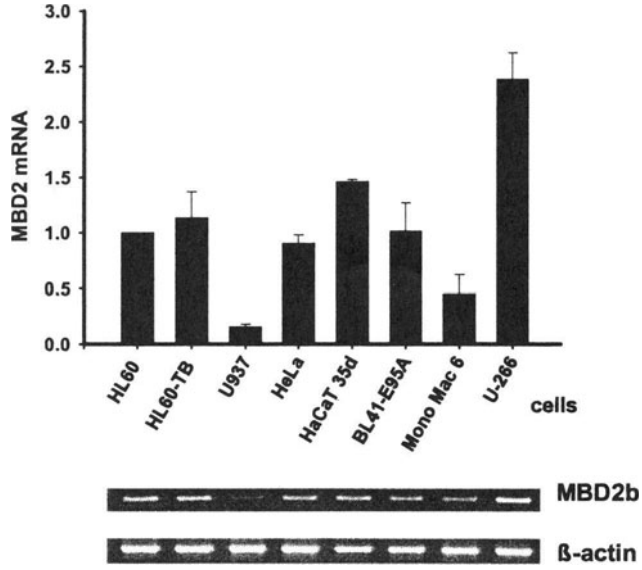

Figure 3. MBD2b mRNA expression in various cell lines. Total RNA was extracted from the indicated cell lines and MBD2b expression was determined by RT-PCR (25 cycles for β-actin and MBD2b, respectively).

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Chapter #36

ISOMERIZATION AND NITRO-OXIDATION OF ARACHIDONIC ACID BY NO_2

Michael Balazy and Jordi López-Fernández

Free radical-mediated oxidation of arachidonic acid leads to formation of iso-eicosanoids, such as isoprostaglandins, isolevuglandins, hydroperoxides, and other products that could function as markers and/or mediators of oxidative stress initiated by a hydroxyl radical. Much less has been known about modifications of arachidonic acid by reactive nitrogen radicals such as NO_2, a major product of NO oxidation *in vivo* and a component of polluted air and smoke. We studied the reaction of NO_2 with arachidonic acid to characterize lipid products and their biological activity and usefulness as markers of NO_2 interaction with biological membranes.

Cis-Trans Isomerization of Arachidonic Acid

The reaction of NO_2 with AA proceeds rapidly in organic solvents and biological buffers and produces a unique pattern of products. One major product was less polar than AA and had a molecular mass of 304, thus it was found to be a mixture of arachidonic acid isomers. Analysis of dimethyloxazoline derivative by GC/MS indicated that the double bond position was the same as in AA [1]. Further conformation of the structures of these products was obtained by comparison of mass spectrometric and chromatographic properties with standards of *trans*-AA isomers synthesized via stereospecific deoxidation of epoxy-AA isomers [2]. The experiments confirmed that the major NO_2-derived product was a mixture of four *trans* isomers of arachidonic acids (*trans*-AA) having one *trans* and three *cis* double bonds.

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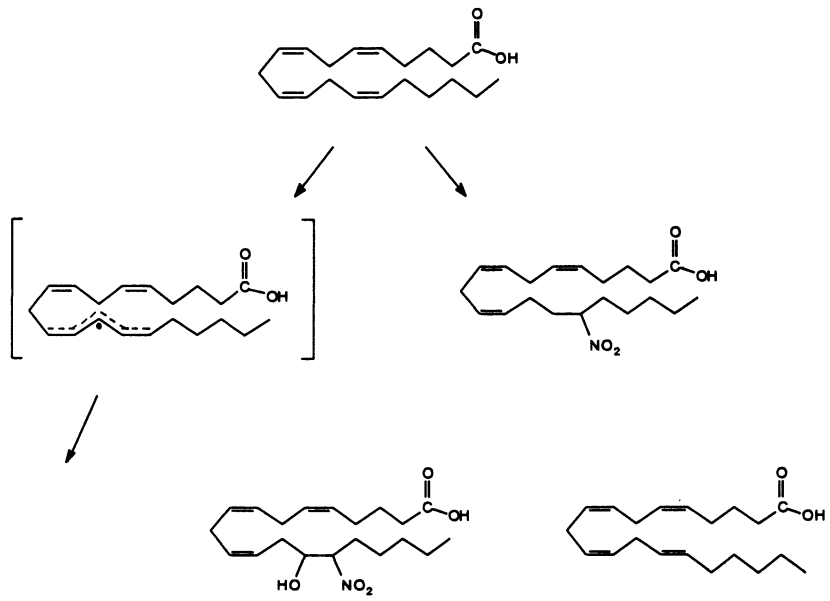

Figure 1. Two major mechanisms are involved in the reaction of NO_2 with arachidonic acid exemplified here with reactions at the C14-C15 double bond. A proton abstraction (*arrow a*) followed by addition of oxygen results in formation of nitrohydroxyeicosatrienoic acids (NO_2AAOH) and other oxidation products whereas a reversible addition of NO_2 causes formation of *trans* arachidonic acid isomers (*trans*-AA) probably via formation of an unstable nitroarachidonyl radical [3] (*arrow b*).

We studied occurrence of the AA/NO_2-derived products *in vivo* using a GC/MS isotopic dilution assay. *Trans*-AA was detected in human (50.3±10 ng/ml, n=8) and rat plasma (78.6±19 ng/ml, n=6). Urinary levels of *trans*-AA were 0.12±0.05 ng/ml in humans and 14.8±5 ng/ml in rats. This suggested that isomerization of AA occurs *in vivo*, possibly via endogenous NO_2 as no other free radical was able to isomerize AA. We hypothesized that in conditions such as inflammation, in which NO synthase is induced there will be more NO_2 available for AA isomerization. The concentration of *trans*-AA in plasma of the rat model of lipopolysaccharide-induced endotoxemia (LPS serotype 0111:B4, infused into the jugular vein at 10 mg/kg/min) increased from 78.6±19 ng/ml to 155.5±33 ng/ml within 5 hrs. LPS treatment resulted in a more than 9-fold increase of *trans*-AA levels in rat cardiac muscle phospholipids. Although biological effects of NO_2 have been previously attributed to lipid peroxidation, our studies also suggest that

isomerization of AA and possibly of other polyunsaturated fatty acids could be an additional mechanism by which NO_2 modifies membrane lipids. NO_2 has been known to induce changes in cell membrane permeability, fluidity and asymmetry [4]. These effects could be mediated by enhanced formation of *trans* fatty acids within the cellular membrane. The *trans*-AA could function as novel lipid markers and mediators of inflammation as they are not readily metabolized by pathways involved in AA metabolism [5]. Preliminary data also suggest that these *trans* AA isomers could function as endogenous inhibitors of cyclooxygenases and lipoxygenases, one implication of which is a stereospecific apoptosis of HL-60 cells induced by 5-*trans*-AA via inhibition of 5-lipoxygenase.

Nitro-Oxidation of Arachidonic Acid

Liquid chromatography/mass spectrometric analysis also revealed novel lipids among the AA/NO_2 reaction products that contained a carbon-nitrogen bond. One group of lipids showed strong negative ions at *m/z* 366 and a fragmentation pattern consistent with the structure of vicinal nitrohydroxyeicosateraenoic acids (NO_2AAOH). All 8 possible isomers of NO_2AAOH have been identified [6]. Two other groups of lipids consisted of nitroeicosatetraenoic acids (NO_2AA) and oxime-arachidonic acids (NOHAA). The AA/NO_2 reaction mixture revealed a potent vasorelaxing activity (69±7% relaxation of bovine coronary artery at 1 μM), which was inhibited by indomethacin and ODQ, an inhibitor of guanylate cyclase. NO_2AAOH was a major vasorelaxing component of the crude mixture that activated guanylate cyclase. The mechanism of relaxation involves a release of NO from NO2AAOH. We further studied the occurrence of the AA/NO_2-derived products *in vivo*. NO_2AAOH was also detected in cardiac tissue (6.8±2.6 ng/g, n=4). Our findings suggest that nitration and isomerization of fatty acids could be additional mechanisms by which NO_2 modifies membrane lipids. These lipids may have actions that modulate multiple biological signaling systems and the potential to function as specific biomarkers of processes involving the interaction of NO_2 with biological membranes. Nitration of lipids by NO-derived nitrative oxidants is an emerging area of research. Recent studies have described nitrolinoleic acid [7] as activator of adenylyl cyclase [8] and its occurrence in human plasma [9]. The nitration of fatty acids is likely to occur in conditions known to induce NO synthase such as inflammation and in individuals exposed to high levels of NO_2 such as chain cigarette smokers.

Acknowledgements

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Chapter #37

PROSTAGLANDIN $F_{2\alpha}$ PROTECTED CULTURED MADIN-DARBY CANINE KIDNEY CELLS FROM THE DEVELOPMENT OF APOPTOSIS INDUCED BY 12-*O*-TETRADECANOYL PHORBOL-β-ACETATE AND STIMULATED SYNERGISTICALLY WITH NORDIHYDROGUAIARETIC ACID

Kohji Nishimura, Hirohumi Tsumagari, Asami Morioka, Shan Lu, Mitsuo Jisaka, Tsutomu Nagaya, and Kazushige Yokota

Introduction

The arachidonate cascade generates a series of lipid mediators to regulate various biological events through cyclooxygenase (COX) and lipoxygenase (LOX) pathways [3,5]. In addition, both COX and LOX metabolites were reported to play a dual role in regulating cell survival and death depending on certain types of cells or signalling molecules [1,4]. However, the cellular mechanism by which these eicosanoids mediate the induction or suppression of apoptosis remains still unclear.

Our laboratory has previously reported that the simultaneous addition of 12-*O*-tetradecanoylphorbol β-acetate (TPA) with calcium ionophore A23187 synergistically induced the delayed production of prostaglandin (PG) E_2 and $PGF_{2\alpha}$ in cultured Madin-Darby canine kidney (MDCK) cells [6-8]. More recently, we observed that nordihydroguaiaretic acid (NDGA), one of nonspecific LOX inhibitors, amplified the apoptosis caused by TPA [2].

