DETERMINANTS OF CYCLOOXYGENASE-2-MEDIATED OXIDATIVE METABOLISM OF THE ENDOCANNABINOID, 2-ARACHIDONOYL GLYCEROL, *IN VITRO* AND *EX VIVO*

By

Joel Musee

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements of

DOCTOR OF PHILOSOPHY

in

Biochemistry

May, 2011

Nashville, Tennessee

Approved:

Lawrence J. Marnett

F.P. Guengenerich

John A. Oates

Ned Porter

Richard N. Armstrong

Tomorrow may never come
For you or me
Life is not promised
Tomorrow may never show up
For you and me
This life is not promised

I ain't no perfect man
I'm trying to do, the best that I can,
With what it is I have

Put my heart and soul into this…
I hope you feel me
From where I am, to wherever you are
I mean that sincerely
Tomorrow may never come
For you and me
Life is not promised
Tomorrow may never appear
You better hold this very moment very close to you (right now)
Very close to you (right now)
So close to you, So- close to you (your moment in history is right now!)
Don't be afraid, to let it shine

My *Umi* said shine your light on the world
Shine your light for the world to see
My *Abi* said shine your light on the world
Shine your light for the world to see

- *Umi Says* by Dante Smith (a.k.a Mos Def)

For Mummy and Daddy.
I hope I let my light shine on the world.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Lawrence J. Marnett, for fostering such a nurturing environment for learning and science. The level of intellectual stimulation that has pervaded the laboratory over the last few years has been intense and will be sorely missed. I am very grateful to the members of my thesis committee, Professors Richard Armstrong, F. Peter Guengerich, John Oates and Ned Porter, for their patience and advocacy. I would also like to especially thank Professors F. Peter Guengerich and Richard Armstrong for allowing me use their equipment and conduct hours upon hours of experiments in their lab space. Thank you for accommodating me. I am also thankful to Professor Bruce Carter, for entertaining my ideas and allowing me to work with his graduate student Jami Schieb. I am extremely glad that I am at Vanderbilt, where this camaraderie seems to be the norm and wonder what would have become of all this work, had it not been for everyone's generosity and willingness to help. These experiences have been formative and I will take them with me wherever I go.

I am also grateful to the entire Marnett lab, in particular the core staff: Brenda Crews, Jashim Uddin, Philip Kingsley and Kebreab Ghebresalasie. None of what I did would have been possible without your help, advice, and willingness to entertain outlandish ideas and for stimulating lunchtime conversations. I am most grateful to Kelsey Duggan, who brought me on board her crystallography project and what a success that has been! I am also thankful to Dan Hermanson for taking what was left of the DRG project and making it his own, and doing such an outstanding job! I am also eternally grateful to Jashim Uddin and Andy Liedtke for spending so much time generating compounds that I pulled out of thin air, and spending so much time on something they

were not sure would work. Thank you. I am also extremely indebted to Carol Rouzer, who supervised me when I was a rotation student in the lab, and showed me how deep the rabbit hole could really go. Carol's careful reading of everything I wrote has helped me improve my technical writing skills. Her words of encouragement during the doldrums of graduate school really made a difference.

I am also thankful to all biochemistry department and VICB administrative staff, especially Marlene Jayne, Peggy Fisher and Celeste Riley. Ensuring I received my course credits and was registered properly, paychecks and access to grant money, respectively. It would have been impossible without their often-unrecognized hardwork.

I am also thankful to my friends and family especially my parents, I cannot express how much it has meant to have the puissance of your goodwill during this time, it has helped me sail through this turbulent sea. I am especially thankful to Professors Maureen McKnabb, Richard Woodruff and Gustave Mbuy at West Chester University, I would not be here if you did not believe in me. Thank you for giving me my first chance to work in a lab almost 9 years ago. I stand on the shoulders of giants. To Professors Dennis Luck, William Fuchsman, Marta Laskowski and Yolanda Cruz, thank you for keeping the embers going, the years at Oberlin were so inspirational thank you for nurturing me.

Lastly, I would like to thank my best friend and wife, where would I be without you? Thank you for taking all the gripe, late nights and weekends in the lab in stride. The journey would not have been as enjoyable without you. Unfortunately, my work schedule will probably get far worse, for this I apologize.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

8-IsoP	8-isoprostane
AG	arachidonylglycerol
AA	arachidonic acid
AEA	anandamide (arachidonoyl ethanolamide)
BODIPY	boron-dipyrromethene
COX	cyclooxygenase
COXib	PGHS-2 selective inhibitor
CNS	central nervous system
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DMEM	Dulbecco's modified Eagle medium
DRG	dorsal root ganglion
-EA	ethanolamide
EPA	eicosapentaenoic acid
ERAD	endoplasmic reticulum associated degradation system
FAH	fatty acyl
FBS	fetal bovine serum
-G	glycerol ester
GPx	glutathione peroxidase
GPCR	G-protein couple receptor
GSH	glutathione
HpETE	hydroperoxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
IFN	interferon
IL	interleukin
IP_3	inositol *tris*-phosphate
LC	liquid chromatography
LOX	lipoxygenase
LPS	lipopolysaccharide
MS	mass spectroscopy
PGHS	prostaglandin H synthase
PL	phospholipase
POX	peroxidase
NArPE	*N*-arachidonoylphosphatidylethanolamine
NMR	nuclear magnetic resonsance
NSAID	non-steroidal anti-inflammatory drug
OVLT	organum vasculosum lamina terminalis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG	prostaglandin
PGES	prostaglandin E synthase
PG-EA	prostaglandin ethanolamide

PG-G	prostaglandin glyceryl ester
PKC	protein kinase C
PNS	peripheral nervous system
PPA	5-phenyl-4-pentenyl alcohol
PPAR	peroxisome proliferator activated receptor
PPHP	5-phenyl-4-pentenyl hydroperoxide
RT	retention time
RVM	rostral ventral medulla
SRM	selective reaction monitoring
THC	tetrahydrocannabinol
TNF	tumor necrosis factor
Tx	thromboxane
UV	ultraviolet
γ- GCS	γ-glutamylcysteine synthase

CHAPTER I

INTRODUCTION

Prostaglandin H Synthases, their Substrates and Products

Historical Overview

Prostaglandin H synthases (PGHS-1 and PGHS-2) also known as cyclooxygenases (COX-1 and COX-2) are enzymes that oxygenate fatty acid substrates (FAH) such as arachidonic acid (AA), leading to the generation of prostaglandins. Prostaglandins mediate a wide array of physiological effects, including pain, inflammation, fever, vascular homeostasis, and parturition. PGHS is the target of non-steroidal anti-inflammatory drugs (NSAIDs), which prevent AA oxygenation and thus prostaglandin generation. The role of PGHS in mediating fever, pain and inflammation has unwittingly made PGHS perhaps the oldest documented drug target known to man. It was documented in Ebers's papyrus in 1500 BC, that the Egyptians used NSAID containing willow bark extract to treat fever (1). The importance of abating fever is evident in the convergent discovery by ancient denizens of separate continents; Romans, Native Americans, Chinese, and South African Hottentots had all established that fever could be reduced by NSAID-containing willow or myrtle leaf extracts (2-4). The first scientific record of NSAID use was by the Reverend Edward Stone, who reported to the Royal Society of London in 1763 that powdered willow bark improved the condition of fifty of his patients who were suffering from maladies manifesting fever and shivering- a condition archaically known as agues (4). Sixty-three years later, the French pharmacist

Henri Leroux isolated the active ingredient in willow bark and myrtle leaves, and named it salicin based on the genus of the willow plant (*Salix*). Leroux further demonstrated that it was salicin that effected willow bark's antipyrexia (5). Salicin is a glycoside that is hydrolyzed to salicylic alcohol, which can be oxidized to form salicylic acid (1) (Figure 1). Thomas MacLagan, a Scottish physician, conducted the first formal clinical trial on salicin in 1874. MacLagan, having experienced no untoward effects after consuming two grams of the compound, administered it to his patient with rheumatic fever and successfully reduced the patient's fever, inflammation, and pain (6). That same year, Lautemann and Kolbe developed a chemical process by which they could generate salicylic acid at a fraction of the cost of extracting it from willow (3). It could even be argued that the rise of NSAIDs led to the birth of the pharmaceutical industry; the first drug factory was built to mass-produce salicylic acid (7). Although salicylic acid soon became the drug of choice for treating fever, pain, and inflammation, its bitterness and gastric side effects led to poor tolerance in some. In 1897, Felix Hoffman- a chemist at the Friedreich Bayer and Company- generated a more palatable form of salicylic acid by acetylating the alcohol on the phenol ring to form acetylsalicylic acid or aspirin (Figure 1) (1). The connection between the effects of NSAID use and the COX enzymes would not be made for another 70 years.

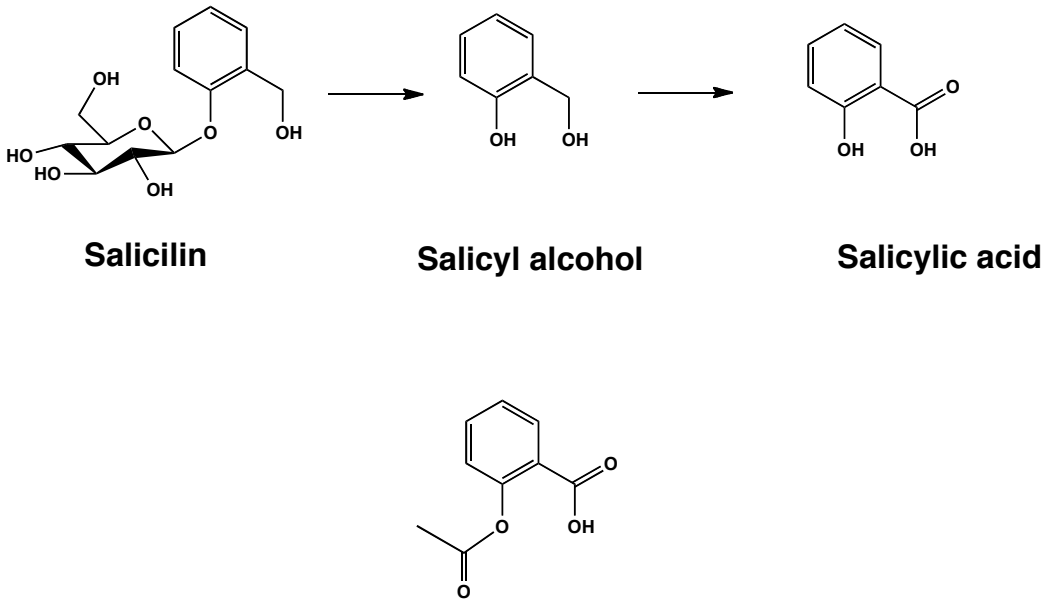


Figure 1. Salicin and its derivatives

Prostaglandins were first discovered in 1930 by two gynecologists Raphael Kurzok and Charles Lieb, as an unidentified substance in semen, that when applied to uterine muscle caused it to contract (8). Over the next few years, Von Euler and Goldbatt independently demonstrated that plasma from semen and vesicular glands possessed an array of potent biological activities (9-13). Von Euler, convinced that the prostate was the source of these substances, christened them prostaglandins. Bergström and Samuelsson elucidated the structures of these new lipid derived molecules, and correctly proposed the mechanisms of their generation. Since the structures they had described contained functionalized cyclopentane rings and the oxygen atoms originated from molecular oxygen, they aptly named the enzyme that catalyzed the reaction cyclooxygenase (COX) (14). A few years later, the connection between NSAIDs and cyclooxygenase was made by Willis and Smith, who demonstrated that treating platelets with aspirin prevented

clotting along with inhibition of prostaglandin formation (15), and the late Sir John Vane, who demonstrated that NSAIDs could diminish the production of prostaglandins in guinea pig lung homogenates (16). Drawing on the well documented action of NSAIDs and these new discoveries, Sir John Vane proposed that inhibition of prostaglandin generation was how NSAIDs mediated analgesia and anti-pyrexia (16). Bergström, Samuelsson, and Vane shared the 1982 Nobel Prize in medicine for their seminal work on prostaglandins and the action of NSAIDs.

The last few decades have seen an explosion in the amount of PGHS-related research. This is especially evident in the early 1990s, when PGHS-2 was discovered as a separate isozyme (17,18). It has become clear that this new protagonist had been surreptitiously engaged in soliloquy; playing an unappreciated role as a mediator of acute inflammation, pain, and cancer progression. Despite our wider understanding of PGHSs and their products, it is now more evident that the roles of prostaglandins in homeostasis is under-appreciated and poorly understood. The lack of understanding is reflected in our inability to explain why taking NSAIDs predisposes patients to increased risk of cardiovascular events (19), despite the fact that the roles of prostaglandins in vascular homeostasis have been well outlined. This indicates that the PGHS field remains a rich loam, awaiting the prepared to sew seeds of discovery.

Prostaglandin H Synthases

Structure and biochemistry

PGHSs are ~70 kDa heme-bearing membrane proteins, that are usually found as ~140 kDa homodimers (20-22). However, a report of functional PGHS-1 and PGHS-2 heterodimers, *in vivo*, has been demonstrated in mice (23). PGHSs bear three domains. Starting at the N- terminus is an epidermal growth factor (EGF)-like domain with an unknown function, a membrane-binding domain that is used to insert the functional homodimer monotopically into the lipid bilayer, and a catalytic domain housing the enzyme's spatially distinct peroxidase (POX) and cyclooxygenase (COX) functionalities (Figure 2) (24,25). PGHSs are evolutionarily related to myeloperoxidase (26). However, unlike myeloperoxidase, PGHSs bear an L-shaped hydrophobic fatty acyl (FAH) binding site that forms the COX active site (27) (Figure 2).

Biochemistry of PGHS

PGHSs carry out the stereo-controlled, *bis*-dioxygenation of polyunsaturated FAHs. Its primary substrate *in vivo* is arachidonic acid (AA), which is released from phospholipid stores by the action of various phospholipases on glycerophospholipids. Oxygenation of AA leads to the formation of an unstable 20 carbon hydroperoxy-endoperoxide known as prostaglandin G_2 (PGG_2), which diffuses out of the COX active site to the peroxidase active site where it is reduced to an endoperoxide alcohol, prostaglandin H_2 (PGH_2) (27) (Figure 4). Formation of PGG_2 in the L-shaped hydrophobic core forming the cyclooxygenase active site (Figure 3), affords stereochemical and regiochemical control

to the addition of the two molecules of oxygen to the FAH backbone, transforming an achiral substrate into one with five chiral centers (Figure 4).