The present study provides novel evidence that the synergistic stimulation of apoptosis with TPA and NDGA is mediated through the generation of reactive oxygen species (ROS) and a caspase-dependent pathway, but independent of the action of LOX metabolites. We describe the protective role of endogenous and exogenous $PGF_{2\alpha}$ in attenuating apoptosis induced by TPA and NDGA.

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Experimental Procedures

For the induction of apoptosis, MDCK cells were cultured and treated with various reagents, and analysed as described before (Nishimura et al., 2002). Caspase activity was also evaluated according to the modified method of CapasAce™ Assay System (Promega).

Results and Discussion

NDGA is widely used as a nonspecific LOX inhibitor. Simultaneous addition of 100 ng/ml TPA with 50 μM NDGA caused marked nuclear condensation. As shown in Figure 1, exposure of MDCK cells to a mixture of TPA and NDGA caused the synergistic activation of caspase by 10-fold after 8 h in the presence of both TPA and NDGA when compared with TPA alone. z-VAD-fmk, a pan-caspase inhibitor, reversed the effect of TPA and NDGA to induce the DNA fragmentation as well as to activate caspase activity.

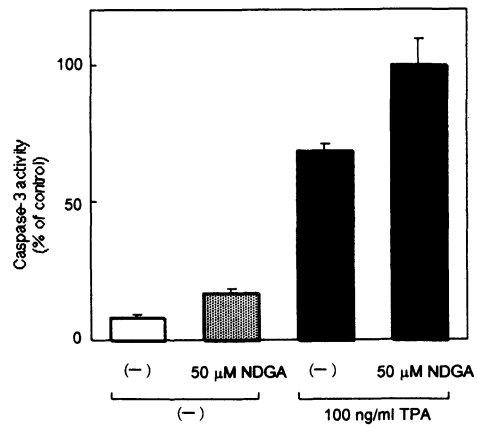

Figure 1. Activation of caspase-3 activity during apoptosis induced by TPA and NDGA. MDCK cells (5 x 10^4 cells/ml) were grown to 80% confluence in Dulbecco's modified Eagle medium (DMEM)-HEPES with 5% fetal calf serum, and then the medium was replaced with fresh DMEM-HEPES containing 2% newborn serum. The cells were pretreated for 1 h with 50 μM NDGA, followed by the addition of 100 ng/ml of TPA for 8 h, and subjected to the analysis of caspase-3 activity. Data represent the mean ± S. D. (n=3).

Effects of NDGA raised the possibility of the involvement of LOX metabolites. However, the LOX pathway was not involved in this apoptosis because the transcripts of LOXs were not detectable by the analysis of reverse transcriptase-polymerase chain reaction. This finding is in sharp contrast with other report that LOX metabolites had roles as survival factors in several cancer cells [4]. The earlier generation of ROS was detected

during the apoptosis induced by TPA and NDGA within 6 h, and pretreatment with 2-mercaptoethanol attenuated the apoptosis [2]. These suggested that the synergistic stimulation of the TPA-induced apoptosis with NDGA appeared to be partly caused by the activation of the ROS-producing enzymes by TPA and NDGA.

Exposure of MDCK cells to A23187 along with TPA for 8 h lowered caspase-3 activity, indicating that endogenous prostanoids would contribute to the protective effect on the TPA-induced apoptosis in an autocrine fashion. Cultured MDCK cells have been shown to synthesize PGE_2 and $PGF_{2\alpha}$ as predominant COX metabolites [6-8]. At a concentration of 50 μM, $PGF_{2\alpha}$ was more effective than PGE_2 to block the progression of chromatin condensation. The addition of $PGF_{2\alpha}$ even at a lower concentration of 0.1 μM was found to be effective to reverse the activated caspase-3 activity (Figure 2). These findings provide evidence that COX metabolites function as survival factors in MDCK cells to attenuate apoptosis stimulated with TPA and NDGA.

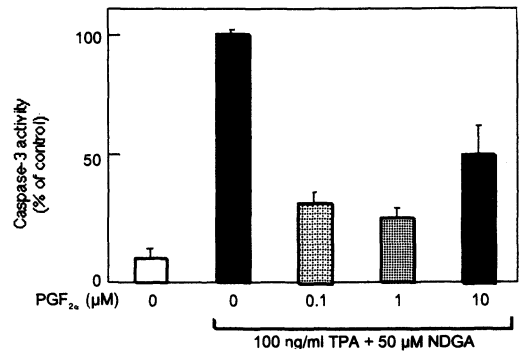

Figure 2. Effect of exogenous prostanoids on apoptosis induced by a mixture of TPA and NDGA in MDCK cells. MDCK cells were cultured as in Figure 1. MDCK cells were pretreated for 1 h with different concentrations of $PGF_{2\alpha}$ before cells were exposed to a mixture of 100 ng/ml of TPA and 50 μM NDGA for 7 h, and then caspase-3 activity was determined. Data represent the mean ± S. D. (n=4).

Conclusion

We found that synergistic stimulation by NDGA of TPA-induced apoptosis involved the production of ROS, and activation of caspase-3, but not LOX pathway. $PGF_{2\alpha}$ potentially serves as a survival factor for apoptosis in MDCK cells induced by TPA and stimulated synergistically with NDGA through an autocrine control mechanism.

Acknowledgements

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Chapter #38

LDL RECEPTOR-RELATED PROTEIN PLAYS AN ESSENTIAL ROLE IN 12/15-LIPOXYGENASE-MEDIATED LDL OXIDATION BY MACROPHAGES

Wanpeng Xu, Yoshitaka Takahashi, Tadao Iwasaki, Hiroaki Hattori, and Tanihiro Yoshimoto

Lipoxygenase incorporates a molecular oxygen regiospecifically and stereospecifically into unsaturated fatty acids. There are four classes of enzymes in mammalian tissues; 5-, 8-, 12-, and 15-lipoxygenases, named by the number indicating oxygenation site of arachidonic acid as a substrate [1,2]. Among the enzymes, 12-lipoxygenases are widely distributed in various tissues and species, and there are three isoforms: platelet, leukocyte and epidermis types. Since the leukocyte 12-lipoxygenase and 15-lipoxygenase-1 are highly related in their primary structures and enzymological properties, these enzymes are collectively called as 12/15-lipoxygenase [3].

It has recently been shown that the 12/15-lipoxygenase plays important pathophysiological roles, especially in atherosclerosis. In the initial stage of this disease, oxidized LDL is incorporated into macrophages via scavenger receptors, and the unlimited accumulation of lipids induces foam cell formation which plays a key role in the development of atherosclerosis [4,5]. However, the detailed mechanism of LDL oxidation *in vivo* is still a subject of investigation. A number of evidences support the role of 12/15-lipoxygenase in LDL oxidation. Namely, purified 12/15-lipoxygenase can oxidize LDL, and the enzyme is present in macrophages co-localized with oxidized LDL in fatty streak lesions. Moreover, the stereospecific oxidation product of cholesteryl linoleate by 12/15-lipoxygenase is found in atherosclerotic lesions. Recent studies on 12/15-lipoxygenase knockout mice and transgenic mice further support the involvement of this enzyme in atherosclerosis [6,7].

In order to investigate precise mechanisms of the 12/15-lipoxygenase-mediated LDL oxidation, we established a mouse macrophage-like J774A1

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cell line overexpressing porcine leukocyte 12-lipoxygenase [8]. The 12/15-lipoxygenase-expressing cells were incubated with native LDL, and the lipids extracted from the medium were subjected to HPLC analysis after alkaline hydrolysis to analyze fatty acid derivatives in the modified LDL. We found the 12/15-lipoxygenase-expressing cells produced larger amount of 13S-hydroxyoctadecadienoic acid (Z, E) than mock-transfected cells. The presence of regio- and stereospecifically oxygenated product of cholesteryl linoleate in the LDL indicates that the 12/15-lipoxygenase is responsible for cell-mediated LDL oxidation. From the fact that auto-oxidized products were also formed in substantial amounts, we assume that the 12/15-lipoxygenase initiates the oxidation reaction followed by the non-enzymatic oxidation to produce oxidized LDL.

The question arises as to how LDL in the extracellular medium can be oxidized by the intracellular 12/15-lipoxygenase. Leakage or secretion of the 12/15-lipoxygenase had been ruled out, because no enzyme activity was detected in the culture medium. We postulated that a certain membrane receptor that binds native LDL is involved in this oxidation process. We found that the cell-mediated LDL oxidation was altered by different preincubation conditions. Namely, LDL oxidation level was reduced by 39% and 19% by the preincubation with LDL and cholesterol, respectively, as determined by TBARS generation. On the other hand, an approximately two-fold increase was observed after preincubation with lipoprotein deficient serum. No apparent change was observed in LDL oxidation level by mock-transfected cells preincubated in these conditions. This result suggests the potential LDL-binding receptor involved in LDL oxidation could be down- or up-regulated by these different culture conditions. Since expression level of the LDL receptor is shown to be altered by these incubation conditions, we assume that the LDL receptor or similar receptors would be involved in the cell-mediated LDL oxidation. In macrophage-like J774A.1 cells, three receptors are known to bind native LDL: the LDL receptor, LDL receptor-related protein (LRP), and scavenger receptor BI (SR-BI). We examined the mRNA level of these receptors under different preincubation conditions by RT-PCR. The LDL receptor and LRP were down-regulated by cholesterol and LDL, and were up-regulated by lipoprotein deficient serum. On the other hand, mRNA level of SR-BI did not significantly change. These results suggest that the LDL receptor and/or LRP are involved in the LDL oxidation by the 12/15-lipoxygenase.

LRP was cloned from human liver by Dr. Herz's group [9], and was demonstrated to bind LDL as well as a variety of ligands including VLDL, remnants, α_2-macroglobulin. Heparin binding protein 44 (HBP44, a mouse homologue of LRP receptor-associated protein) is a universal antagonist of LDL receptor family including LRP and the LDL receptor. The HBP-44 dose-dependently inhibited LDL oxidation by the 12/15-lipoxygenase-expressing cells, indicating that the LDL receptor and/or LRP are responsible for LDL oxidation by the 12/15-lipoxygenase-expressing cells. In order to determine which receptor is involved in the LDL oxidation, we

examined effects of the antibodies against the LDL receptor and LRP on mouse peritoneal macrophages and the 12/15-lipoxygenase-expressing J774A1 cells. The anti-LRP antibody inhibited the LDL oxidation (Fig.1), but the anti-LDL receptor antibody was not effective. Next, we synthesized 20-mer antisense oligodeoxyribonucleotides complement with the each receptor. The mRNA level of each receptor was markedly decreased by respective antisense oligodeoxyribonucleotides preincubation, but was not changed by sense oligodeoxyribonucleotides. These cells were incubated with LDL, and the LDL oxidation level was determined. Suppression of LRP expression decreased LDL oxidation, while the suppression of the LDL receptor did not cause significant changes. These results are in good agreement with the reports showing that peritoneal macrophage prepared from LDL receptor knockout mice oxidized LDL at the same extent as the wild-type mice [10]. The results taken together indicate that LRP is essentially involved in 12/15-lipoxygenase-mediated LDL oxidation by macrophages.

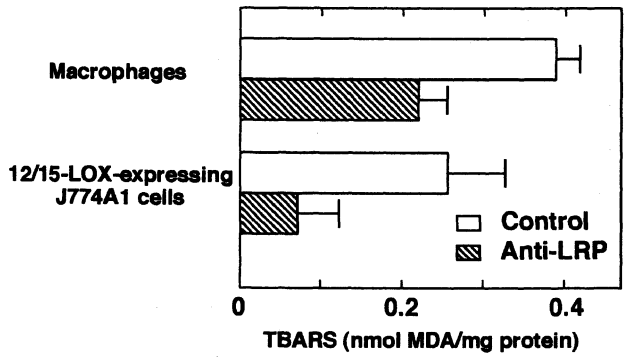

Figure 1. Effects of anti-LRP antibody on LDL oxidation.

The next question is why the LRP, but not LDL receptor, is the key receptor in the LDL oxidation process, although both of which can bind to LDL. Other than well-known receptor-mediated endocytosis, there is another pathway called "selective uptake". In this mechanism cholesteryl ester in the lipoprotein is selectively transferred to the cell membrane without uptake or degradation of the lipoprotein particle. This mechanism was mainly studied in the uptake of cholesteryl ester of HDL mediated by SR-BI. A recent report indicated that LRP also mediated selective uptake of cholesteryl ester in LDL [11]. In order to demonstrate that the cholesteryl ester in LDL is selectively transferred to the plasma membrane of 12/15-lipoxygenase-expressing cells, a fluorescent dye, dioctadecylindocarbocyanine (DiI) was employed. It is known that SR-BI-positive cells incubated with DiI-labeled HDL produce a diffuse staining pattern over the entire cell surface, because the DiI-labeled particles still stay on the cell surface without endocytosis. On the other hand, when the DiI-labeled LDL was incubated with wild-type

Chinese hamster ovary cells which express the LDL receptor, the cells produced a punctate staining pattern. The result is known to indicate endocytosis of the fluorescent DiI-labeled LDL particles which are transferred to endosomes and lysosomes and degraded [12]. When we applied DiI-LDL to mouse resident macrophages, which only express LRP, they exhibited a diffuse staining pattern indicating "selective uptake". Furthermore, the 12/15-lipoxygenase-expressing J774A1 cells with the DiI-LDL, in the presence of anti-LDL receptor antibody, produced same staining pattern. These results strongly suggest that cholesteryl ester in LDL is selectively transferred to the plasma membrane via LRP without endocytosis. Further investigations are necessary to elucidate the detailed molecular mechanism on LRP-mediated LDL oxidation by intracellular 12/15-lipoxygenase expressed in macrophages.

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Chapter #39

EFFECTS OF IN VITRO EXPOSURE TO CARBON DIOXIDE ON THE EXPRESSION OF APOPTOSIS-ASSOCIATED GENES IN HUMAN PERITONEAL FIBROBLASTS

Ângela Mara Bentes de Souza, Christine May Briton-Jones, Chi Chiu Wang, Christopher John Haines, and Michael Scott Rogers

Introduction

The peritoneum is composed of a superficial layer of mesothelial cells with a submesothelial layer that contains fibroblasts, macrophages, and blood vessels. Fibroblasts invade wounds in the first few days of healing. They have multiple functions in wound repair, including collagen synthesis, extracellular matrix reorganization, and wound contraction leading to mature scar formation [1].

Carbon dioxide (CO_2) is the most common gas used to create the pneumoperitoneum during laparoscopic surgery. The standards for use during laparoscopy are defined in the United States Pharmacopoeia and National Formulary, and require 99% purity [2]. This concentration of CO_2, induces a drop in the peritoneal cavity pH with transient impairment in cytokine and tumor necrosis factor release by macrophages in peritoneal fluid [3]. It may also induce mesothelial hypoxia, a co-factor in adhesion formation [4].

The objective of this study was to use an in vitro model with conditions that are close to those during routine laparoscopic surgery, and to observe whether hypoxia in association to acidosis have any influence on the gene expression of Bcl-2 and Bax in human peritoneal fibroblasts.

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Material and Methods

CELL ISOLATION AND CULTURE

Fibroblasts were isolated from normal human peritoneum harvested from three patients undergoing hysterectomy for uterine fibroids. All of them were informed about the procedure, and gave written consent to tissue collection. Harvested tissue samples were immediately rinsed with pre-warmed DMEM, and placed in a digesting solution with 0.2% collagenase type I for 3 hs at 37°C. Samples were then centrifuged for 5 minutes at 1000x rpm, transferred into a 4-well dish with pre-warmed growth medium and placed in a 37°C incubator with 5% CO_2.The outgrowth of fibroblasts took 10 days on average. When confluence was achieved, the cells were transferred to 90-mm tissue culture dish. Studies were performed using cells from the third and fourth passage with 60% confluence.

CELL CHARACTERIZATION

Immunofluorescence staining was used to identify cells in culture. The primary antibody used was mouse anti-human fibroblasts. To detect non-fibroblast cell types the following antibodies were utilized: mesothelial cells, mouse anti-human cytokeratin 19; macrophages, mouse anti-human CD68. The secondary antibody was FITC-labeled rabbit anti-mouse.

HYPOXIC TREATMENT

Experiments were performed using a modular chamber as previously described [3]. The chamber was deoxygenated by a positive infusion of humidified 100% CO_2 for 10 min. Thereafter, the chamber was tightly-sealed and kept inside a 37°C incubator for 4 hours. After this period plates were transferred to standard conditions and harvested at four different time points: immediately, 6, 12 and 24 hours after CO_2 exposure. Parallel cultures were placed in standard conditions for all time points to act as controls. Cell pellets were snap-frozen in liquid nitrogen, and stored at -80°C.

ANALYSIS OF BCL-2 AND BAX EXPRESSION BY RT-PCR

Messenger RNA extraction was performed using the Oligotex direct mRNA kit. All mRNA samples were treated with 1U of DNase inactivated by incubation at 37°C for 15 min + 5 min at 90°C. One hundred nanograms of mRNA was used for cDNA synthesis with Multiscribe reverse transcriptase, and 5 μl of the resultant cDNA was then used for PCR with Amplitaq Gold DNA polymerase. Beta-actin was coamplified with Bcl-2 and Bax to provide a semiquantitative internal control for RNA quantity and PCR reaction efficiency. Forward and reverse primers specific for Bcl-2, Bax and B-actin cDNA were designed from the published cDNA sequence

[5]. The density ratio between the PCR-amplified Bax and Bcl-2 products with the simultaneously amplified control β-actin, was obtained for each sample.

FLOW CYTOMETRIC ANALYSIS OF APOPTOTIC PROFILES

Apoptotic cells were quantified by flow cytometry using the Annexin V kit I following the manufacturer's protocol. The green (FITC) and red (PI) fluorescence intensities of cells were measured using an Epics Elite ESP flow cytometer.

Statistical analysis

A one-way ANOVA with Scheffe post hoc comparisons were conducted using SPSS, version 10.0 for Windows. Statistical significance was determined at $p<0.05$.

Results

Immunofluorescence staining of adherent cells showed that more than 93% of the cells were fibroblasts. Exposure to 100% CO_2 caused an important drop in the culture medium pH from 7.4 to 6.2 after 5 minutes incubation. This value remained steady during the incubation period, and gradually returned to baseline levels after placing the plates in standard conditions.

Comparison of Bcl-2/b-actin intensity ratio between control and experimental group showed significant increase in the experimental group at the six-hour time point ($p = 0.03$). No difference was found immediately after exposure ($p=0.57$), at 12-hour ($p=0.20$) or at 24-hour ($p=0.68$) time points. Comparison of Bax/b-actin intensity ratio between control and experimental groups showed no difference immediately after exposure ($p=0.88$), at 6-hour ($p=0.06$), 12-hour ($p=0.11$) or at 24-hour ($p=0.34$) time points. Flow cytometric analysis showed no significant difference in apoptosis at any harvesting time point in both control and experimental groups.

Discussion

The Bcl-2 family is implicated in the mechanism of hypoxia-induced apoptosis and proliferation in a variety of tissues. High levels of Bcl-2 protein enhance cell survival when exposed to various adverse stimuli. It forms a heterodimer with a related protein, Bax, which has pro-apoptotic functions. The current study demonstrates that in vitro exposure to CO_2 induced an up-regulation on Bcl-2-mRNA levels in peritoneal fibroblasts without causing any change in Bax-mRNA levels. The Bcl-2 up-regulation

was not observed immediately after the hypoxic stimuli, but only few hours after returning the cells to standard conditions, suggesting that reoxygenation may be required to the up-regulation of this gene. Expression of Bcl-2 gene is associated with the inhibition of apoptosis mediated by glucose withdrawal, membrane peroxidation and free radical injury [6], indicating this gene is likely to play a role in reperfusion injury. We have previously reported that CO_2-pneumoperitoneum induces lipid peroxidation in peritoneal membrane during laparoscopic surgery [7]. Thus, it is possible that hypoxia-reoxygenation, leading to oxidative stress, may be responsible for the up-regulation of Bcl-2 observed in this study.