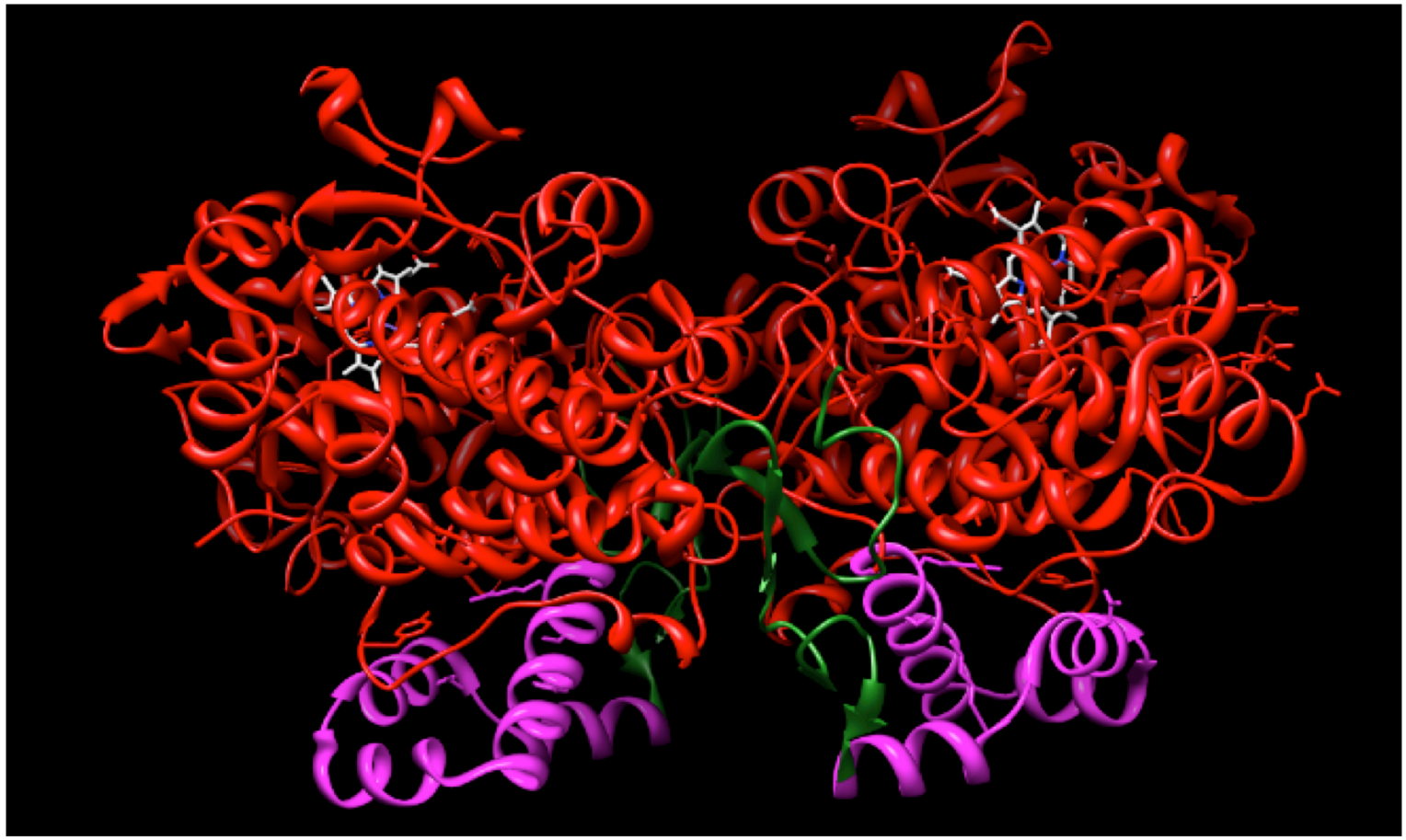


Figure 2. X-ray crystal structure of murine PGHS-2 with annotation of the domains- Red-Catalytic domain, bearing both the heme-associated peroxidase active site and the structurally distinct oxygenase active site, Green- EGF like domain, Lilac-Membrane binding domain, serves to anchor the protein monotopically in the lipid bilayer

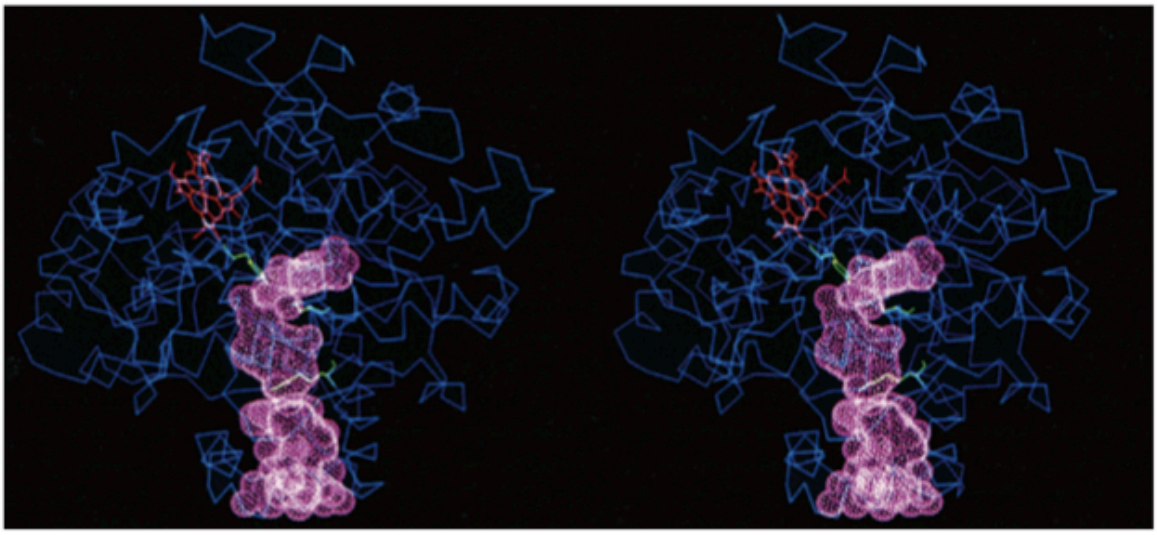


Figure 3. Stereodiagram of PGHS-1's hydrophobic channel demarcating the COX active site. reproduced(27)

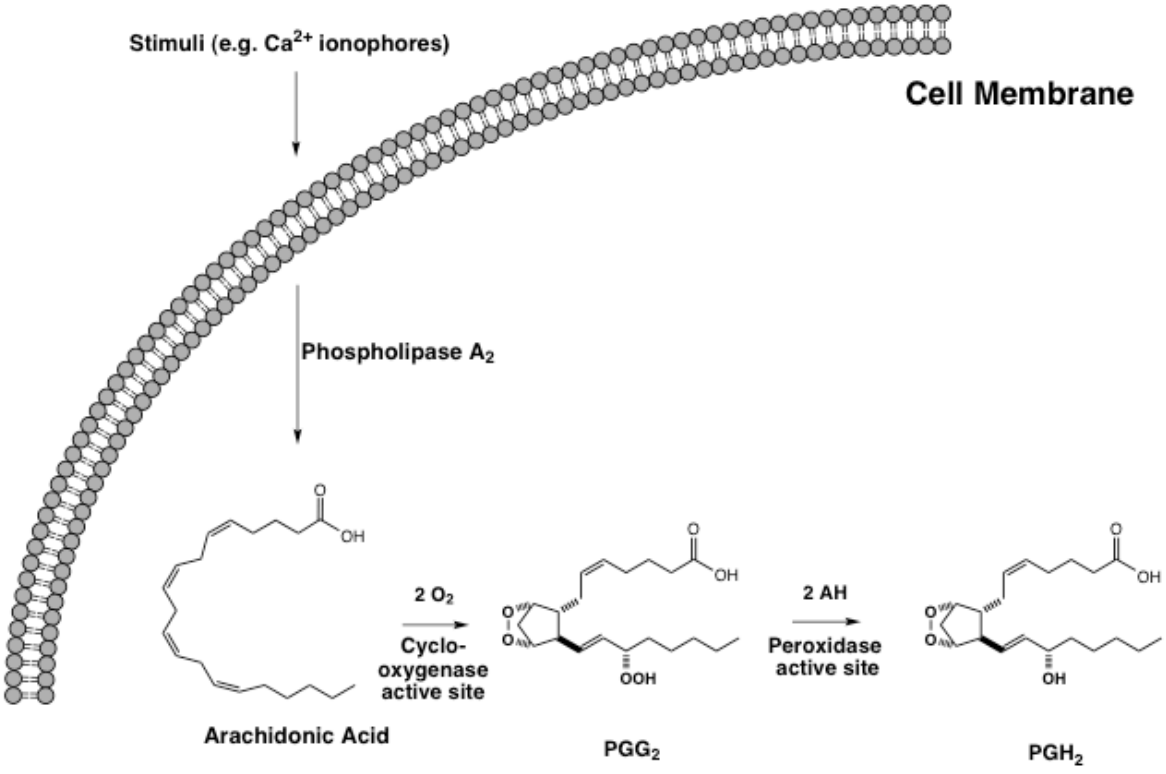


Figure 4. The prostanoid synthetic pathway

The mechanistic linkage of the POX and COX active site was proposed as the branched chain mechanism by Dietz et al. (1988) (28). This mechanism, which gives PGHS elegant control over the process of FAH oxygenation, has been validated by the majority of experimental observations and is represented in its simplest form, for substrate AA, in Figure 5. The POX active site can function independently of the COX active site (29); however, PGHS's COX activity is latent until the peroxidase active site heme [(PPIX)Fe^{3+}] undergoes a two-electron oxidation, leading to the formation of an ferryloxoprotoporyphin cation radical [(PPIX)$^{\bullet+}Fe^{4+}$ O] (Figure 5, Step A). Similar to other peroxidases, two subsequent one-electron reductions of the ferryloxoprotoporyphin cation radical [(PPIX)$^{\bullet+}Fe^{4+}$ O] return the heme to its resting state, [(PPIX)Fe^{3+}] (Figure 5). Reducing co-substrates, such as phenol, epinephrine, and ascorbate, are oxidized in this process, and it is this necessity for a reducing co-substrate that links the POX and COX active sites (30). The catalytic residue in the COX active site, Tyr-385, can serve as the reductant (E_o'= 0.9 V) for the first one-electron reduction of the ferryloxoprotoporyphin cation radical [(PPIX) $^{\bullet+}Fe^{4+}$ O] (E_o = 1.0 V), a thermodynamically favorable reaction, leading to the formation of a tyrosyl radical at Tyr-385 (Figure 5, Step B)(31). This step is *the* critical connection between the POX and the COX active sites of PGHS. The Tyr-385 radical is critically positioned in the COX active site, enabling it to abstract a hydrogen atom from the 13-*pro*-(*S*) position of the backbone of AA (E_o 0.6 V) (Figure 5, Step C), another thermodynamically favorable event (32,33). The formation of a radical at the 13-position followed by a series of radical rearrangements along the AA backbone, allows the addition of two molecules of oxygen,

leading to the formation of the 15-hydroperoxyl radical of PGG_2, which is then reduced by Tyr-385, regenerating the Tyr-385 radical (Figure 5, Step D).

Note that, once formed, the Tyr-385 radical can carry out COX functionality, independently of the POX active site (34). However, a requirement for hydroperoxides for continuous COX activity has been observed (35-37). It appears that the Tyr-385 radical can be reduced to its latent state, a reaction competing with the abstraction of the 13-*pro*-(*S*) hydrogen from the AA backbone (Figure 5, Step E). This indicates that the continuous presence of hydroperoxides in necessary to reoxidize the heme as needed to reactivate the Tyr385 catalytic residue.

Studies examining the paradoxical existence of a peroxide-depleting functionality (POX) and a peroxide-dependent functionality (COX) in PGHS led to the demonstration that the POX activity of PGHS is easily suppressed in the presence of competing peroxide reducing glutathione peroxidase (GPx), while the COX activity of PGHS is refractory to this suppression (35,38,39). This was the first indication that there was a distinction between the concentration of peroxide required for half-maximal activity (K_m) of the POX activity of PGHS and the concentration of peroxide required to activate the COX functionality of PGHS. This latter concentration, is consequently much lower than the K_m for the POX active site and has been coined K_p (39). The K_p is a relative value that is determined by factoring in the K_m of the peroxide in question for the POX active site of PGHS, and the slope of the decay of oxygenation in the face of increasing GPx concentrations. These values have been measured for PGHS-1 with AA as the COX substrate and PGG_2 as the POX substrate. Whereas the K_m for PGG_2 at the POX active site of PGHS-1 is about 2 μM, the K_p for AA oxygenation is 0.2 μM (39). Part of the

work described herein describes how this phenomenon can come into play when examining the metabolism of non-AA substrates.