Conclusion

Four-hour in vitro exposure of peritoneal fibroblasts to 100% CO2 caused an up-regulation of Bcl-2 expression 6 hours after returning cells to standard conditions. Whether hypoxia-reoxygenation resulting in oxidative stress is the factor responsible for Bcl-2 up-regulation is not clear. Further investigation is required to answer this question.

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Chapter #40

IDENTIFICATION, CHARACTERIZATION, AND PROPERTIES OF A CLASS ALPHA MICROSOMAL GLUTATHIONE S-TRANSFERASE

K. Sandeep Prabhu, Padala V. Reddy, Andrew D. Liken, Emily C. Jones, Hemant P. Yennawar, and C. Channa Reddy

Cytosolic glutathione (GSH) S-transferases (GST) are dimeric enzymes that exhibit both GSH-dependent conjugation and selenium-independent GSH-peroxidase (NonSe-GPX) activities [1]. Of the various GST supergene families, class Alpha GST isozymes have attracted a lot of attention for their role in the conjugation and reduction of cellular lipid peroxidation products, including 4-hydroxy nonenal and lipid hydroperoxides. Morgenstern and his colleagues reported a non-homologous GST, designated as MGST, in rat and human liver microsomes that exhibited both these activities and differed from the cytosolic GSTs in primary structure [2].

Earlier studies in our laboratory showed that vitamin E-independent inhibition of lipid peroxidation by GSH and glutathione disulfide (GSSG) was observed in rat and human liver microsomes [3,4]. S-Hexyl GSH, a potent inhibitor of GSTs, blocked this inhibition [3], indicating that the microsomes contained an additional GST activity.

Isolation of the microsomal Ya-GST

Solubilized GST activity from sheep liver microsomes was purified by affinity chromatography on S-hexylGSH-linked Sepharose 6B (Figure 1). The affinity bound GST fraction was eluted, concentrated, and analyzed as described earlier from our laboratory [5,6]. The affinity purified microsomal GST fraction revealed one major 25 kDa band and two minor 26 and 27 kDa bands on an SDS-PAGE. The 25, 26 and 27 kDa were designated as Ya-, Yb- and Yc-class GSTs, respectively. Interestingly, Western immunoblot analyses indicated that Morgenstern's MGST was found in the flow-through

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fractions and not bound to the affinity column (data not shown). The Ya-GST was further purified to electrophoretic homogeneity by anion-exchange (DE-52) column chromatography and sequenced at N- and C-terminal sequences were determined. Based on the deduced amino acid sequences as well as the isoelectric point (pI of 9.6), the sheep liver microsomal Ya-GST was classified as a class Alpha GST isozyme. The Ya-GST was cloned and heterologously expressed in a bacterial system (6). Similarly, human liver microsomes also contained Ya, Yb- and Yc-sized GSTs [7]. The human liver microsomal Ya-GST peptide sequence, performed at the C-terminus, indicated about 70 % amino acid homology with other reported class Alpha GSTs from human liver.

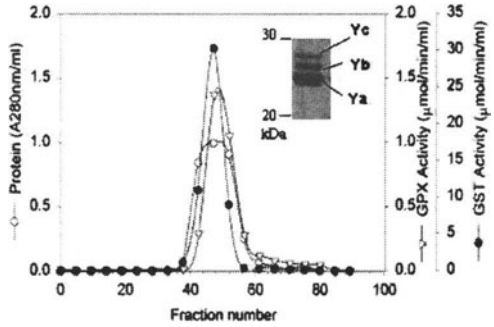

Figure 1. Elution profile of microsomal GSTs from the S-hexylGSH-linked Sepharose 6B affinity column. Inset: Sodium dodecyl sulfate-PAGE profile of the affinity eluate.

Substrate specificity of the Ya-GST

The microsomal Ya-GST purified from sheep and human liver exhibited significant conjugation activity with 1-chloro-2,4-dinitrobenzene (CDNB) as well as 4-hydroxy 2-nonenal, a lipid peroxidation product known to form adducts with DNA and proteins [6,7]. In addition, the microsomal Ya-GST also reduced hydroperoxides of linoleic acid, 13-S-HPODE and 9-S-HPODE, as well as arachidonic acid hydroperoxides, 5-S-HPETE and 15-S-HPETE. Microsomal Ya-GST also exhibited moderate LTC_4 synthase activity. Compared to the human liver cytosolic class Alpha GSTs and microsomal (MGST) GSTs, the human microsomal Ya-GST exhibited several folds higher NonSe-GPX activity towards lipid hydroperoxides [1,8-10]. These results suggest that the microsomal Ya-GST could be involved in the protection of membranes from peroxidative damage and its function is greatly affected by its localization in the membranes.

Structural studies of the microsomal Ya-GST

On-going studies in our laboratory suggest that the Ya-GST is localized to the lumenal (inner) side of the microsomal membranes. Alkaline sodium

carbonate treatment, limited proteolysis with trypsin, and EDTA treatment of microsomes confirmed that the Ya-GST was tightly bound to the lumenal-side of the microsomal membrane [6]. In an attempt to understand if some secondary structural components were responsible for its localization, we crystallized the recombinant sheep liver microsomal Ya-GST using the hanging-drop technique.

The three-dimensional structure of Ya GST deduced at 1.5 Å suggests that the Ya-GST monomer has a two-domain structure with an N-terminal αβ domain (glutathione-binding domain) and a α-helical domain. The overall structure of the microsomal Ya-GST is similar to class Alpha, Mu and Pi GSTs [11]. The most striking difference is the presence of disordered loop in the C-terminus, which allows binding hydrophobic substrates better. Furthermore, a large number of α-helices and helix-loop-helix structures are also seen. Although the characteristic membrane binding domains are absent in the microsomal Ya-GST, it is possible that the protein is embedded in some way into the membrane via the helices, as in the case of cyclooxygenases [12]. Based on these studies, a hypothetical model for the microsomal Ya-GST localization is proposed in Figure 2.

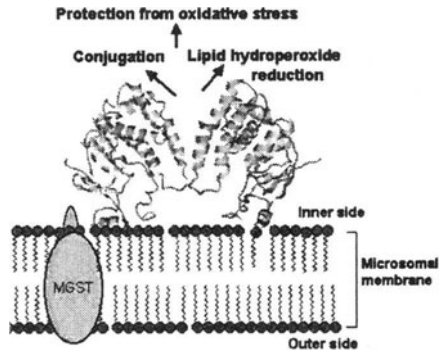

Figure 2. Hypothetical model of the localization and function of microsomal Ya-GST.

Conclusions

In summary, we have isolated a unique cationic Ya-GST from sheep and human liver microsomes that exhibits significantly high conjugation and NonSe-GPX activities, especially against the products of lipid peroxidation. Based on these studies, we hypothesize that the membrane-associated Ya-GST may be a likely candidate for protection against peroxidative damage to biological membranes and its localization further aids in the function.

Acknowledgments

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Chapter #41

CYCLOOXYGENASE (COX) FUNCTION IN THE DUCTUS ARTERIOSUS: ANOTHER LOOK

Flavio Coceani, Barbara Baragatti, Francesca Brizzi, Silvia Barogi and Cameron Ackerley

Introduction

It is well accepted that patency of the ductus arteriosus in the fetus is an active process being sustained primarily by prostaglandin E_2 (PGE_2) but conceivably involving other agents, nitric oxide (NO) in particular [1]. Previous investigations, using lamb and pig, have shown that cyclooxygenases (COXs) develop unevenly within the ductus wall, with COX1 preceding COX2 through the last third of gestation [2-4]. Therefore, both enzymes have been implicated in PGE_2 synthesis at term, while COX1 has been assigned a greater role in the premature [4]. In contrast with these findings, however, it has recently been reported that the term mouse ductus is normally missing COX1 and may, accordingly, fail to constrict to indomethacin (given to the mother) once its COX2 has also been deleted [5]. A separate investigation, on the other hand, could not demonstrate any significant evidence of either enzyme in the same vessel [6].

Aims and Methods

The present study was carried out to resolve these apparent inconsistencies among species and to gain, concomitantly, a better insight into the functional organization of the COX system in the ductus. Three complementary lines of research were pursued, using comparatively wild-type and COX1- or COX2-deficient mice [7]. Their specific aims are listed below.

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(i) To examine occurrence and subcellular distribution of isoforms of COX (COX1 and COX2) and PGE synthase (cytosolic and microsomal; cPGES and mPGES) in muscle cells of the term fetal ductus by immunogold electronmicroscopy. The procedure followed a published protocol [4], and preparations from the lamb and guinea pig were used as a reference. The guinea pig was included in this comparative analysis since, in a peculiar departure, its ductus differs from that of other species, lamb included, in not being contracted by non-steroidal antiinflammatory drugs (NSAIDs) [8].

(ii) To determine the effect of the dual COX1/COX2 inhibitor, indomethacin, on the isolated term ductus at a Po_2 mimicking the fetal (2.5% O_2) or neonatal (12.5% O_2) condition. The mouse preparation was set up as previously described [9].

(iii) To assess the extent of prenatal patency and timing of postnatal closure of the mouse ductus in vivo with a whole-body freezing technique [9].

Results and Discussion

Examination of ductus muscle cells by transmission electronmicroscopy coupled with immunogold labelling showed that, contrary to the aforecited reports [5-6], the mouse resembles the lamb [4] in having both COX enzymes expressed. Furthermore, in the mouse as in the lamb, enzymes are located in the perinuclear rather than the peripheral region of the cytoplasm. Both species, on the other hand, differ from the guinea pig where COX1/COX2 staining is uniformly low and without any particular selectivity in its distribution across the cytosol. Hence, when taking the findings in NSAID-responsive (lamb) and NSAID-unresponsive (guinea pig) species as a reference, one may conclude that COX enzymes not only are present in the mouse ductus, but are also expressed within the muscle cells in a pattern congruent with functional competence. The same cells are also endowed with both isoforms, cytosolic and microsomal, of PGE synthase. However, PGE synthases, unlike COX enzymes, are diffusely distributed inside the cell and show no obvious change across the three species examined. In the latter respect, the ductus is peculiar, because mPGES has preferentially a perinuclear location [10].

To confirm that COX enzymes are functional, separate experiments were performed with the isolated ductus from wild-type and COX-deleted animals. Outwardly, vessels belonging to the three groups had similar morphological features. Nevertheless, their contraction to indomethacin (2.8 μM) varied in pattern and reversibility. While wild-type and COX1(-/-) preparations contracted rapidly after an initial short delay (peak in about 10 min), the response of COX2(-/-) preparations was delayed in onset and gradual (peak in about 60 min). Once attained, however, peak contraction (about 0.9 mN/mm) showed only minor differences among genotypes, regardless of the Po_2 of the medium, while in contrast the extent of the

reversal after washing out the inhibitor varied with the genotype [COX1(-/-) > wild-type > COX2(-/-)]. Despite these differences in vitro, COX1(-/-) and COX2(-/-) mice could not be distinguished from their corresponding wild-type in vivo. Prenatally, the ductus was widely patent. Likewise, the timing of postnatal closure was the same with any genotype. Summing up, the mouse ductus is endowed with a full complement of COX/PGES enzymes. Hence, previous investigations [5, 6] reportedly showing the absence of COX enzymes in the vessel was not confirmed. Significantly, COX1 and COX2 differ in their susceptibility to indomethacin, and this particular feature could explain the apparent lack of effect of the drug on the COX2 (-/-) ductus when given to the pregnant mother [5].

Our findings in vivo, on the other hand, may indicate that COX enzymes, despite their uneven susceptibility to indomethacin, are able to substitute each other in keeping the ductus patent. Alternatively, or coincidentally, the same findings may mean that COX suppression results in upregulation of complementary relaxing mechanism(s), specifically the NO-based mechanism [1]. Indeed, in a separate set of experiments we have found that the contraction of the isolated mouse ductus to the NO synthesis inhibitor, N^{w}-nitro-L-arginine methylester (L-NAME, 100 μM) increases 3- to 4-fold (from a basal value of about 0.3 mN/mm) following COX1, or COX2, deletion.

From the foregoing data, we conclude that both COX1 and COX2 sustain PGE_2 synthesis in the ductus arteriosus and, hence, contribute to prenatal patency of the vessel. The relative importance of COX isoforms at term gestation remains unsettled. Results with indomethacin point to a predominant role of COX2 over COX1, while the rebound in NOS activity upon COX deletion, with its even magnitude regardless of the isoform being removed, would argue against any such differential arrangement. Nevertheless, it is confirmed that PGE_2 and NO can function synergistically in keeping the ductus patent.

Acknowledgements

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Chapter #42

EVIDENCE FOR ENHANCED OXIDATIVE STRESS IN CORONARY HEART DISEASE AND CHRONIC HEART FAILURE

Roswitha Wolfram, Anthony Oguogho, Barbara Palumbo, and Helmut Sinzinger

Introduction

Oxidation injury has been claimed as one of the key factors in the development of coronary heart disease and chronic heart failure [1,2]. The isoprostane 8-epi-$PGF_{2\alpha}$ is gaining increasing interest as reliable marker of in-vivo oxidation injury [3]. As it causes among others coronary vasoconstriction [4] its pathophysiological role may become even more important. We therefore assessed the isoprostane 8-epi-$PGF_{2\alpha}$ in arteries, veins, heart valves and myocardial tissue immunochemically as well as by immunohistochemistry. Furthermore, 8-epi-$PGF_{2\alpha}$ was determined in plasma and urine of patients with CHD compared to patients with dilated and ischemic CMP.

Material and Methods

Blood samples were anticoagulated with 2% EDTA and 1 mg/ml (final blood volume) of acetylsalicylic acid. After centrifugation at 4°C at 1000g for 10 minutes plasma was removed and stored in liquid nitrogen for no longer than 2 weeks. Interassay variability was 5.3 ± 1.6%, intraassay variability 2.4 ± 0.7%.

Urine was collected for 24 hours. Aliquots adjusted to pH 4 were extracted. The eluate was subjected to silicic acid chromatography for further elution. The final eluate was dried, recovered in buffer and assayed after dilution. Values are given in pg 8-epi-$PGF_{2\alpha}$/ml creatinine. Interassay variability was 6.6 ± 2.2%, intraassay variability 2.9 ± 0.9%, normal values 150 – 250 mg creatinine. Immunohistochemistry was done on histological sections derived from transplant donors using ox-LDL and 8-epi-$PGF_{2\alpha}$

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antibodies [5,6] and subsequently quantified according to a scale given in table 1.

Table 1. Involved area assessed by planimetry (0 – 100 %) intensity (estimated units – eu)

0 ... no activity
1 ... minimal
0 ... no activity
1 ... minimal
2 ... slight
3 ... intensive
4... very intensive

Table 2. Immunohistochemistry reveals abnormally increased extent and intensity of positively stained areas

ARTERIES: CHD > CMP > Co
IP vs. oxLDL ($p < 0.001$)
IP (HISTO) vs. IP (CHEM) ($p < 0.001$)
IP EXTENT vs. IP INTENSITY ($p < 0.001$)
HEART VALVES: PULM > AORTIC
CHD > CMP > Co

IP: isoprostane

Results

Immunohistochemistry reveals abnormally increased extent and intensity of positively stained areas (Table 2). There is a strong correlation between staining intensity and extent (Table 3) as well as biochemical analysis [5,6].

Table 3. Comparison between the % area or intensity (eU) from the isoprostane 8-epi-$PGF_{2\alpha}$ in the intima vs. media in all three patient groups[6]

Patient group	8-epi-$PGF_{2\alpha}$ (intima vs. media)	p-value
Control	% area	= 0.0061
	intensity (eU)	= 0.101
CMP	% area	< 0.0001
	intensity (eU)	< 0.0001
CHD	% area	< 0.0001
	intensity (eU)	< 0.0001

Estimation of 8-epi-$PGF_{2\alpha}$ in plasma [7] exhibited the highest values in ischemic CMP and dilative CMP, followed by CHD and the controls. A comparable finding was also obtained in urinary samples. Interestingly, 8-epi-$PGF_{2\alpha}$ showed a significant correlation to the NYHA-stage and an inverse one to the left ventricular ejection fraction. As a matter of fact the risk factors (heavy cigarette smoking and diabetes) were associated with significantly higher 8-epi-$PGF_{2\alpha}$ values as compared to the respective controls. No difference in 8-epi-$PGF_{2\alpha}$ between dilative vs. ischemic CMP could be found.

Conclusion

The findings reflect an enhanced oxidation injury in CHD and CMP in tissues and body fluids. These findings indicate the role of oxidative stress in the pathogenesis of the disease and suggest that a modulation of disease progression might be possible. The effect of an antioxidative therapeutic intervention is investigated at present.

Table 4. 8-epi-$PGF_{2\alpha}$ in vascular and valve tissue in CMP

Co	CMP	CHD	tissue
107.4 ± 16.3	289.4 ± 66.5	551.5 ± 86.3	LCA*
112.6 ± 24.5	295.7 ± 71.6	536.4 ± 80.2	RCA*
104.6 ± 20.5	269.5 ± 63.2	484.6 ± 69.6	sinus*
157.9 ± 30.6	286.5 ± 57.3	347.8 ± 63.3	aortic valve**
149.6 ± 25.5	260.4 ± 60.5	322.6 ± 65.9	pulm. valve **

Data are mean ± SD of 20 samples each, * pg 8-epi-$PGF_{2\alpha}$/mg wet weight, ** pg 8-epi-$PGF_{2\alpha}$/mg protein, LCA: left coronary artery, RCA: right coronary artery, pulm: pulmonary.

Table 5. Immunohistochemical tissue staining

CMP		CHD		tissue
extent	int	extent	int	
56.8 ± 15.7	36.9 ± 14.5	206.4 ± 41.5	109.6 ± 32.7	I (RCA)
53.7 ± 16.5	40.2 ± 15.9	203.1 ± 50.6	126.4 ± 44.3	I (LCA)
39.6 ± 12.8	47.5 ± 16.8	27.9 ± 11.6	36.4 ± 18.4	M (RCA)
40.7 ± 15.0	50.6 ± 14.3	25.4 ± 10.3	42.5 ± 15.6	M (LCA)

Values as % increase vs. controls, data are mean ± SD of 16 samples each, I: intima, M: media, LCA: left coronary artery, RCA: right coronary artery.

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Chapter #43

THE LEUKOTRIENE RECEPTOR CYSLT$_1$ AND 5-LIPOXYGENASE ARE UPREGULATED IN COLON CANCER

Christian K Nielsen, John F Öhd, Katarina Wikström, Ramin Massoumi, Sailaja Paruchuri, Maria Juhas, and Anita Sjölander

Introduction

The metabolites of arachidonic acid are well connected to pathological situations such as inflammation, cancer and asthma. Sheng et al. [7] found that COX-2 is upregulated in colon cancer tissue and tumor cell lines indicating that COX-2 is involved in colon cancer. This is supported by studies showing that patients treated with nonsteroidal anti-inflammatory drugs, inhibitors of COX-2, exhibit a lower frequency of colon cancer [8]. When the non-transformed intestinal epithelial cell line, Int 407 was stimulated with LTD_4 or LTB_4 we observed an accumulation of COX-2 in membrane fractions as well as an increased production of prostaglandin E_2 [5]. Treatment of these cells with the COX-2 inhibitor NS-398 caused apoptosis and this effect could be prevented by LTD_4 [5] or LTB_4 [4]. Similar results were obtained when cell viability with LTD_4 or LTB_4 in the presence or absence of NS-398 was assayed [4,5]. The results demonstrate that these leukotrienes can suppress the NS-398 induced apoptosis in intestinal cells.