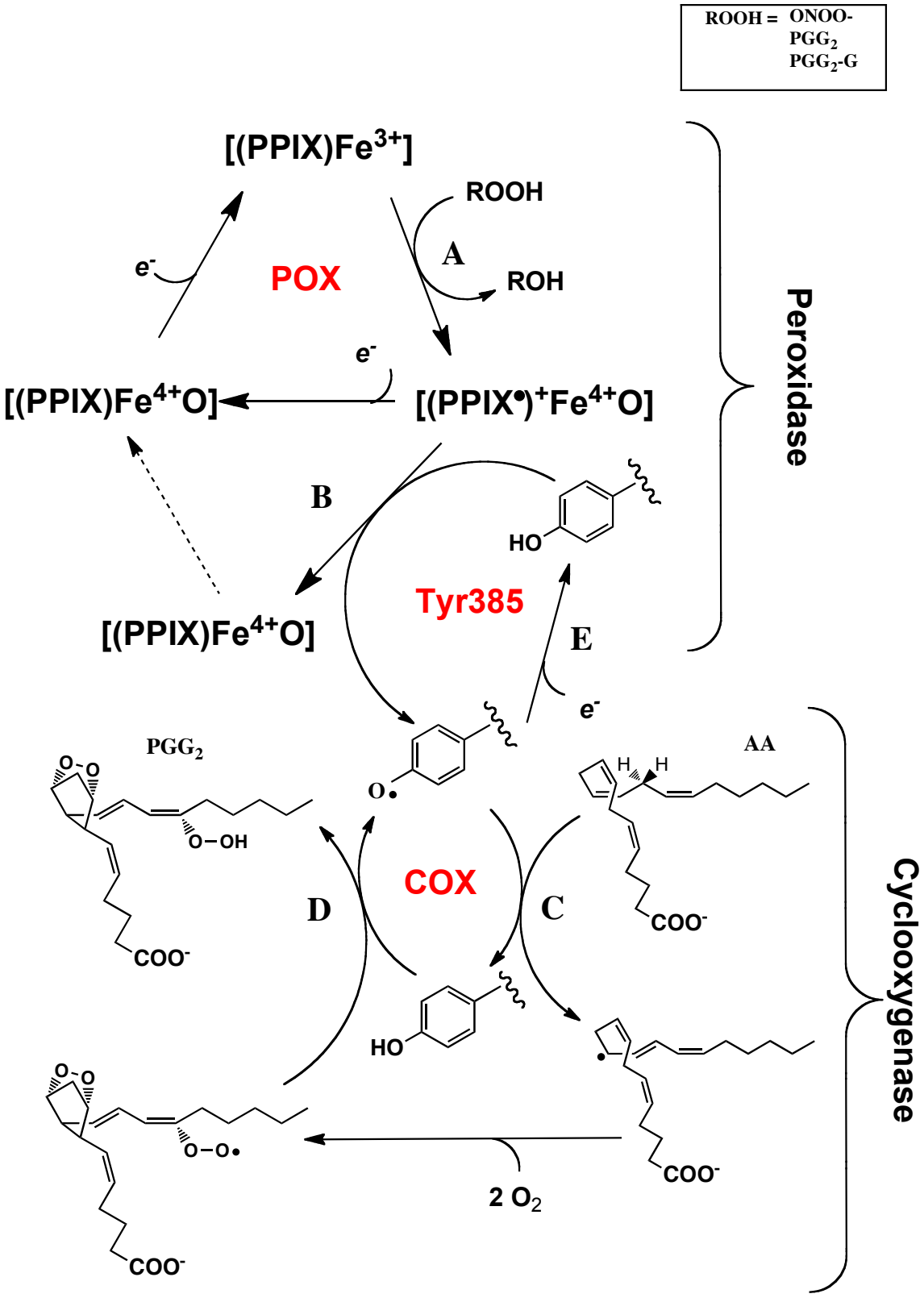


Figure 5. The Branched Chain Mechanism and POX and COX mechanism of PGHS

Interestingly, PGHS also undergoes a first-order irreversible inactivation that is turnover-dependent (40). This phenomenon is poorly understood and is thought to represent a reaction of the protein with a catalytic intermediate, leading to modification of the enzyme (41). The nature of the modification is yet to be identified; however the culprit in the inactivation of the peroxidase active site has been identified as the ferryloxoprotoporyphin cation radical and not the Tyr-385 radical (42). Regardless, it is uncertain whether inactivation is a factor *in vivo*, since reactions with PGHS *in vitro* are carried out with excess substrate and low concentrations of PGHS. Furthermore, the cellular milieu of PGHS differs markedly from the *in vitro* conditions under which this phenomenon is observed. In fact, the presence of reductants has been shown to protect PGHS from oxidative inactivation (30).

There are two isoforms of PGHS, PGHS-1 and PGHS-2. The discovery of the second isoform in the 1990s was exciting and complicated the prostanoid field even further. PGHS-1 and PGHS-2 from the same species share 60-65% sequence identity, and similar isoforms from different species are 85-90% identical. As would be expected from such high sequence homology, the X-ray crystal structures of PGHS-1 and PGHS-2 are virtually superimposable; the backbone atoms demonstrate a meager 0.9-Å root mean square deviation of (25). Despite the high degree of sequence homology, key structural differences between PGHS-2 and PGHS-1 lead to functional differences between the two enzymes. For example, PGHS-2 (599 amino acids) is 19 amino acids larger than PGHS-1 (580 amino acids) as the result of an extra 19 amino acids in PGHS-2 located at the C-terminus of the protein. This short stretch of amino acids direct PGHS-2 to the endoplasmic reticulum associated degradation system (ERAD), where proteins that are

toxic or misfolded are ubiquitinated and subsequently degraded via the *26 S* proteasome pathway (43). Thus, PGHS-1, which lacks this sequence has a half-life ($t_{1/2}$) > 12 hours in NIH/3T3 fibroblasts, whereas PGHS-2 has a $t_{1/2}$ of about 2 hours (43). PGHS-1 and PGHS-2 also differ in their post-translational modifications. PGHS-1 is N-glycosylated at three positions, whereas PGHS-2 can be variably glycosylated at two to four positions. One of these, Asn-594, is associated with the ERAD degradation pathway discussed above. Mutation of this residue to an alanine (Asn594Ala) decreases the turnover of PGHS-2 via the ERAD pathway (44).

PGHS-1 and PGHS-2 also differ in the regulation of their expression. PGHS-2 is undetectable under normal conditions in most tissues, but is inducible by a number of stimuli, including, inflammatory cytokines (e.g. TNF, IL-1β), and growth factors (e.g. PDGF, EGF) and tumor promoters (e.g. phorbol ester, benzo[α]pyrene) (27,45), implying a key role in the mediation of fever and inflammation. PGHS-1 is expressed constitutively in most tissues. Thus, PGHS-1 is ascribed the role of homeostatic prostanoid generation, such as gastric cytoprotection and platelet aggregation. However, PGHS-2 is also expressed constitutively in the kidney, brain and spinal cord.

A third important structural and perhaps functional difference between the isoforms is that the PGHS-2 COX active site is about 25% larger than PGHS-1's (24,25,46). This difference in size is due to a single amino acid substitution; PGHS-2 has a less bulky Val-523 versus an Ile-523 in PGHS-1. This affords a space extending off of the AA binding site that is coded in all PGHS-2 genes sequenced to date. This space allowed for the development of PGHS-2 selective inhibitors, the diarylheterocycles, which were 1000-fold more selective for PGHS-2 than PGHS-1. By sparing PGHS-1

inhibition, the diarylheterocycles had reduced gastrointestinal side effects compared to non-selective traditional NSAIDs, but were potent inhibitors of PGHS-2 mediated pain and inflammation. Site-directed mutagenesis of Val-523 leading to the elimination of the side-pocket leads to a loss of inhibition by the diarylheterocycles. The reverse is true, with creation of a side-pocket in PGHS-1 leading to a sensitization to diarylheterocycle inhibition (46-49).

PGHS-1 and PGHS-2 are both acetylated by aspirin at Ser-530 (50,51). Although PGHS-1 is rendered inactive by aspirin acetylation, acetylated PGHS-2 can still oxygenate AA to form 15*(R)*-hydroxyeicosatetraenoic acid, instead of PGH_2 (52,53). Site-directed mutagenesis eliminating the PGHS-2 COX side-pocket abrogates the ability of asprin-acetylated PGHS-2 to oxygenate AA(54). This indicates that aspirin-acetylated PGHS-2 accommodates AA in its COX active site by taking advantage of the additional space afforded by the side-pocket (54).

An interesting functional difference between PGHS-1 and PGHS-2 is seen in their their sensitivity to activation; PGHS-2 is activated by concentrations of peroxide 10-fold lower than those required to activate PGHS-1 (35). Although the structural foundation for this remarkable difference is not known, it indicates that PGHS-2 can be active at low peroxide concentrations while PGHS-1 remains latent, a phenomenon that may provide differential control of catalysis between the two isoforms (35).

Prostaglandins

PGH_2 is a substrate for five downstream synthases that lead to the generation of the prostanoids, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TxA_2 (Figure 6). Prostanoids act in an

autocrine and paracrine fashion, through separate G-protein coupled receptors (GPCRs), eliciting a broad array of effects that are cell and tissue dependent (Figure 6). A brief description of the major *in vivo* actions of the prostanoids follows.

Pyrexia, Pain and Inflammation

The role of prostaglandins in effecting pyrexia was discovered when an infusion of a PGE_2 analog, PGE_1, into the third ventricle of the cat brain was shown to cause elevations in body temperature(55). PGE_2 has been shown to act through four EP receptors ($EP_{1\text{-}4}$), in mediating its downstream effects. PGE_2, which is the major AA oxygenation product, can agonize abundantly expressed EP_3 receptors in the organum vasculosum lamina terminalis (OVLT) an area connected to the anterior hypothalamus, and devoid of the blood-brain barrier (56,57). This area is critical to regulation of the body's temperature set point, and EP_3 agonism leads to an elevation in the body's temperature set point. Fever soon ensues, and what is known as the febrile response. A key role for PGHS-2 is effecting the febrile response. While PGHS-2 expression in the brain is mostly constitutive, endothelia in the brain's vasculature and glia in the brain can be induced to up-regulate PGHS-2 and PGE synthase (PGES) production in response to blood-borne pathogens and inflammatory cytokines, leading to the generation of PGE_2 (58-60). Convincing evidence for PGHS-2's role in mediating pyrexia is the fact that PGHS-2 selective inhibitors, such as the diarylheterocylces, are anti-pyretic (61). Furthermore, PGHS-2-null and EP_3-null mice *cannot* mount the febrile response in response to cytokines or bacterial lipopolysaccharides, while the febrile response remains intact in PGHS-1-null mice (56,57,61).

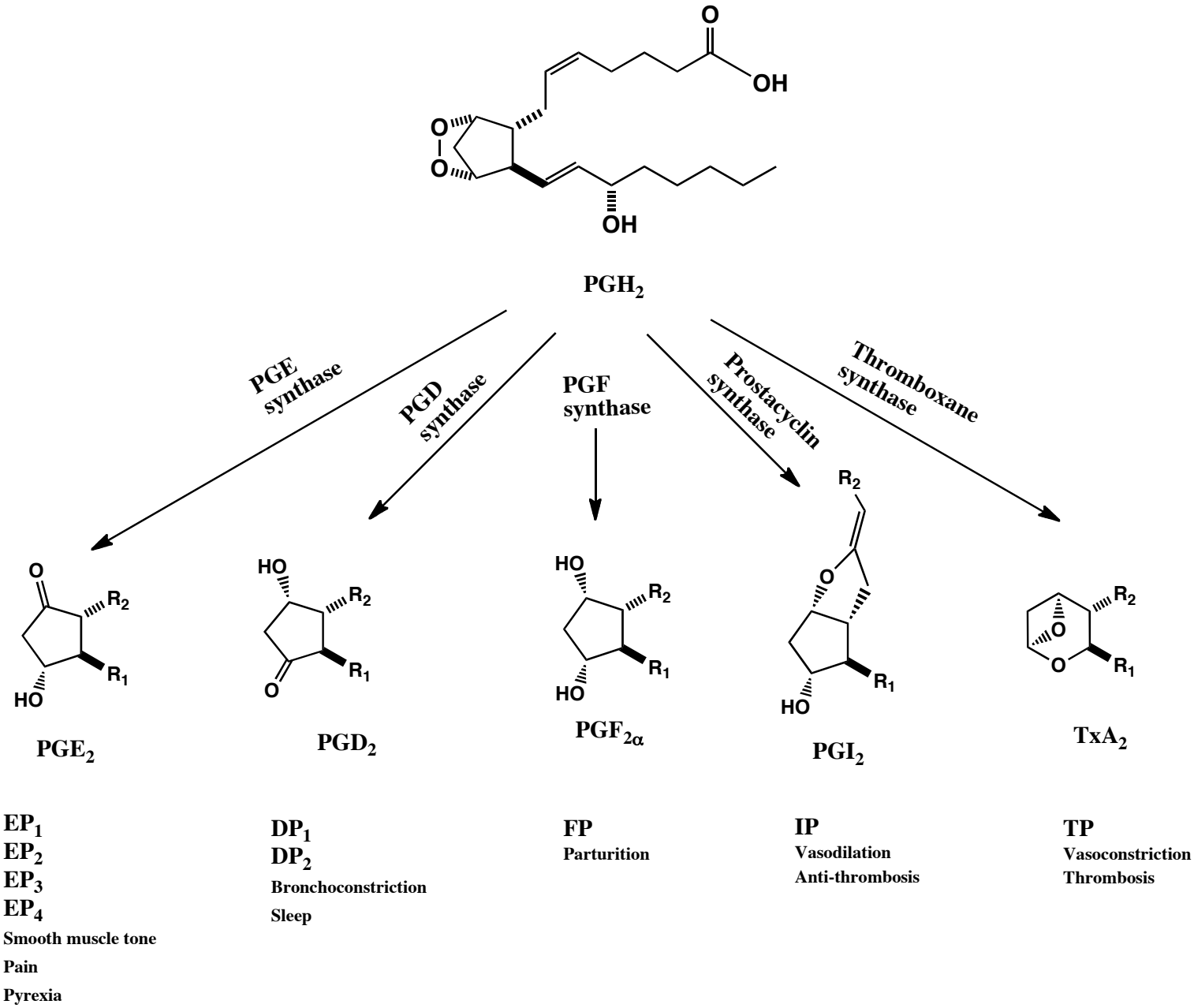


Figure 6. Prostanoid biosynthesis, downstream GPCRs and selected effects of GPCR agonism

Following the demonstration that NSAIDs inhibited prostanoid biosynthesis, Sir John Vane concluded that the production of PGE_2 or a similar prostanoid in the brain was responsible for the febrile response. He further proffered that the analgesic action of NSAIDs was also through the inhibition of prostanoid production, despite the fact that there was direct evidence that local adminstration of prostanoids did not cause much more than a "weal and flare"(16). While it may have been difficult to demonstrate that prostanoids directly effected pain, he correctly ascribed local inflammation to PGE_2, as it had been isolated from wound exudate from carrageenan inflammation in the rat (62). This said, inflammation usually precedes pain, and it has been recently demonstrated that prostanoids, especially PGE_2, cause a sensitization of both peripheral and central pain pathways (63,64). Tissue damage can cause the release of inflammatory cytokines such as TNFα and IL-1β, that cause an induction of PGHS-2 expression which leads to the generation of PGE_2, eventuating in increased peripheral synaptic excitability and firing (64-66). In a feed-forward mechanism, increased peripheral firing, induced by PGE_2, causes an induction of excitoxicity in upstream neurons in areas critical for the modulation of pain; the dorsal root ganglia and the dorsal horn of the spinal cord (64,67). This series of events leads to hyperalgesia, which is an increase in the gain in the pain system leading to an exaggerated pain response, and allodynia, which is a pain sensation in response to non-pain evoking stimuli (63). The involvement of the PGHS pathway in the amplification of pain will be revisited in the discussion of the endocannabinoid system.