Bcl-2 and Bcl-X_L are cell survival promoting members of the Bcl-2 protein family. When intestinal epithelial cells were stimulated with LTD_4 it induced accumulation of Bcl-2 in a membrane fraction. This effect could be impaired by NS-398, indicating a role for COX-2 and Bcl-2 in the ability of LTD_4 to prevent programmed cell death [5]. Bcl-X_L has been shown to be the predominantly expressed anti-apoptotic protein in colon cancer cells [1].

When examining the interaction between cells and the extra cellular matrix we found that, LTD_4 induces the accumulation of vinculin in focal adhesions and an increased adhesion through activation of β_1-integrins [2]. In colon cancer cells, LTD_4 induced increased adhesion to the extracellular matrix mediated by COX-2 (unpublished data).

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Besides the involvement of LTD_4 in cell survival it was recently shown in our lab that LTD_4 induced cell proliferation in Int 407 cells [6] via two separate pathways, one Ras dependent and one pathway independent of Ras but dependent on PKCε and ERK1/2.

5-LO and CysLT$_1$R are Upregulated in Colon Cancer

To investigate a possible role for LTD_4 in the development of colon cancer we compared samples of non-transformed and cancerous colon tissue. Proteins from these samples were subjected to electrophoretic separation and Western blotting and blotted for 5-LO, COX-2 or CysLT$_1$R after which the levels of proteins were quantified. The results revealed a pronounced upregulation of CysLT$_1$R in colon cancer cells compared to normal cells. Upon comparison of 5-LO, COX-2 and CysLT$_1$R levels in control and cancerous colon tissues from single patients, there was a trend indicating a correlation between elevated levels of 5-LO and COX-2 and an increased level of CysLT$_1$R (see figure 1).

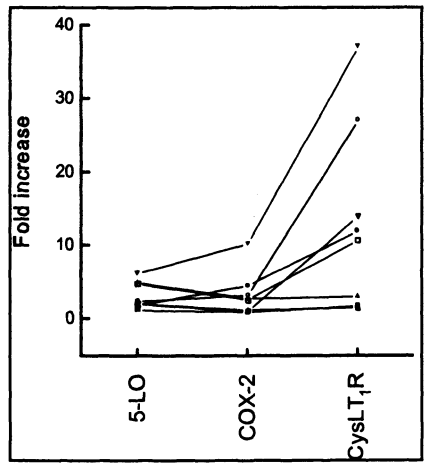

Figure 1. Samples of cancerous and normal colon tissue were taken from patients undergoing surgery. Proteins were isolated and separated on SDS-polyacryl amide gels, transferred onto PVDF membranes by electrophoresis, blotted for the 5-LO, COX-2 or CysLT$_1$R and detected by the aid of enhanced chemiluminescence. Levels of 5-LO, COX-2 and CysLT$_1$R in single patients were quantified and plotted. Data represents samples from eight patients, each graph representing one individual.

These results correspond to our findings from immunohistochemical staining for 5-LO, COX-2, and CysLT$_1$R of paraffin embedded normal mucosa and colorectal cancer specimens as well as results from non-transformed epithelial and colon cancer cell lines [3].

Summary and conclusion

We have shown that LTD_4 induces increased cell survival and adhesion mediated by COX-2. We identified a contemporary upregulation of 5-LO, COX-2 and $CysLT_1R$ in colon cancer compared to control colon cells in clinical samples as well as in cell lines. These results indicate that colon cancer cells not only have the ability to over express leukotrienes and prostaglandins due to upregulated levels of 5-LO and COX-2 but also have potentially increased sensitivity towards LTD_4 due to the higher levels of $CysLT_1R$.

Based on our present and earlier findings we believe that LTD_4 can play a role in the development of colorectal carcinoma by affecting cell survival, proliferation, and cell adhesion, particularly in a LTD_4 enriched environment as can be found in inflammatory bowel diseases (see figure 2).

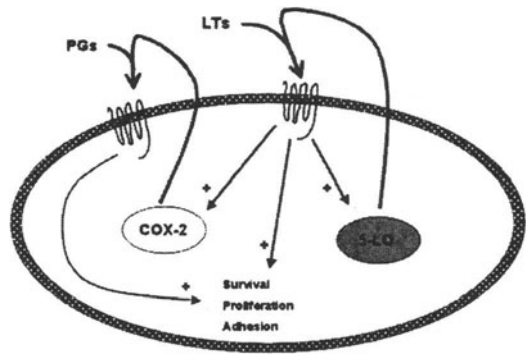

Figure 2. The effect of leukotrienes and prostaglandins in intestinal epithelial cells.

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Chapter #44

INCREASED EXPRESSION OF 5-LIPOXYGENASE IN GLIOBLASTOMA MULTIFORME

Mladen Golubic, Richard A. Prayson, Linda Vargo, Judy Bondar, and Gene H. Barnett

Introduction

Gliomas are the most common primary brain tumors and account for 40% of all central nervous system neoplasms. Glioblastoma multiforme (GBM) is the most malignant glioma and is characterized by highly infiltrative and neurologically destructive growth patterns. Despite new advances in surgical intervention, radiotherapy, chemotherapy, and a variety of molecular target specific therapies, the median survival of patients with GBM did not change in the last two decades. Today, most GBM patients still die in less than a year [1]. Therefore, it is essential to identify novel molecular targets that may be suitable for treatment and prevention of GBM.

An important group of molecular changes that could serve as therapeutic target and is often encountered in glioblastoma cells is the increased metabolism of arachidonic acid (AA), a nutritionally relevant polyunsaturated ω-6 fatty acid, into biologically active eicosanoids. First, concentrations of AA and its precursor, linoleic acid (LA) are significantly higher in GBM than in normal brain tissue [2,3]. Second, GBM cells also overexpress cyclooxygenase-2 (COX-2) [4]. Third, both COX- and 5-lipoxygenase (5-LO)-derived eicosanoids are overproduced by GBM cells [5,6]. 5-LO catalyzes the first two reactions in the biosynthesis of leukotrienes, which are potent mediators of inflammatory and allergic reactions [7], as well as tumor growth and promotion [8,9].

In contrast to recent findings of overexpression of COX-2 protein and the positive correlation of COX-2 levels with a shorter survival of patients with GBM [4], it is not known whether 5-LO is expressed in GBM. Boado et al., reported that high levels of 5-LO mRNA were detected in two GBM samples (of two examined), while 5-LO was not expressed in grade II glioma and

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barely detectable levels were found in one grade III tumor [10]. The aim of this study was to determine whether 5-LO protein is expressed in surgical specimens of human GBM.

Material and Methods

A retrospective examination of 5-LO expression of frozen tissue sections of GBM was performed by immunohistochemical analysis with the monoclonal anti-5-LO IgG (BD Biosciences) as described [11]. Recombinant 5-LO protein was used for preincubation of antibodies to ensure the specificity of 5-LO signal. Immunoreactive complexes were detected through utilization of the enhanced 3,3'-diaminobenzidene (DAB) detection chemistry. Double staining of frozen section was performed with anti-CD-68 antibody (Dako), a macrophage specific marker, and anti-5-LO IgG. Secondary, biotin-coupled antibodies were detected by anti-avidin antibodies with bound horseradish peroxidase for DAB detection or alkaline phosphatase for staining of CD-68 containing immunocomplexes. Monoclonal anti-5-LO antibody and polyclonal anti-5-LO antiserum (Cayman Chemical) were used for immunoblotting analysis of protein extracts from surgical GBM specimens and established GBM cell lines.

Results and Discussion

Boado et al., reported that high levels of 5-LO mRNA were present in two GBM samples (of two examined) [10]. To determine if 5-LO protein is expressed in human GBM, we performed a retrospective examination of 5-LO expression in frozen tissue sections of 37 surgical GBM specimens by immunohistochemical analysis with anti-5-LO monoclonal antibody. This analysis revealed cytoplasmic staining of neurons in the "normal" brain tissue from an epilepsy patient undergoing temporal lobectomy. In contrast, the white matter of the same specimen stained very weakly (less than 1% of cells) for 5-LO. Anti-5-LO antibody, however, positively stained cells (mostly nuclear staining) in 32 GBMs out of 37 samples analyzed. In 16 samples (43%), up to 50% of cells were positive, while less than 5% of cells stained in the remaining 16 tumors (43%). Nuclear localization of 5-LO is likely to indicate that 5-LO is activated in GBM cells and translocated to nuclear membrane, the major site for leukotriene synthesis [12].

To determine whether tumor cells or the tumor-infiltrating macrophages express 5-LO protein, we performed a double staining study of four GBM samples. Double staining with anti-CD-68 antibody, a macrophage specific marker, and anti-5-LO IgG shows that 5-LO protein is expressed by both CD-68–negative GBM cells and CD-68-positive macrophages. CD-68-positive cells were often found in perinecrotic areas.

To confirm and extend these findings, immunoblotting analysis was performed with protein extracts prepared from six GBM surgical samples and three GBM cell lines with anti-5-LO antibodies. The strong 5-LO-

specific band of about 80 kDa was detected in all six surgical GBM specimens and two established GBM cell lines.

Our data clearly indicate that 5-LO protein is expressed at aberrantly high levels in most GBM specimens compared with cells of the non-tumorous white brain matter tissue where precursor cells for GBM are located. At present, it is not clear whether 5-LO protein expression levels are associated with clinically relevant parameters. Nevertheless, the results obtained in this study suggest that 5-LO could serve as a potential target for treatment of GBM by specific 5-LO inhibitors [13,14]. These inhibitors exert a potent cytotoxic activity against a variety of human epithelial cancer cells [15] and as shown by others and us, brain tumor cells [16], including GBM cells *in vitro* [17,18] and *in vivo* [19]. 5-LO overexpression has been recently demonstrated in human lung [20], prostate [11], pancreatic [21], and colon cancer [22] specimens. Our results indicate that GBM is a new member of a growing list of human malignancies that aberrantly overexpress 5-LO. It is, therefore, necessary to explore whether the adjuvant use of 5-LO inhibitors may be beneficial for patients suffering from this incurable disease.