PGHS-2 has been found to be elevated in the mucosa of asthmatics, leading to exuberant amounts of PGD_2 in lung tissue and the pathognomonic hyper-reactive airways

upon allergen exposure. PGD_2 agonism at DP_1 receptors effects contraction of smooth muscle cells found in lung airways leading to the airway constriction characteristic of asthma. This is evident in mice lacking the DP_1 receptor that are resistant to allergens that mediate asthma (68). Unfortunately, NSAIDs do little to ameliorate asthma, and in fact, make symptoms worse in 10% of asthmatics (69) . It is accepted that interaction of the prostanoid pathway with another AA consuming pathway, the leukotriene pathway, explains why diminution of PGD_2 levels in the lung does little to prevent asthma (69).

Reproduction

While the prostanoids were initially discovered via their action on reproductive tissue, we are now just now beginning to appreciate the complex role of the prostanoid pathway in reproduction. In mammals, PGE_2 is important for the effective maintenance of pregnancy; in fact, mice lacking the EP_2 receptor manifest diminished fertility (70-72). While a clear connection between the agonism of EP_2 receptors by PGE_2 and fertility is not as clear, PGE_2 is produced in the hypothalamus and can modulate the production of the gonadotropin, luteinizing hormone, which surges during the menstrual cycle leading to ovulation (70). $PGF_{2\alpha}$ is critical for the birth of live young. Its ability to contract uterine tissue was discovered when semen was shown to induce uterine contractions (8). $PGF_{2\alpha}$ levels in pregnant women are greatly elevated just prior to parturition and its administration to women at the term of their gestation leads to induction of labor (73,74). Mice lacking the FP receptor bear defects in parturition (75). The production of $PGF_{2\alpha}$ at the term of gestation leads to the dissolution of the corpus luteum, which is critical for the maintenance of pregnancy to term. The dissolution of the corpus luteum leads to a drop in

progesterone and the induction of the oxytocin receptor in the uterus. This leads to increased sensitivity of the uterine myometrium to the induction of contractions in response to oxytocin. Interestingly, PGHS-2–null female mice are infertile (76), while PGHS-1–null females demonstrate normal fertility. However, while PGHS-2–null mice demonstrate defects in ovulation, fertilization and implantation, PGHS-1–null mice lack these defects but exhibit a higher than usual perinatal morbidity (77,78). Thus, a simplistic view of the roles of the two isoforms in reproduction is appreciated. PGHS-2 is involved in the early events in pregnancy, while PGHS-1 plays a role in later events, especially those around parturition.

Cardiovascular and Renal

The prostanoid pathway's role in maintenance of vascular homeostasis is widely appreciated. This was first evident following the discovery that PGE_2 and PGE_1 could cause platelet aggregation (79-81). It has now been demonstrated that the actions of vascular endothelium-derived PGI_2 and platelet-generated TxA_2 strike a balance between vasodilation/anti-thrombosis and vasoconstriction/thrombosis that maintains patency of the body's vasculature under normal conditions. Mice deficient in the TxA_2 receptor (TP) demonstrate increased bleeding times and cannot induce platelet aggregation in response to appropriate agonists (82). However, mice deficient in the PGI_2 receptor (IP) do not demonstrate any immediate defects in bleeding times; however, vascular endothelial damage dramatically increases thrombogenesis (83). Platelets are anuclear cell fragments of the hematological precursor cell, the megakaryocyte. Platelets therefore do not posses the machinery to express more protein than they already contain. This leads to their

relatively short $t_{1/2}$ of 5-9 days. Furthermore, platelets do not contain any PGHS-2 so PGHS-1 is the sole source of the TxA_2 generated in platelets. PGI_2 is made upon damage to vascular endothelium, chiefly from the action of PGHS-2. The finding that un-challenged PGHS-2–null mice demonstrate no defects in bleeding further supports this. The use of controlled release, low-dose aspirin leads to selective and irreversible inhibition the PGHS-1 in platelets; this results in inhibition of TxA_2 production for the life of platelet and systemic anti-thrombosis due to the shift in the vascular prostanoid profile, favoring the production of PGI_2 over that of TxA_2. Controlled release, low-dose aspirin is a gold standard for prophylaxis of thrombosis in susceptible cohorts, such as those who have suffered myocardial infarction (84,85).

PGE_2 is involved in maintenance of vascular tone related to salt intake (72). EP_2–null animals manifest profound hypertension in response to a high salt diet. This suggests that aberrations in PGE_2 might be to blame for salt sensitive hypertension (72).

PGE_2 and possibly PGI_2 play a role in vascular remodeling at birth. Closure of the ductus arteriosus (DA) in a newborn fetus is required to block the shunt between the pulmonary artery and the aorta. This shifts oxygenation of blood in the baby from the placenta to the lung. Premature closure of the shunt can lead to congestive heart failure, and failure to close *postpartum* leads to mixing of oxygenated and deoxygenated blood, and a decreased capacity to effectively oxygenate blood. NSAIDs can be used to initiate the closure of the DA or conversely, patency can be maintained by use of PGE_1 (Alprostadil) (86). The role of PGE_2 in vascular remodeling is demonstrated by the fact that EP_4–null mice demonstrate a patent DA at birth (87).

While prostanoids have been known to play a major role in renal physiology, there has been no real delineation of the exact mechanism by which this occurs. Their importance is highlighted by the roles of vasodilatory PGE_2 and PGI_2 under conditions of compromised renal function as occur in renal insufficiency, glomerular disease, liver cirrhosis, and congestive heart failure (88,89). Under these conditions, NSAIDs have been shown to cause renal ischemia leading to renal failure by blocking the synthesis of the vasodilatory prostanoids, which serve to maintain renal blood flow (88,89). Thus, the prostanoid pathway acts along with the renin-angiotensin system in maintaining renal physiology especially in the presence of underlying pathology. It is worth mentioning that PGHS-2–null mice have renal developmental problems, with kidneys demonstrating few, poorly developed nephrons and mesenchyma (76,78,90).

Neoplasia

The potential role of PGHS in neoplasia was first demonstrated by the finding that NSAIDs were chemopreventative in animal models of colon cancer (91,92). These findings were extended in humans with the demonstration that sulindac, an NSAID, reduced the burden of precancerous polyps in patients with familial adenomatous polyposis coli, a genetic condition that invariably leads to colorectal cancer (93,94). Retrospective studies on the use of aspirin in a very large cohort (660,000) led to the clear demonstration of a link between aspirin use and a decrease in mortality from colon cancer (95). Other NSAIDs have also been shown to decrease mortality from colorectal cancer (96-98). Furthermore, it has been demonstrated that PGE_2 is elevated in colorectal tumors and in the urine of cancer patients (99-101). This suggests that the prostanoid

pathway in colorectal tumors is deranged, allowing for the production of large amounts of PGE_2, consistent with the finding of constitutive over-expression of PGHS-2 in these tumors (102). PGHS-2 generates PGE_2, which has been shown to enhance the growth of colorectal cancer cells by promoting angiogenic endothelial cell growth and blood vessel formation (103,104). Targeting the overexpression of PGHS-2 in colon cancer led to the initiation of clinical trials of PGHS-2 selective inhibitors (COXibs) for the prevention of colonic polyp development or recurrence (105-107). The results were as expected; the COXibs caused a reduction in colon polyp recurrence of up to 24% (105). As exciting as these findings were, they were overshadowed by an almost 2-fold increase in the risk of cardiovascular events in the trial participants who were on COXibs, relative to study participants on placebo (19). This led to the misconception that COXibs carried an increased risk of cardiovascular events over that of non-selective NSAIDs and ultimately led to the premature withdrawal of almost all the COXibs on the market. This fanfare was short-sighted as it has now been shown that ALL NSAIDs carry as great a risk of cardiovascular events as the COXibs (19).

So, despite the wealth of knowledge about the actions of prostanoids, our knowledge about their complex interactions under both homeostatic conditions and pathology is still incomplete. This has recently come to light with the COXib fiasco, where despite attractive hypotheses, it is still not clear why NSAID use leads to increased cardiovascular effects(19).

Glutathione Peroxidases

Structure and biochemistry

There are over 30 different mammalian selenoproteins (108). Of these, four are glutathione peroxidases (GPxs), which carry out the reduction of peroxides using the tripeptide glutathione (GSH, Glu-Cys-Gly) as the reductant (Figure 7).

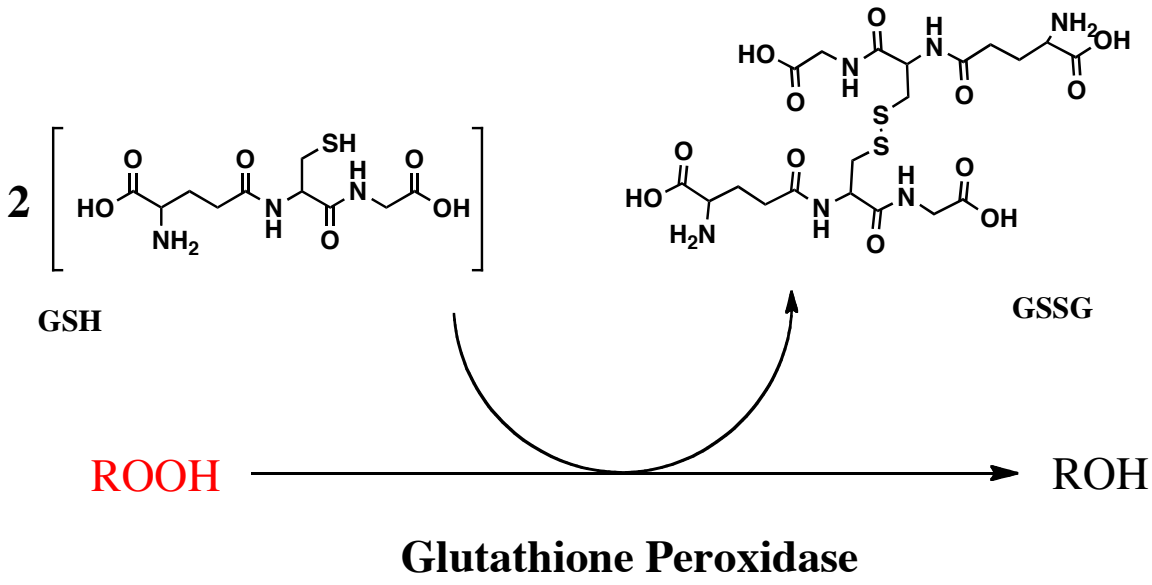


Figure 7. The reduction of a peroxide by glutathione peroxidase

The four mammalian isoforms of glutathione peroxidase are; the classical or cytosolic glutathione peroxidase (GPx1 or cGPx), the gastro-intestinal glutathione peroxidase (GPx2 or GI-Gpx), the plasma glutathione peroxidase (GPx3 or pGPx) and the phospholipid hydroperoxide glutathione peroxidase (GPx4 or PHGPx). Their expression is varied and tissue specific, leading to confusion about each isoform's specific function (109). There is general agreement in the field that they serve to protect the cell from

oxidant stress. Interestingly, there are several chronic degenerative conditions, such as Alzheimer's and Parkinson's Diseases, that are associated with depletion of the GPx reducant, GSH(110).

GPx1

GPx1 was the first glutathione peroxidase to be characterized. Discovered in 1957, GPx1's importance in protecting heme in the erythrocyte from oxidative damage (111) was quickly recognized, and it has subsequently been shown to be expressed at high levels in tissues that have a high rate of peroxide production, such as liver, kidney, and lung (109). GPx1 expression has also been shown to follow metabolic activity. In the newborn rat lung, GPx1 expression is high, especially following exposure to high oxygen tension (112). Interestingly GPx1–null mice develop normally, and only succumb to very high level of oxidant stress (113,114). It appears as though the peroxide detoxification systems in the cell have evolved redundancies to ensure the cell is protected from oxidant stress. Breast, kidney, and prostate cancers demonstrate reduced GPx1 activity (115-118), so despite the apparent lack of an overt phenotype in the GPx1–null mouse, loss of GPx1 functionality is associated with malignancy. Apparently, loss of GPx-1, while not being directly causative, is permissive of other processes that have been shown to be important for tumorigenesis. These include the association of redox stress with high PGHS activity, which as discussed earlier promotes tumor growth and vascularization through the production of angiogenic PGE_2 (99-101,115). The association of increased redox stress with prostaglandin generation is examined as part of this work.

GPx2

GPx2 is expressed exclusively in the gastro-intestinal (GI) system, and is restricted to the epithelium (119). It was initially thought to be the first barrier against ingested hydroperoxides (119), but has now been shown to associate with the highly proliferative cells in GI crypts, where it plays a role in the regeneration of GI epithelium (120). Although GPx2 is upregulated in some malignancies, knock-out mice are more susceptible to tumors induced by UV- irradiation (121) and a combined GPx1 and GPx2 knock-out leads to an increased incidence of intestinal tumors (122). In this model, GPx2's role in the development of intestinal tumors was preceded by the development of an inflammatory condition (colitis), leading to the supposition that GPx2 plays a role in modulating inflammation (122). Transcriptional control of GPx2 expression occurs via the Nrf2 pathway, which is activated by xenobiotics and oxidative stress, serving to induce several cytoprotective enzymes such as γ-glutamylcysteine synthase (γ- GCS)- the enzyme that generates GSH, and heme oxygenase- an enzyme involved in the metabolism of heme (123-125). GPx2 has been hypothesized to antagonize PGHS-2-mediated PGE_2 production, and in fact, GPx2 and PGHS-2 are both upregulated in the intestinal epithelium of patients with sporadic colorectal cancer (126). Furthermore, stable siRNA knock-down of GPx2 in a colorectal cancer cell line enhances PGHS-2 and PGE synthase (PGES) expression. It is noteworthy that PGE_2 can act in a paracrine loop, inducing PGHS-2 expression (126). While its exact role is still not known, GPx2's importance both in oxidant homeostasis and cancer is now apparent.

GPx3

GPx3 is the most poorly understood glutathione peroxidase. It is found in plasma where the low GSH concentrations (30 μM) can only support a limited number of catalytic turnovers (127). Its role is therefore unclear. GPx3 is mainly produced in the kidney by proximal tube epithelium and parietal cells in Bowman's capsule (128). Spurring a renewed interest in GPx3's function is the finding that it can use thioredoxin and glutaredoxin as reductants, in lieu of GSH (129). This means that the low plasma GSH levels might not be limiting to its unknown, specific function.

GPx4

The phospholipid hydroperoxide glutathione peroxidase is perhaps the most important GPx of all four. It is the only one required for life, since homozygous GPx4–null mice are embryonic lethal (130). Compared to wild-type littermates, GPx4–null embryos show defective gastrulation and almost no indication of the development of ectoderm, mesoderm, or endoderm (130). GPx4 was initially discovered as a factor in preventing lipid peroxidation (131) and is strongly associated with the lipid bilayer, congruent with its role as the only glutathione peroxidase capable of reducing phospholipid-bound hydroperoxides (132-134). Over-expression of GPx4 in rat basophilic leukemia cells is protective against oxidative and metabolic stressors, such as *t*-BOOH, rotenone, and potassium cyanide (135). Overexpression of GPx-4 has also been shown to reduce apoptotic markers such as mitochondrial cytochrome c release and caspase activation (136). Mice deficient in the enzyme that generates GSH, γ-glutamylcysteine synthase (γ-GCS), die at the same stage of development as GPx4 –null embryos, suggesting that

GPx4 is perhaps the limiting GSH-utilizing enzyme. (137). Primary embryonic fibroblasts from conditional GPx4-null mice demonstrate overt lipid peroxidation, compared to wild-type cells, that is associated with a high rate of apoptosis. The lethality of the mutation of GPx4 is also associated with the metabolism of AA by enzymes known as lipoxygenases (LOX). LOXs are a diverse group of non-heme iron-containing enymes that can carry out regio and streospecific oxygenation of FAH such as AA, forming hydroperoxyeicosatetraeinoic acid (HpETE). LOXs are not restricted to free FAH and can oxygenate phospholipid esterified FAHs (138,139). In a conditional knockout of GPx4, apoptosis is induced by lipid peroxidation that requires the presence of functional LOX (110). Furthermore, specific LOX inhibitors were effective in preventing cell death due to GPx4 depletion. Overt lipid peroxidation and the ensuing apoptosis in GPx4-null fibroblasts were prevented by lipid-soluble α-tocopherol, while water-soluble anti-oxidants could prevent neither lipid peroxidation nor apoptosis. (110).

Of interest to us, is the fact that GPx4 is involved in the modulation of AA metabolism by PGHS (140). As previously discussed, PGHS requires peroxide to activate its oxygenase activity. Depletion of GPx4 in human epidermoid carcinoma cells leads to massive increases in oxidant stress and a doubling of the metabolism of AA by PGHS (140). Conversely, over-expression of GPx4 in rat basophilic leukemia cells leads to a four-fold reduction in stimulable PGD_2 levels relative to wild-type cells (141). This work reveals the most direct connection of a specific GPx to its ability to modulate PGHS activity. Previous work had predicted this finding; rudimentary measurements of the levels of PGHS activity compared to those of GPx in tissue homogenates demonstrated that they were inversely correlated (38). While this work was conducted before the

discovery of PGHS-2 and therefore did not consider the inducibility of PGHS-2, it still established a correlation that has held to date. Tissues that generate large quantities of prostanoids such as monocytes, possess the least total GPx activity, while those that do not generate large amounts of prostanoids, such as the liver, possess the highest total GPx activity (38). Furthermore, the ability of GPx to suppress PGHS has been studied extensively *in vitro*. The relationship between PGHS activation and GPx4 is explored further in this work.

The Endocannabinoid System

Historical Overview

The *Cannabis* plant is a dioecious, flowering herb, indigenous to central and south Asia. Its use, at least for fiber, dates back to 10000 B.C as depicted in this pictogram of two hemp plants, under a shelter (Figure 8) (142). The medicinal use of *Cannabis* in Egypt is documented in Eber's papyrus, dating back to 1500 B.C. (143), but its use in Europe was similar as in Asia, mostly for rope making and fabric. *Cannabis*' alternative applications first started attracting attention when Napoleon's troops returned from Egypt and reported its psychotropic activity; they described it as intoxicating and narcotic (144).

Figure 8. Chinese pictogram of two hemp plants under a shelter, dating back to 10,000 B.C.

Soon thereafter, *Cannabis* extracts were introduced into the medical community and in 1848, the British Pharmacopoeia heralded its analgesic and antispasmodic activities (144). The active constituent of *Cannabis*, a terpenoid derivative Δ^9-tetrahydrocannabinol (THC), was finally identified in 1964 (Figure 9) (145).

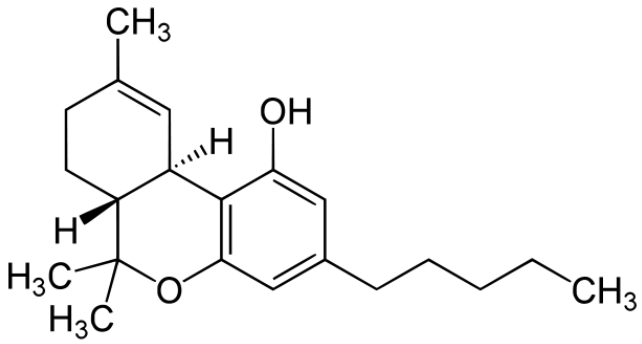


Figure 9. Cannabis' primary psychotropic constituent, Δ^9-tetrahydrocannabinol

THC's structure did not hint at any exact mechanism of action, and it was thought to modulate membrane fluidity, due to its lipophilic nature. The generation of analogs of THC led to a description of areas of the brain that were receptive of the active compounds (146). The cannabinoid receptor (CB_1) was finally cloned from rat brain in

1990 (147). A second isoform, CB_2, that is primarily expressed in the immune system, was cloned by sequence homology three years later (148). The search for an endogenous ligand for the CB receptors led to the discovery of a lipid, which was identified as an ethanolamine amide of AA (arachidonoyl ethanolamine, AEA) (Figure 10) (149). It was christened anandamide from the Sanskrit word for bliss, *ananda* (149). Soon thereafter, another endogenously produced lipid CB ligand was discovered. It was a glyceryl ester of AA, 2-arachidonoyl glycerol (2-AG) (Figure 10) (150). Although a number of other lipids produced *in vivo* have been shown to agonize CB receptors, AEA and 2-AG remain the best understood and most physiologically relevant (144).

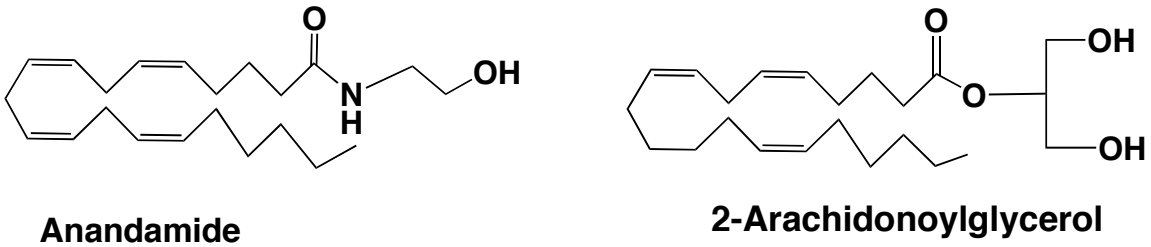


Figure 10. Structures of the endocannabinoids

Biochemistry of the Endocannabinoid System

AEA Biosynthesis and Metabolism

AEA is formed in neurons in a calcium channel-dependent mechanism that is activated when neurons fire. This leads to the activation of a phospholipase D that cleaves the phospholipid precursor N-arachidonoyl phosphatidylethanolamine (NArPE), releasing anandamide and phosphatidic acid (Figure 11) (144,151). NArPE is regenerated by an N-acetyltransferase (NAT) that catalyses the transfer of AA from the *sn-1* position of

phosphatidylcholine to the head group of phosphatidylethanolamine (Figure 11) (144). NAT is inactive in the absence of Ca^{2+}, and increasing concentrations of Ca^{2+} during synaptic transmission have been shown to activate latent NAT. The activity of NAT is enhanced upon phosphorylation by protein kinase A. Protein kinase A's activity, in turn, is regulated by levels of the second messenger cyclic AMP (cAMP) (152). Therefore, despite low basal amounts of NArPE in the brain (20-40 pmol/g), continuous synaptic transmission ensures that Ca^{2+} levels stay high enough to maintain NAT activity (144). Upon its generation in neurons, AEA passively diffuses through the post-synaptic cell membrane owing to its lipophilic nature, exposing it to its intracellular catabolic enzymes (153).

AEA is also specifically trafficked by a transporter, modulation of which has been shown to vary intracellular AEA levels (154). Upon entry into the cell, AEA is subject to hydrolysis by a membrane-bound serine hydrolase, fatty acid amide hydrolase (FAAH) (155). Hydrolysis of AEA leads to the formation of AA and ethanolamine and the termination of endocannabinoid signaling. AEA is also subject to oxidative metabolism by PGHS, LOXs, and cytochrome P450 enzymes (CYPs) (156). The oxygenation of AEA by PGHS-2 leads to the formation of PGE_2-EA at a rate comparable to that of PGE_2 from AA, but with AEA's K_m for PGHS-2 about 4-fold higher than AA's (157). PGH_2-EA is as an efficient substrate for the majority of the downstream synthases responsible for prostaglandin synthesis, save for TxA_2 synthase (Figure 12). Interestingly, AEA is an extremely poor substrate for PGHS-1, suggesting that the oxygenation of neutral AA derivatives is a possible distinction in function between PGHS-1 and PGHS-2. TxA_2

demonstrates substrate specificity that is associated with the carboxy terminus of PGH_2, indicating a neutral amide like PGH_2-EA would form a poor substrate (158).

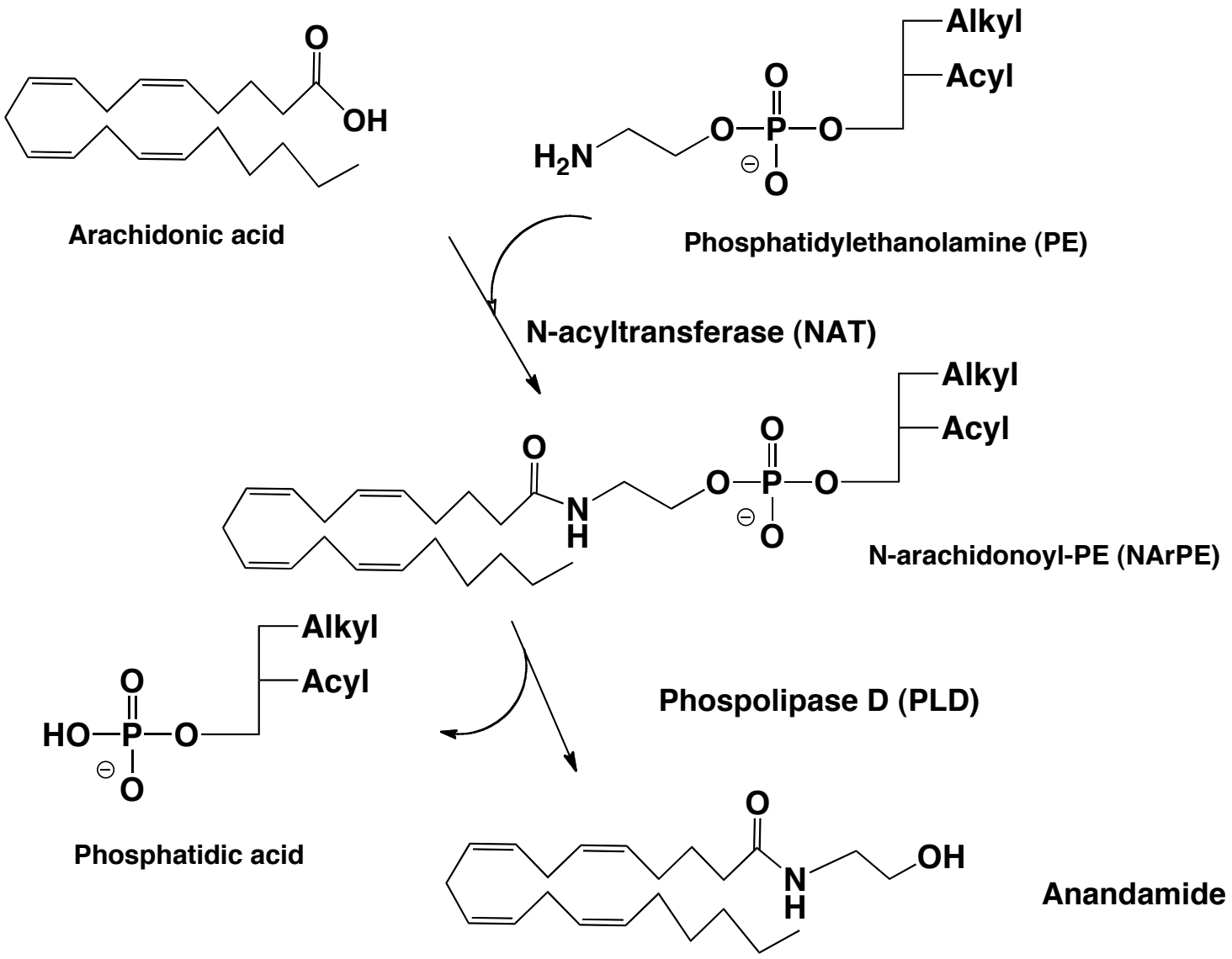


Figure 11. Biosynthesis of Anandamide

While it is interesting that AEA is substrate for PGHS-2, AEA is only present at very low levels, even in the presence of a stimulus, peaking in the nanomolar range, while its K_m for PGHS-2 is 26 μM (159,160). It remains to be seen whether the oxygenation of AEA by PGHS-2 is physiologically relevant. The generation of the FAAH- null mouse has revealed the AEA levels are tightly regulated by this enzyme, with the mice showing high sensitivity to administered AEA (161). This has thus far established FAAH as the chief metabolizing enzyme for AEA.