Acknowledgments

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Chapter #45

VARIABLE INFLUENCE OF STATINS ON ISOPROSTANES IN HYPERLIPIDEMIA

Helmut Sinzinger and Anthony Oguogho

Although statin therapy under normal circumstances reduces oxidation injury, the isoprostane 8-epi-$PGF_{2\alpha}$ may increase various time intervals after initiation of treatment in certain patients for unknown reason. All statins seem to be equally affected. Stopping statin therapy causes reversal within about 2 weeks. During and after exercise the 8-epi-$PGF_{2\alpha}$ increase is more frequent. Isoprostanes may become relevant to discover a statin side effect. The pathophysiological and clinical consequences of this finding need to be elaborated.

Introduction

Muscle related side effects are the major group of adverse events occurring during statin treatment (for review see [1]). While treatment of hyperlipidemia is associated with a decrease in markers of oxidative stress, in some patients an increase of 8-epi-$PGF_{2\alpha}$ (an isoprostane in well recognized as a marker of in-vivo oxidation injury) has been reported during the last conference for the first time [2].

This increase on certain statins was described to occur immediately when starting and normalizing after 2 weeks after the withdrawal of the respective compound. We focused our interest on the behavior of 8-epi-$PGF_{2\alpha}$ during statin therapy.

Results

The normal response outlined in 100 patients (Table 1) indicates that already 1 month after starting therapy there is a decrease in isoprostane 8-

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epi-$PGF_{2\alpha}$ which is found in plasma (P), serum (S) and urine (U), the values achieved persisting stable thereafter for 6 months and even more.

Table 1. 8-epi-$PGF_{2\alpha}$ in smokers (SM) vs. non-smokers (NS) not showing an increase in isoprostane levels (normal response)

	p	1	3	6	
NS	28.3±7.3	24.1±5.0	23.7±4.6	23.8±4.7	P
(61)	286.7±37.2	262.4±29.8	257.8±31.4	259.4±27.9	S
	351.4±42.5	294.3±36.2*	288.5±34.6	290.7±35.2	U
SM	36.4±8.9	30.7±7.2	30.4±6.8	29.6±6.9	P
(39)	457.3±48.2^{+}	335.0±39.2*$^{+}$	341.7±40.6*$^{+}$	334.2±39.1*$^{+}$	S
	486.5±56.2^{+}	408.2±57.0*$^{+}$	411.9±60.2*$^{+}$	400.9±51.3*$^{+}$	U

Values in mean ± SD; *$p < 0.01$ (vs. prevalue); $^{+}p < 0.01$ (vs. non-smokers)

While the actual values are higher in cigarette smokers vs. non-smokers, the behavior is comparable in both the groups. In 11 patients, however (for characteristics see Table 2) there was an increase rather than a decrease persisting throughout the monitoring period of 6 months (Tables 3 and 4).

Table 2. Patients' characteristics

	A			B		
Groups	total (100)	m (56)	f (44)	total (11)	m (7)	f (4)
Age (a)	19 – 58	19 – 54	22 – 58	24 – 51	24 – 51	24 – 50
SM/NS	29/61	20/36	9/35	4/7	2/5	2/2
FH since a	0 – 12	0 – 10	1 – 11	0 – 12	1 – 8	0 – 12
ATH +/–	24/76	15/41	9/35	3/8	2/5	1/3
Other drug	37/63	22/34	15/29	3/8	1/5	2/3

A: total group of 100; B: subgroup showing an increase in 8-epi-$PGF_{2\alpha}$; SM: smokers; NS: non-smokers; FH: familial hypercholesterolemia; ATH: clinical manifestation of atherosclerosis; m: males; f: females; a: years.

In contrast, in another subgroup of patients there was a decline in isoprostanes occurring not immediately but rather late after initiating statin treatment. Increase of 8-epi-$PGF_{2\alpha}$ is sometimes but not always associated with muscle symptoms and only rarely with CK-elevation. An eventual increase was always reversible when the drug was withdrawn. Analyzing the capacity of various statins at different doses in the range of 10–40 mg (Table 5; the patients numbers are given in parentheses) reveals no dose-dependency in the investigated range. There was also no correlation to the potency of the respective drugs.

Table 3. Characteristics of patients showing an increase in 8-epi-$PGF_{2\alpha}$

No	initials	sex m/f	age (a)	height (cm)	weight (kg)	FH since(a)	ATH +/–	SM +/–	drug +/–	statin
1	DS	m	41	181	77	8	–	–	–	L
2	HF	f	46	169	60	9	–	–	+	S
3	RD	m	24	187	80	4	–	+	–	A
4	LO	m	37	163	55	7	–	–	–	F
5	WD	m	36	177	77	1	–	–	–	S
6	GM	f	43	158	53	10	–	–	–	P
7	FO	f	24	162	60	0	–	+	–	A
8	SR	m	51	174	76	7	+	+	+	A
9	AO	m	42	179	73	5	+	–	–	S
10	PN	f	50	159	54	12	+	+	+	A
11	EI	m	33	191	87	3	–	–	–	P

L: lovastatin; S: simvastatin; A: atorvastatin; F: fluvastatin; P: pravastatin (always used at the lowest available dose)

Table 4. 8-epi-$PGF_{2\alpha}$ in pg/ml (plasma, serum) and pg/mg creatinine (urine) values in patients with abnormal response

No	Plasma				Serum				Urine			
1	27	36	42	39	262	348	380	397	353	497	521	530
2	24	40	43	42	256	371	395	368	331	477	502	486
3	38	75	73	80	457	694	731	712	497	726	851	783
4	28	40	37	37	271	365	370	364	362	469	471	486
5	24	36	43	47	265	362	384	371	316	462	484	465
6	26	46	49	45	290	381	362	387	327	498	483	492
7	35	53	56	57	428	596	561	612	473	514	564	570
8	42	63	61	66	477	612	641	670	483	609	625	621
9	29	37	56	54	284	375	542	508	342	486	554	570
10	39	44	40	39	329	446	425	429	409	502	461	462
11	29	51	47	54	308	575	544	569	344	542	536	579
Interval	p	1	3	6	p	1	3	6	p	1	3	6

p: prevalue; time interval is given in months

Table 5. Influence of various statins on 8-epi-$PGF_{2\alpha}$

Statins / dose	10 mg	20 mg	40 mg
Atorvastatin	- 24.7 (17)	- 25.3 (24)	- 27.9 (16)
Fluvastatin	–	- 18.7 (19)	- 23.2 (14)
Lovastatin	–	- 20.6 (17)	- 21.4 (12)
Pravastatin	- 18.9 (25)	- 24.2 (18)	- 23.6 (16)
Simvastatin	- 25.6 (14)	- 24.8 (12)	- 28.2 (9)

Data reflect mean (%) lowering of 8-epi-$PGF_{2\alpha}$ (number of patients inparenthesis)

Conclusion

These findings indicate that statin therapy in general decreases LDL-oxidation and in-vivo oxidation injury as outlined by a drop of 8-epi-$PGF_{2\alpha}$ [1]. In a small subpopulation, however, this decrease starts only several months after initiating the therapy. There is no apparent difference between the various statins on the market as there is no apparent dose-dependency up to the range of 40 mg. A paradoxical increase in 8-epi-$PGF_{2\alpha}$ [2] may reflect a side effect of the drug. Only very few patients show an isoprostane increase on all statins. Earlier it has been reported that the paradoxical increase is not necessarily associated with the onset for muscle pains or an increase in creatinine phosphokinase[1]. Whether this increase in isoprostane 8-epi-$PGF_{2\alpha}$ is linked to an oxidation injury at the mitochondrial level [3] in the striated muscle still needs to be assessed. Histochemical findings reveal an oxidation injury in muscle biopsy in patients with exercise-induced statin myopathy[3] pointing into this direction. For clinical consequence it remains unclear whether an increase in 8-epi-$PGF_{2\alpha}$ should be considered as an adverse event and the drug discontinued. Further investigations are necessary to elucidate this very complex isoprostane response during statin treatment.

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Chapter #46

INCREASED ISOPROSTANES IN CHILDREN OF SMOKING PARENTS

Helmut Sinzinger, Anthony Oguogho, and Heidemarie Pilz

Introduction

Experimental tobacco smoking (ETS) is attracting increasing attention [1] as a potential risk factor for the development of atherosclerosis [2,3]. Assessment of ETS under real life conditions of biochemical changes is difficult. We therefore assessed the isoprostane 8-epi-$PGF_{2\alpha}$ in children of parents diagnosed for familial hyperlipoproteinemia at the receptor level with the aim to clarify whether parental smoking may have an influence.

Material and Methods

Blood was drawn from a total of 203 healthy children (107 boys, 96 girls, both aged up to 18 years). The children were divided into the groups whether mother or father or both of them were smoking compared to a non-smoking control group. The number of cigarettes was defined as total number of cigarettes smoked at home per day throughout the last 8 weeks before blood withdrawal by using a special questionnaire.

Blood was drawn from the cubital vein using a 1,2 mm diameter butterfly needle after an at least 30 minutes rest and avoiding venous occlusion. Blood was anticoagulated with 2% sodium EDTA under addition of 10 mg acetylsalicylic acid/ml processed and centrifuged at 4°C. Plasma was removed and stored in liquid nitrogen. This blood withdrawal took place in the morning after an at least 12 hours overnight fasting period.

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Results

If one of the parents was smoking cigarettes, 8-epi-$PGF_{2\alpha}$ was higher as compared to the respective controls. If both parents were smoking or with an increased number of cigarettes smoked at home, 8-epi-$PGF_{2\alpha}$ showed a further increase (Table 1). Age and/or sex, however, had no significant influence. Data from 127 children (parents smoking > 20 and > 40 cigarettes/day, respectively) are not shown.