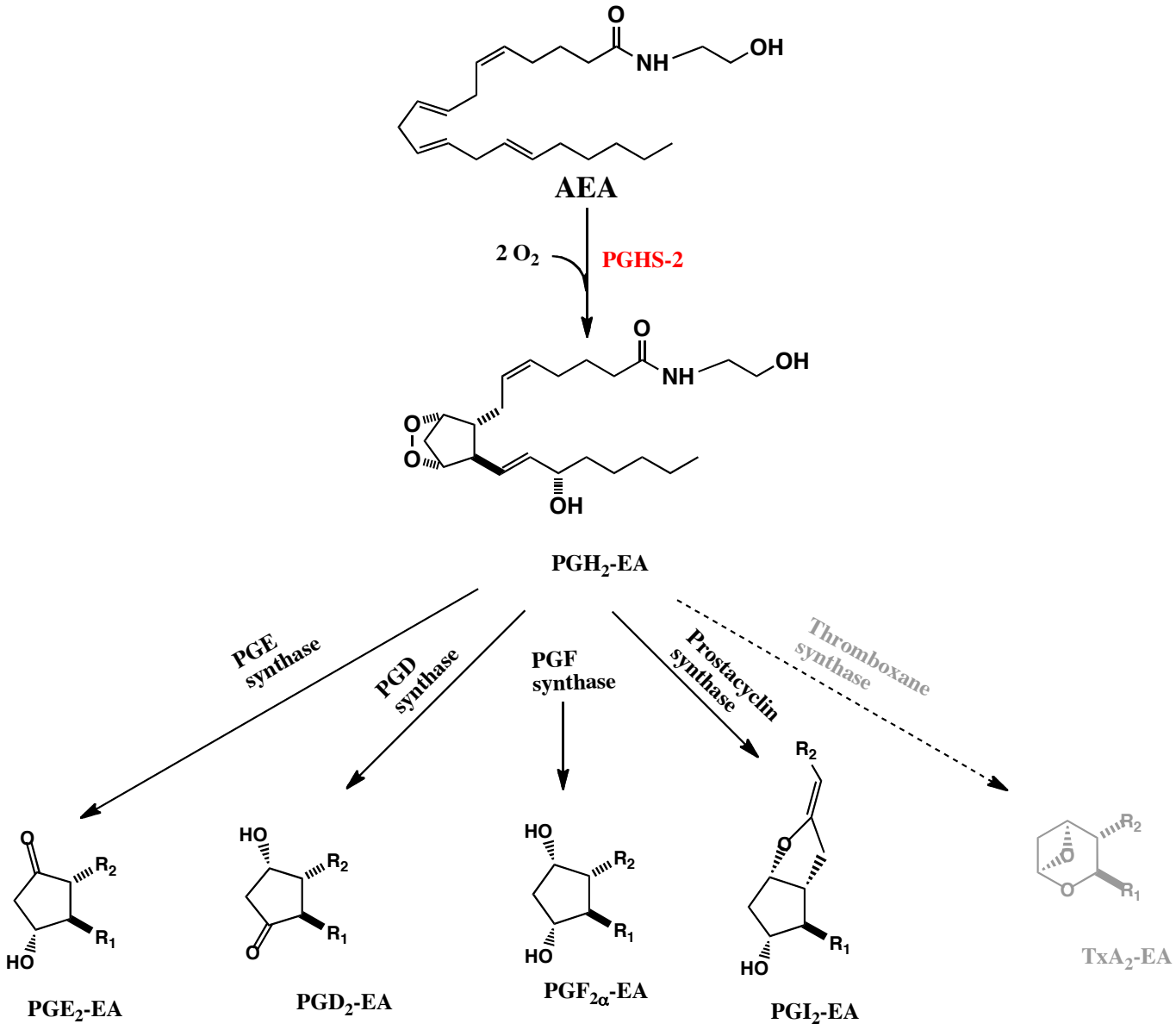


Figure 12. Oxidative Metabolism of AEA by PGHS-2

2-AG Biosynthesis and Metabolism

2-AG is at the crossroads of multiple metabolic pathways, which has hampered efforts to study its biosynthesis (144). Like AEA, the biosythesis of 2-AG is initiated by increasing Ca^{2+} levels. Increased intracellular Ca^{2+} activates phospholipase C (PLC) which hydrolyzes phosphatidyl inositol (PI), liberating 1,2 diacylglycerol (DAG). DAG is then hydrolyzed to 2-AG, by DAG lipase (DGL) (Figure 13) (162). An alternative pathway for the generation of 2-AG begins with the generation of 2-Arachidonoyl lysophospholipid, mediated by phospholipase A1(163,164). 2-rachidonoyl lysophospholipid may be hydrolyzed by lyso PLC, forming 2-AG (Figure 13). There is no direct evidence for this second proposed pathway; however PLA A1 is highly expressed in the brain, making this pathway possible (162,163).

Upon its post-synaptic release, 2-AG can passively diffuse into the intracellular compartment, or be rapidly and selectively transported by carrier systems present in neurons (153,154). Once in the intracellular compartment, 2-AG is subject to various metabolic pathways. Similar to AEA, 2-AG is subject to hydrolytic inactivation, generating AA and glycerol, primarily by a serine hydrolase known as monoacylglycerol lipase (MAGL)(165). MAGL is widely distributed in the central nervous system and its activity leads to a termination of 2-AG mediated endocannabinoid signaling (166). This has made it a suitable pharmacological target, as a tool to evaluate the role of the endocannabinoid system. Indeed, Long et al. (2009) stepped up to this challenge with the development of a powerful new inhibitor design tool, activity based protein profiling (ABPP) (167), which allowed them to generate potent specific inhibitors. Their best compound, JZL184, selectively inhibits MAGL with an IC_{50} of 8 nM and inhibits up to

85% of total rat brain hydrolysis of 2-AG. The rest of the hydrolysis of 2-AG is mediated by FAAH and other serine hydrolases such as ABHD6 (168). JZL184 also inhibits FAAH, with an IC_{50} of 4 μM, increasing its utility in dissection of the endocannabinoid signaling pathway (169). The inhibition of 2-AG hydrolysis by JZL184 in mice leads to an 8-fold increase in 2-AG levels and recapitulates CB_1-mediated behavioral and physiological effects, including analgesia, hypothermia, and hypomotility (170). The ability to pharmacologically modulate 2-AG levels has opened avenues of research for applications in a number of neurological and physiologic disorders where 2-AG-mediated endocannabinoid signaling has been shown to be important, such as pain, depression, and obesity. Recently, it has been shown that aggressive cancers of the skin (melanoma), breast, and ovaries overexpress MAGL and hijack this pathway, to generate strongly pro-tumorigenic terminal free fatty acid metabolites like PGE_2 and lysophosphatidic acid. Pharmacologic inhibition of MAGL with JZL184 or siRNA-mediated depletion of MAGL led to a loss of aggressiveness in cultured melanoma, breast, and ovarian cancer cells lines, indicating that MAGL inhibitors might have an application in cancer treatment (171,172).

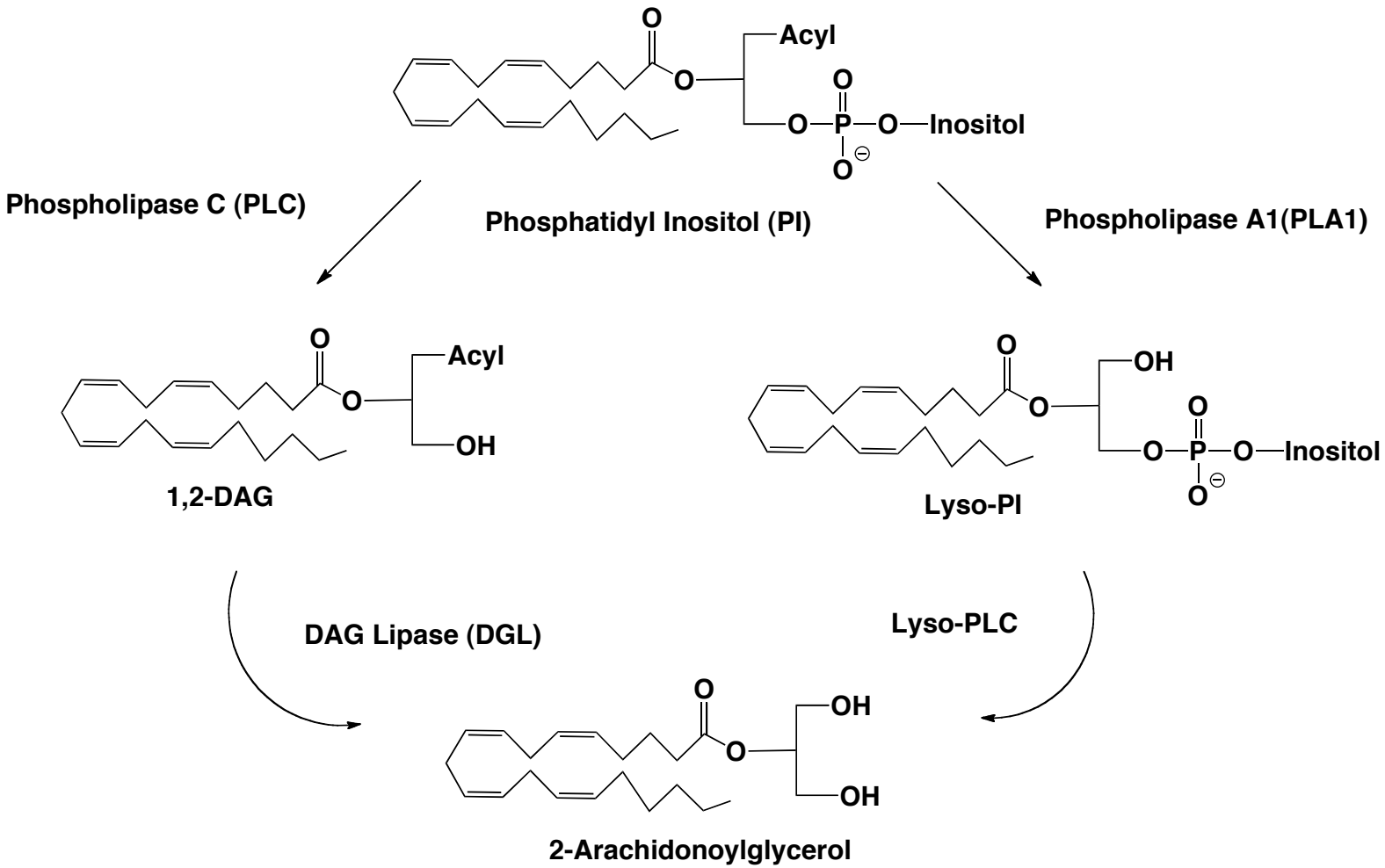


Figure 13. Biosynthesis of 2-arachidonoyl glycerol

2-AG is also subject to oxidative metabolism by the same spectrum of oxygenases that metabolize AEA. Action of human 15-lipoxygenase (15-LOX) on 2-AG generates 15-hydroxyeicosatetraeinoic acid glyceryl ester (15-HETE-G) which is a potent PPARα agonist (173). The oxygenation of 2-AG by PGHS-2 is as efficient as that of AA by hPGHS-2, *in vitro* (174). In cell culture, the action of PGHS-2 on 2-AG leads to virtually the same array of prostanoids that are generated from AA, known as prostaglandin glyceryl esters (PG-Gs), and similar to PGH_2-EA, PGH_2-G is a poor substrate for TxA_2 synthase (Figure 14)(175). Like AEA, 2-AG is an extremely poor substrate for PGHS-1, suggesting that the oxygenation of neutral AA derivatives is a possible distinction in function between PGHS-1 and PGHS-2. TxA_2 demonstrates substrate specificity that is associated with the carboxy terminus of PGH_2, indicating a neutral ester like PGH_2-G would form a poor substrate (158). Mouse peritoneal macrophages pre-treated with the inflammatory stimulus LPS and followed by zymosan phagocytosis lead to the formation of PGE_2-G and PGI_2-G (176). While the levels of 2-AG released in response to agonist were only 10 fold lower than those of AA, the ratio of the total PGs:PG-Gs detected was 1000:1, indicating that PG-Gs were formed inefficiently (177). Determinants of the inefficient oxygenation of 2-AG are examined in this work.

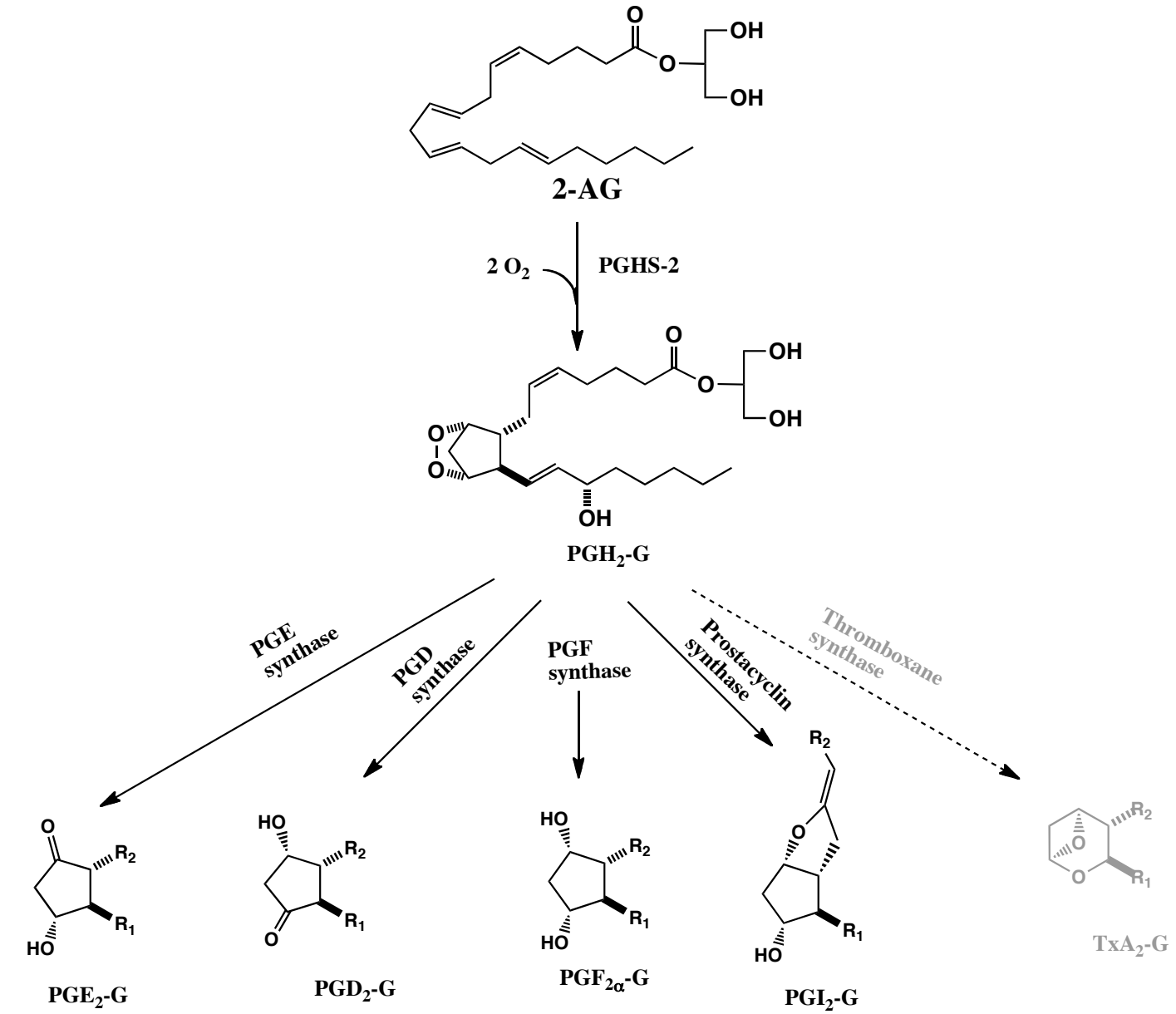


Figure 14. Oxidative Metabolism of 2-AG by PGHS-2

PGE_2-G has a $t_{1/2}$ of 14 seconds in rat plasma and 7 minutes in human plasma (177). The rapid hydrolysis of PG-Gs has hampered the validation of the PGHS-2/2-AG pathway; however, recent exciting work has demonstrated that the PGHS-2/2AG pathway is worth considering. PGE_2-G has been shown to act at a yet to be identified unique receptor, independent of the traditional PG receptors, mediating Ca^{2+} mobilization in RAW264.7 macrophages and inducing aberrant pain sensations and (allodynia) and increased sensitivity to pain (hyperalgesia) in rats (178,179). Further validation of this pathway is described herein.

Cannabinoid Signaling and Biology

As mentioned before, two-cannabinoid receptors have been cloned. The CB_1 receptor is the most abundant GPCR in the mammalian brain, highlighting the importance of endocannabinoid signaling. CB_1 receptor expression primarily in the hippocampus, basal ganglia, cerebellum, brain stem, spinal cord, and dorsal root ganglia (180) explains the action of CB_1-mediated signaling. Furthermore, the four symptoms defining cannabinoid intoxication in rat models, hypothermia, hypomobility, and analgesia, are absent in CB_1 –null mice (181). The pertussis toxin-sensitive $G_{i/o}$ CB_1 mediates these effects by inhibiting voltage gated N- and P/Q type voltage-activated Ca^{2+} channels and K^+ channels. The β-γ subunits of the $G_{i/o}$ bind to both the Ca^{2+} and K^+ channels and depress the signaling at GABA and glutaminergic synapses in the CNS (144). This depressive effect on synaptic transmission is the hallmark of cannabinoid signaling. The CB_2 receptor is expressed in immune cells, such as macrophages and B-cells (148). The role of endocannabinoid signaling in the immune system has not been adequately explored. This is reflected in the limited number of reports of CB_2-mediated actions in the

immune system. However, 2-AG has been shown to posses anti-inflammatory activity and can alter cytokine production (182).

While 2-AG is a full agonist at both CB_1 and CB_2 receptors (183,184), AEA is only a partial CB_1 receptor agonist and does not agonise the CB_2 receptor at all (185,186). In addition to being an endocannabinoid, AEA also binds and activates the transient receptor potential vanilloid receptor (TRPV1), which was discovered for its sensitivity to capsaicin. AEA binding to the TRPV1 receptor in the nerves of blood vessel walls elicits vasodilation (187). TRPV1 is expressed in the CNS and peripheral nervous system (PNS), where its role is thought to be the detection of noxious stimuli such as heat and low pH (188). Thus AEA is also considered an endovanilloid. For the rest of the discussion and for the sake of pertinence to the work presented herein, we will focus our attention on 2-AG which is considered a pure endocannbinoid and is more abundant and ubiquitous across the CNS and PNS with brief mention of significant AEA biology in the literature (144).

The physiological actions of AEA and 2-AG

The involvement of the endocannabinoid system in the modulation of pain is one of the most attractive features of this pathway. The potential medical applications are evident in the embracing of medicinal marijuana by a number of states in the U.S. and across the world. Current treatment modalities, primarily with opiates, are habit forming and can be dangerous, whereas cannabinoid-based therapies bear promise due to their safety and low potential for habit formation. There are a number of issues associated with marijuana use, such as the exact chemical composition of *Cannabis* extracts, which can vary by strain and plant, and the health risks associated with the chief mode of administration, smoking. The realization that modulation

of the endogenous cannabinoid system could mediate the same effects as *Cannabis*- bypassing the socioeconomic, legal issues, and smoking health risk associated with *Cannabis* consumption- is exciting and warrants fervent investigation (189).

Pain and Inflammation

In the brain, endocannabinoids have been shown to tonically modulate neuronal transmission in the rostral ventral medulla (RVM), the periaqueductal grey, and the spinal trigeminal areas critically involved in the promotion of central analgesia (190-192). Supporting the importance of these areas in the modulation of pain is the fact that localized treatment with a CB_1 receptor antagonist leads to the induction of hyperalgesia, in an opioid–independent manner. Noxious and painful stimuli from the periphery create neuronal hyperexcitability in the dorsal horn of the spinal cord. This hyperexcitability leads to increased glutaminergic signaling and excitotoxicity and induces the expression of PGHS-2 (67). Recent work has demonstrated that spinally applied PGHS-2 inhibitors prevent this hyperexcitability by preserving the levels of neuronal 2-AG leading to analgesia (193). This suggests that PGHS-2-dependent metabolism of 2-AG may play an important role in the regulation of nociception. Interestingly, intraperitoneally injected 2-AG has been shown to prevent the induction of PGHS-2 by inflammatory (LPS) and excitotoxic stimuli (glutamate) in the mouse hippocampus, in a dose-dependent manner (67). These actions, which were absent in CB_1–null mice and in the face of CB_1 antagonism, were mediated by β-γ subunits associated with CB_1-activated G proteins. 2-AG-mediated signaling in this model attenuated phosphorylation of p38 MAP kinase and NF-κB leading to decreased expression of PGHS-2. The inhibition of hydrolysis of endogenously produced 2-AG using MAGL inhibitors recapitulated the effects of exogenous 2-AG, indicating that preservation of

endogenous levels of 2-AG can prevent the induction of PGHS-2 in the hippocampus, even in the presence of inflammatory insult (67). Lending credence to the PGHS-2/2AG axis in pain-related signaling is the finding that PGE_2-G elicits neuronal hyperexcitability that is associated with allodynia and hyperalgesia (67,179). This has made understanding and targeting the PGHS-2/2-AG axis an important avenue for inquiry and is part of the work presented herein.

Addiction and Food Intake

The endocannabinoids have also been shown to play a role in reward based addictive disorders and behaviors such as obesity, tobacco and, alcohol abuse (144). The reward pathway in the nucleus accumbens (NA) is critical for habit formation, and has been shown to be involved in an array of reward-based activities. Recently, levels of 2-AG in the NA have been shown to be elevated in rats self-administering ethanol, in a dose-dependent manner (194). Supporting a role for 2-AG in ethanol addiction is the fact that rats chronically self-administering ethanol demonstrated potentiated increases in 2-AG levels in the NA of these rats upon each instance of ethanol consumption (195). Antagonism of the CB_1 receptor using SR141716 (Rimonabant) led to a reduction in self-administration of ethanol in chronically self-adminstering rats. This was the first indication that modulation of cannabinoid signaling could abate alcohol addiction. Similar effects have been reported for heroin abuse, also associated with the NA-associated reward pathway (194). Smoking and eating addiction are also encoded in the same NA dependent pathways through which ethanol and narcotic addiction are mediated. Interestingly, in 2001, the hormone leptin was shown to reduce hypothalamic levels of 2-AG and AEA, in a dose-dependent manner (196). Leptin is a key modulator of nutritional status and food intake, and its actions lead to reduced food intake. Leptin–null mice (db/db) which are obese, demonstrate significantly elevated levels of 2-AG and AEA in the hypothalamus. Similar to the action of

rimonabant on ethanol self-adminstration, rimonabant led to reduced food intake and significant weight loss in db/db mice, relative to vehicle treated mice (196). These findings led to the institution of clinical trials for rimonabant for the treatment of obesity, along with a hypocaloric diet and exercise. Cohorts in the rimonabant phase II clinical trial demonstrated weigh loss greater than four-fold over those on placebo (4.5 Kg- rimonabant versus 1 Kg – placebo) (144). Furthermore, cohorts in the trial on rimonabant had significant lowering of fasting blood glucose and triglycerides and an increase in HDL cholesterol; leading to a reduction in cardiovascular risk factors in trial participants (197,198). This led to separate clinical trials, to evaluate the ability of CB_1 antagonism to protect from cardiovascular effects resulting from elevated triglycerides and cholesterol (199). Unfortunately, rimonabant was withdrawn from the European market in early 2009, citing the increased incidence of suicide among patients taking the drug (200). All the current rimonabant trials have also been halted due to the same safety concerns (199). Despite being heralded as a miracle drug, the future of CB_1 antagonism comes into question as a result of our poor understanding of the role of the endocannabinoid system in mood.

So far, the only cannabinoid approved by the FDA is synthetic THC, Marinol, which is indicated for appetite stimulation (orixogenia) in treating anorexia associated with AIDS and in the treatment of nausea associated with chemotherapy.

Motor function

Endocannabinoids have also been shown to play an important role in locomotion. The CB_1 receptor is highly expressed in the basal ganglia, an area in the forebrain that plays a critical role in motor behavior (144). The actions of endocannabinoids in the basal ganglia are mediated by

the inhibition of the release of the neurotransmitter GABA. This eventuates in a dimunition of dopaminergic signaling, suppressing motor activity (201). These findings indicate that the endocannabinoid system may play an important role in debilitating diseases caused by dysfunctional dopaminergic signaling such as Parkinson's and Alzheimers diseases. Further work is needed to determine the potential of endocannabinoid modulation in the mitigation of these debilitating conditions.

Dissertation Aims

The research described herein encompasses efforts to determine the substrate specific determinants of the endocannabinoid 2-AG's oxygenation by PGHS-2 *in vitro* and *ex vivo*. Oxidative metabolism of 2-AG by PGHS-2 has been recognized as a pathway for the creation of novel prostaglanoid ligands that have unique actions in modulating pain and lead to termination of 2-AG-mediated analgesia. These efforts were prompted by findings indicating that factors not reflected in steady state parameters may govern the metabolism of 2-AG in the cellular milieu. Importantly, the interaction of 2-AG-derived peroxides with PGHSs has not been evaluated. These are described in Chapter II; here I undertook an exhaustive study of the disposition of 2-AG derived hydroperoxides as POX substrates and activators of PGHSs' oxygenase functionality, compared to AA derived hydroperoxides. Further than this, I determined the peroxide requirements for activation of PGHS-2 mediated 2-AG oxygenation, relative to AA. As an extension of the findings in Chapter II, the *ex vivo* determinants of 2-AG oxygenation will be presented in Chapter III, here I demonstrate that the factors determined in Chapter II may hold true in the cellular environment. Chapter IV will highlight my efforts to apply the findings presented in the preceding chapters; with the preliminary development of substrate-selective inhibitors of PGHS-2-mediated 2-AG oxygenation. Finally, Chapter V will describe the development of a robust new screening platform for the development of agents targeting the metabolism of AEA and 2-AG by PGHS-2, in a neuronal context.

In total, the results presented here provide new information on the determinants of 2-AG oxygenation by PGHS-2 and present a case for the relevance of this pathway in endocannabinoid

metabolism and foray into the pharmacological modulation of this pathway. The implications of these findings will be discussed in Chapter VI.

References:

1. Blobaum, A. L., and Marnett, L. J. (2007) *J Med Chem* **50**, 1425-1441

2. Vane, J. R., Flower, R. J., and Botting, R. M. (1990) *Stroke* **21**, IV12-23

3. Jack, D. B. (1997) *Lancet* **350**, 437-439

4. Stone, E. (1763) *Philos Trans R Soc Lond* **53**, 195-200

5. Leroux, M. (1830) *J Chim Med* **6**, 341

6. Maclagan, T. J. (1876) *Lancet* **1**, 342-343

7. Brune, K., and Hinz, B. (2004) *Arthritis Rheum* **50**, 2391-2399

8. Kurzrok, R. a. L., C. (1930) *Proc. Soc. Exp. Biol. N.Y.* **28**, 268-272

9. Von Euler, U. S. (1935) *Kiln. Wschr.* **14**, 1182-1183

10. Von Euler, U. S. (1936) *J. Physiol.* **88**, 213-234

11. Von Euler, U. S. (1939) *Skand. Arch. Physiol.* **81**, 65-80

12. Goldblatt, M. W. (1935) *Biochem J* **29**, 1346-1357

13. Goldblatt, M. W. (1935) *J Physiol* **84**, 208-218

14. Hamberg, M., and Samuelsson, B. (1967) *J Biol Chem* **242**, 5336-5343

15. Smith, J. B., and Willis, A. L. (1971) *Nat New Biol* **231**, 235-237

16. Vane, J. R. (1971) *Nat New Biol* **231**, 232-235

17. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. (1991) *J Biol Chem* **266**, 12866-12872

18. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc Natl Acad Sci U S A* **88**, 2692-2696

19. Marnett, L. J. (2009) *Annu Rev Pharmacol Toxicol* **49**, 265-290

20. Yu, Y., Fan, J., Chen, X. S., Wang, D., Klein-Szanto, A. J., Campbell, R. L., FitzGerald, G. A., and Funk, C. D. (2006) *Nat Med* **12**, 699-704

21. van der Ouderaa, F. J., Buytenhek, M., Slikkerveer, F. J., and van Dorp, D. A. (1979) *Biochim Biophys Acta* **572**, 29-42

22. Roth, G. J., Siok, C. J., and Ozols, J. (1980) *J Biol Chem* **255**, 1301-1304

23. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and Van Dorp, D. A. (1977) *Biochim Biophys Acta* **487**, 315-331

24. Picot, D., Loll, P. J., and Garavito, R. M. (1994) *Nature* **367**, 243-249

25. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) *Nat Struct Biol* **3**, 927-933

26. Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988) *J Biol Chem* **263**, 3550-3553