Table 1. Differences in percent in plasma isoprostane values between children with non-smoking parents (Co) and children with smoking parents (data from 76 children)

cig./day	SM par.	Gender of child	n	plasma (pg/ml)	p-value vs. Co
0	0	m	26	7.36±1.70	0 m
0	0	f	28	7.25±1.84	0 f
< 20	mother	m	18	9.98±1.96	0.002
< 20	mother	f	12	10.21±1.83	0.000
< 20	father	m	11	9.93±1.37	0.003
< 20	father	f	16	9.46±1.40	0.002
< 20	both	m	12	10.77±1.88	0.000
< 20	both	f	7	10.64±1.76	0.000

m: male; f: female; SM: smoking; Co: Control

Discussion

It has already been shown under experimental conditions that environmental tobacco smoking is associated with an activation of platelet function [4], increased thromboxane formation and a decrease in platelet sensitivity [5] to the antiaggregatory prostaglandins (PGI_2, PGE_1 and PGD_2). Isoprostanes have already been shown to be reliable in-vivo oxidative stress markers in active cigarette smoking [6].

In this study only children with normal lipid profile have been incorporated as it has been shown before, that hypercholesterolemia is associated with an increased 8-epi-$PGF_{2\alpha}$ [7]. ETS apparently induces a significant increase in 8-epi-$PGF_{2\alpha}$ (Table 1) by about one third. Interestingly single exposure to ETS in an earlier experimental study we performed [8] did not alter 8-epi-$PGF_{2\alpha}$ while continuous exposure to ETS apparently exerts a significant influence. This methodological approach is of particular interest as it reflects true to life conditions. Earlier studies [3,4] only have been performed in people being exposed under experimental conditions to ETS in a closed room for a shorter period of time with well defined conditions concerning smoke.

Conclusion

These findings clearly document that long-term exposure to ETS induces significant in-vivo oxidation injury with all its eventual deleterious consequences. A screening study going on in school children to verify these findings is being performed at present.

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Chapter #47

THE DINOR ISOPROSTANE PATHWAY IN PLANTS

Christane Loeffler, Ingeborg Thoma, Markus Krischke, and Martin J. Mueller

Introduction

Isoprostanes are products of free radical-catalyzed oxidation of arachidonic acid (20:4) in mammals [1]. However, isoprostanoids can be formed virtually from any natural polyunsaturated fatty acid that contains at least three double bonds. α-Linolenic acid (18:3) fulfills that criterion, and thus, can be oxidized to a variety of C-18 isoprostane classes (dinor isoprostanes) [2]. Dinor isoprostanes in animals are either derived from ß-oxidation of C20 isoprostanes or peroxidation of γ-linolenic acid (18:3) but not from α-linolenic acid which is only a trace fatty acid in mammals [3,4]. In contrast, higher plants generally are devoid of arachidonate and biosynthesize α-linolenate as one of the major polyunsaturated fatty acids. Since α-linolenate derived dinor isoprostanes differ in structure from mammalian dinor isoprostanes, plant dinor isoprostanes have been termed phytoprostanes [5].

Biosynthesis and Occurrence of Phytoprostanes

The biochemical pathway that leads to the formation of phytoprostanes is analogous to the isoprostane pathway in mammals (Figure 1). Free radical catalyzed oxidation and cyclization of α-linolenic acid yields two regioisomeric phytoprostanes G_1 (type I and type II), each of which is theoretically comprised of 16 isomers. Phytoprostanes G_1 are unstable molecules that readily decompose to phytoprostanes D_1, E_1 and F_1. Moreover, phytoprostanes D_1 and E_1 may dehydrate and isomerize to cyclopentenone phytoprostanes with the A_1, B_1 and deoxy-J_1 ring system.

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Analysis of phytoprostanes D_1, E_1, F_1, A_1, B_1 and deoxy-J_1 in plants by GC-MS [5-7] and

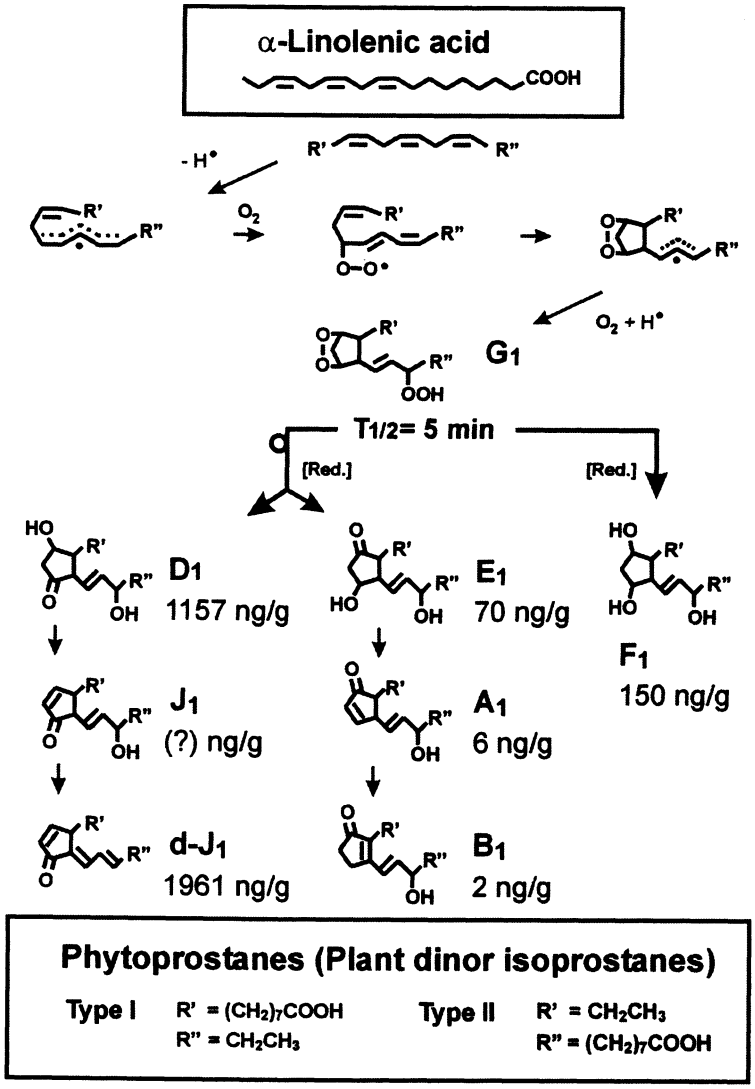

Figure 1. The phytoprostane pathway in plants. Levels of phytoprostanes in a representative plant (tomato, leaves) are indicated.

HPLC-fluorescence detection [8] revealed that phytoprostanes occur apparently ubiquitously in the plant kingdom [5-8]. Interestingly, levels of phytoprostanes in plants are generally several orders of magnitude higher than levels of isoprostanes in humans. For instance, the level of free isoprostanes F_2 in human blood (0.035 ng/ml) is 2 to 3 orders of magnitude

lower than phytoprostane F_1 levels in plant leaves (4 – 150 ng/g) [5]. Notably, phytoprostane levels in dried plant materials are generally in the μg/g range [5,6,8]. Free cyclopentenone isoprostanes could so far not be detected in mammals, possibly due to the rapid metabolism of these compounds [9]. In contrast, cyclopentenone phytoprostanes A_1, B_1 and deoxy-J_1 are clearly detectable in plants (Figure 1) and their levels are in the range of enzymatically formed, prostaglandin-like hormones of the jasmonate family that mediates plant defense reactions [10-13].

Phytoprostanes: Induction by Oxidative Stress and Biological Activities

In plants, abiotic (peroxides, heavy metals) as well as biotic stresses (wounding, pathogens) associated with increased free radical formation have all been shown to increase phytoprostane levels *in vivo*. For instance, peroxides and heavy metals may induce an up to 15-fold increase of phytoprostanes E_1, F_1, B_1 and A_1 in plant suspension cultures [7, 14]. Infection of tomato leaves with a necrotrophic fungus, *Botrytis cinerea,* that induces a massive oxidative burst and free radical production in many plant species triggered also phytoprostane E_1, A_1 and B_1 formation (3- to 4-fold induction) [7]. Oxidative stress imposed by *t*-butyl hydroperoxide (1 mM) on tobacco cell suspension cultures (*Nicotiana tabacum*, Solanaceae) induced a transient increase of an antimicrobial and antioxidative secondary metabolite, scopoletin, in the medium of the cell culture. Maximum levels (12-fold induction) were observed after 4 h [7]. The same treatment also transiently induced a 6 to 10-fold increase of endogenous phytoprostanes E_1 and A_1/B_1 with maximum levels between 1 and 2 h while jasmonic acid levels remained unchanged [7]. When jasmonic acid as well as phytoprostanes E_1, A_1, B_1 and deoxy-J_1 were applied to tobacco cell cultures at a 10 μM concentration, scopoletin transiently accumulated in the medium of the cell culture (6- to 8-fold induction) with a similar time course as seen after butyl hydroperoxide treatment [7]. Phytoprostanes A_1 and B_1 not only induce antimicrobial metabolites in tobacco but also benzophenantridine alkaloids in *Eschscholzia californica* (Papaveraceae) and isobavachalcone in *Crotalaria cobalticola* (Fabaceae) [7]. Results suggest that phytoprostanes may have a profound stimulatory effect on secondary metabolism in at least three taxonomically distant plant species similar to jasmonic acid. Thus, phytoprostanes in plants may have a function as mediators of oxidative stress in plants.

Outlook

Data collected so far indicates that phytoprostanes accumulate to high levels in plants post harvest due to autoxidation of endogenous linolenate. Therefore, it will be interesting to investigate whether or not phytoprostanes in human diet, pharmaceutical plant preparations and parenteral nutrition

(soybean oil emulsions) may exert a pharmacological effect on humans. Since at least one member of the human dinor isoprostanes (= isomers of phytoprostanes) displays biological activity in the nanomolar concentration range [3], it is likely that members of the phytoprostane family may also be pharmacologically active. In plants, preliminary data suggests that phytoprostanes do not only have structural but also functional features in common with jasmonates. Yet, the precise mechanism of action, effects on global gene expression and function of phytoprostanes in plants remain to be established.

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