27. Rouzer, C. A., and Marnett, L. J. (2003) *Chem Rev* **103**, 2239-2304

28. Dietz, R., Nastainczyk, W., and Ruf, H. H. (1988) *Eur J Biochem* **171**, 321-328

29. Marnett, L. J., Wlodawer, P., and Samuelsson, B. (1975) *J Biol Chem* **250**, 8510-8517

30. Markey, C. M., Alward, A., Weller, P. E., and Marnett, L. J. (1987) *J Biol Chem* **262**, 6266-6279

31. DeFelippis, M. R., Murthy, C. P., Faraggi, M., and Klapper, M. H. (1989) *Biochemistry* **28**, 4847-4853

32. Koppenol, W. H. (1990) *FEBS Lett* **264**, 165-167

33. Tsai, A., and Kulmacz, R. J. (2000) *Prostaglandins Other Lipid Mediat* **62**, 231-254

34. Goodwin, D. C., Rowlinson, S. W., and Marnett, L. J. (2000) *Biochemistry* **39**, 5422-5432

35. Kulmacz, R. J., and Wang, L. H. (1995) *J Biol Chem* **270**, 24019-24023

36. Hemler, M. E., Graff, G., and Lands, W. E. (1978) *Biochem Biophys Res Commun* **85**, 1325-1331

37. Hemler, M. E., Cook, H. W., and Lands, W. E. (1979) *Arch Biochem Biophys* **193**, 340-345

38. Marshall, P. J., Kulmacz, R. J., and Lands, W. E. (1987) *J Biol Chem* **262**, 3510-3517

39. Kulmacz, R. J., and Lands, W. E. (1983) *Prostaglandins* **25**, 531-540

40. Wu, G., Wei, C., Kulmacz, R. J., Osawa, Y., and Tsai, A. L. (1999) *J Biol Chem* **274**, 9231-9237

41. Hemler, M. E., and Lands, W. E. (1980) *J Biol Chem* **255**, 6253-6261

42. Wu, G., Rogge, C. E., Wang, J. S., Kulmacz, R. J., Palmer, G., and Tsai, A. L. (2007) *Biochemistry* **46**, 534-542

43. Mbonye, U. R., Wada, M., Rieke, C. J., Tang, H. Y., Dewitt, D. L., and Smith, W. L. (2006) *J Biol Chem* **281**, 35770-35778

44. Mbonye, U. R., Yuan, C., Harris, C. E., Sidhu, R. S., Song, I., Arakawa, T., and Smith, W. L. (2008) *J Biol Chem* **283**, 8611-8623

45. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) *Annu Rev Biochem* **69**, 145-182

46. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) *Nature* **384**, 644-648

47. Wong, E., Bayly, C., Waterman, H. L., Riendeau, D., and Mancini, J. A. (1997) *J Biol Chem* **272**, 9280-9286

48. Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M., and Seibert, K. (1996) *J Biol Chem* **271**, 15810-15814

49. Guo, Q., Wang, L. H., Ruan, K. H., and Kulmacz, R. J. (1996) *J Biol Chem* **271**, 19134-19139

50. Roth, G. J., Stanford, N., and Majerus, P. W. (1975) *Proc Natl Acad Sci U S A* **72**, 3073-3076

51. Shimokawa, T., and Smith, W. L. (1992) *J Biol Chem* **267**, 12387-12392

52. Holtzman, M. J., Turk, J., and Shornick, L. P. (1992) *J Biol Chem* **267**, 21438-21445

53. Meade, E. A., Smith, W. L., and DeWitt, D. L. (1993) *J Biol Chem* **268**, 6610-6614

54. Rowlinson, S. W., Crews, B. C., Goodwin, D. C., Schneider, C., Gierse, J. K., and Marnett, L. J. (2000) *J Biol Chem* **275**, 6586-6591

55. Milton, A. S., and Wendlandt, S. (1970) *J Physiol* **207**, 76P-77P

56. Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., and Narumiya, S. (1998) *Nature* **395**, 281-284

57. Saper, C. B., and Breder, C. D. (1994) *N Engl J Med* **330**, 1880-1886

58. Ek, M., Engblom, D., Saha, S., Blomqvist, A., Jakobsson, P. J., and Ericsson-Dahlstrand, A. (2001) *Nature* **410**, 430-431

59. Breder, C. D., Dewitt, D., and Kraig, R. P. (1995) *J Comp Neurol* **355**, 296-315

60. Breder, C. D., and Saper, C. B. (1996) *Brain Res* **713**, 64-69

61. Li, S., Wang, Y., Matsumura, K., Ballou, L. R., Morham, S. G., and Blatteis, C. M. (1999) *Brain Res* **825**, 86-94

62. Willis, A. L. (1969) *Prostaglandins, peptides and amines* **31**

63. Woolf, C. J., and Salter, M. W. (2000) *Science* **288**, 1765-1769

64. Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V., and Woolf, C. J. (2001) *Nature* **410**, 471-475

65. Vikman, K. S., Hill, R. H., Backstrom, E., Robertson, B., and Kristensson, K. (2003) *Pain* **106**, 241-251

66. Fehrenbacher, J. C., Burkey, T. H., Nicol, G. D., and Vasko, M. R. (2005) *Pain* **113**, 113-122

67. Zhang, J., and Chen, C. (2008) *J Biol Chem* **283**, 22601-22611

68. Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y., Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., and Narumiya, S. (2000) *Science* **287**, 2013-2017

69. Bennett, A. (2000) *Thorax* **55 Suppl 2**, S54-56

70. Ojeda, S. R., and Campbell, W. B. (1982) *Endocrinology* **111**, 1031-1037

71. Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. (1999) *Proc Natl Acad Sci U S A* **96**, 10501-10506

72. Kennedy, C. R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C. D., Magnuson, M. A., Oates, J. A., Breyer, M. D., and Breyer, R. M. (1999) *Nat Med* **5**, 217-220

73. Karim, S. M. (1968) *Br Med J* **4**, 618-621

74. Karim, S. M., Trussell, R. R., Patel, R. C., and Hillier, K. (1968) *Br Med J* **4**, 621-623

75. Sugimoto, Y., Yamasaki, A., Segi, E., Tsuboi, K., Aze, Y., Nishimura, T., Oida, H., Yoshida, N., Tanaka, T., Katsuyama, M., Hasumoto, K., Murata, T., Hirata, M., Ushikubi, F., Negishi, M., Ichikawa, A., and Narumiya, S. (1997) *Science* **277**, 681-683

76. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M., and et al. (1995) *Nature* **378**, 406-409

77. Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) *Cell* **91**, 197-208

78. Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chulada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., Kluckman, K. D., Kim, H. S., and Smithies, O. (1995) *Cell* **83**, 483-492

79. Shio, H., and Ramwell, P. (1972) *Nat New Biol* **236**, 45-46

80. Shio, H., and Ramwell, P. W. (1972) *Science* **175**, 536-538

81. Shio, H., Ramwell, P. W., and Jessup, S. J. (1972) *Prostaglandins* **1**, 29-36

82. Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H., and Coffman, T. M. (1998) *J Clin Invest* **102**, 1994-2001

83. Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., and Narumiya, S. (1997) *Nature* **388**, 678-682

84. Patrono, C., and Rocca, B. (2009) *J Thromb Haemost* **7 Suppl 1**, 258-261

85. Collins, R., Peto, R., Baigent, C., and Sleight, P. (1997) *N Engl J Med* **336**, 847-860

86. Smith, G. C. (1998) *Pharmacol Rev* **50**, 35-58

87. Nguyen, M., Camenisch, T., Snouwaert, J. N., Hicks, E., Coffman, T. M., Anderson, P. A., Malouf, N. N., and Koller, B. H. (1997) *Nature* **390**, 78-81

88. Patrono, C., and Dunn, M. J. (1987) *Kidney Int* **32**, 1-12

89. Oates, J. A., FitzGerald, G. A., Branch, R. A., Jackson, E. K., Knapp, H. R., and Roberts, L. J., 2nd. (1988) *N Engl J Med* **319**, 761-767

90. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A., and Smithies, O. (1995) *Cell* **83**, 473-482

91. Pollard, M., and Luckert, P. H. (1981) *Science* **214**, 558-559

92. Kudo, T., Narisawa, T., and Abo, S. (1980) *Gann* **71**, 260-264

93. Waddell, W. R., and Loughry, R. W. (1983) *J Surg Oncol* **24**, 83-87

94. Waddell, W. R., Ganser, G. F., Cerise, E. J., and Loughry, R. W. (1989) *Am J Surg* **157**, 175-179

95. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. (1991) *N Engl J Med* **325**, 1593-1596

96. Peleg, II, Maibach, H. T., Brown, S. H., and Wilcox, C. M. (1994) *Arch Intern Med* **154**, 394-399

97. Smalley, W., Ray, W. A., Daugherty, J., and Griffin, M. R. (1999) *Arch Intern Med* **159**, 161-166

98. Kune, G. A., Kune, S., and Watson, L. F. (1988) *Cancer Res* **48**, 4399-4404

99. Smith, B. J., Wills, M. R., and Savory, J. (1983) *Ann Clin Lab Sci* **13**, 359-365

100. Oka, M., Inaba, A., Uchiyama, T., Hazama, S., Shimoda, K., Suzuki, M., and Suzuki, T. (1994) *Am J Surg* **167**, 264-267

101. Seyberth, H. W., Segre, G. V., Morgan, J. L., Sweetman, B. J., Potts, J. T., Jr., and Oates, J. A. (1975) *N Engl J Med* **293**, 1278-1283

102. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* **107**, 1183-1188

103. Hanahan, D., Christofori, G., Naik, P., and Arbeit, J. (1996) *Eur J Cancer* **32A**, 2386-2393

104. Hanahan, D., and Folkman, J. (1996) *Cell* **86**, 353-364

105. Baron, J. A., Sandler, R. S., Bresalier, R. S., Quan, H., Riddell, R., Lanas, A., Bolognese, J. A., Oxenius, B., Horgan, K., Loftus, S., and Morton, D. G. (2006) *Gastroenterology* **131**, 1674-1682

106. Bertagnolli, M. M., Eagle, C. J., Zauber, A. G., Redston, M., Solomon, S. D., Kim, K., Tang, J., Rosenstein, R. B., Wittes, J., Corle, D., Hess, T. M., Woloj, G. M., Boisserie, F., Anderson, W. F., Viner, J. L., Bagheri, D., Burn, J., Chung, D. C., Dewar, T., Foley, T. R., Hoffman, N., Macrae, F., Pruitt, R. E., Saltzman, J. R., Salzberg, B., Sylwestrowicz, T., Gordon, G. B., and Hawk, E. T. (2006) *N Engl J Med* **355**, 873-884

107. Arber, N., Eagle, C. J., Spicak, J., Racz, I., Dite, P., Hajer, J., Zavoral, M., Lechuga, M. J., Gerletti, P., Tang, J., Rosenstein, R. B., Macdonald, K., Bhadra, P., Fowler, R., Wittes, J., Zauber, A. G., Solomon, S. D., and Levin, B. (2006) *N Engl J Med* **355**, 885-895

108. Behne, D., Kyriakopoeulos, A., Weiss-Nowak, C., Kalckloesch, M., Westphal, C., and Gessner, H. (1996) *Biol Trace Elem Res* **55**, 99-110

109. Brigelius-Flohe, R. (1999) *Free Radic Biol Med* **27**, 951-965

110. Seiler, A., Schneider, M., Forster, H., Roth, S., Wirth, E. K., Culmsee, C., Plesnila, N., Kremmer, E., Radmark, O., Wurst, W., Bornkamm, G. W., Schweizer, U., and Conrad, M. (2008) *Cell Metab* **8**, 237-248

111. Mills, G. C. (1957) *J Biol Chem* **229**, 189-197

112. Clerch, L. B., and Massaro, D. (1993) *J Clin Invest* **91**, 499-508

113. Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M., and Funk, C. D. (1997) *J Biol Chem* **272**, 16644-16651

114. de Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998) *J Biol Chem* **273**, 22528-22536

115. Durak, I., Beduk, Y., Kavutcu, M., Ozturk, S., Canbolat, O., and Ulutepe, S. (1997) *Int Urol Nephrol* **29**, 5-11

116. Zachara, B. A., Szewczyk-Golec, K., Tyloch, J., Wolski, Z., Szylberg, T., Stepien, S., Kwiatkowski, S., Bloch-Boguslawska, E., and Wasowicz, W. (2005) *Neoplasma* **52**, 248-254

117. Gladyshev, V. N., Factor, V. M., Housseau, F., and Hatfield, D. L. (1998) *Biochem Biophys Res Commun* **251**, 488-493

118. Esworthy, R. S., Baker, M. A., and Chu, F. F. (1995) *Cancer Res* **55**, 957-962

119. Chu, F. F., and Esworthy, R. S. (1995) *Arch Biochem Biophys* **323**, 288-294

120. Florian, S., Wingler, K., Schmehl, K., Jacobasch, G., Kreuzer, O. J., Meyerhof, W., and Brigelius-Flohe, R. (2001) *Free Radic Res* **35**, 655-663

121. Walshe, J., Serewko-Auret, M. M., Teakle, N., Cameron, S., Minto, K., Smith, L., Burcham, P. C., Russell, T., Strutton, G., Griffin, A., Chu, F. F., Esworthy, S., Reeve, V., and Saunders, N. A. (2007) *Cancer Res* **67**, 4751-4758

122. Esworthy, R. S., Aranda, R., Martin, M. G., Doroshow, J. H., Binder, S. W., and Chu, F. F. (2001) *Am J Physiol Gastrointest Liver Physiol* **281**, G848-855

123. Banning, A., Deubel, S., Kluth, D., Zhou, Z., and Brigelius-Flohe, R. (2005) *Mol Cell Biol* **25**, 4914-4923

124. Kobayashi, M., and Yamamoto, M. (2005) *Antioxid Redox Signal* **7**, 385-394

125. Singh, A., Rangasamy, T., Thimmulappa, R. K., Lee, H., Osburn, W. O., Brigelius-Flohe, R., Kensler, T. W., Yamamoto, M., and Biswal, S. (2006) *Am J Respir Cell Mol Biol* **35**, 639-650

126. Banning, A., Kipp, A., Schmitmeier, S., Lowinger, M., Florian, S., Krehl, S., Thalmann, S., Thierbach, R., Steinberg, P., and Brigelius-Flohe, R. (2008) *Cancer Res* **68**, 9746-9753

127. Flohe, L. (1978) *Ciba Found Symp*, 95-122

128. Yoshimura, S., Watanabe, K., Suemizu, H., Onozawa, T., Mizoguchi, J., Tsuda, K., Hatta, H., and Moriuchi, T. (1991) *J Biochem* **109**, 918-923

129. Bjornstedt, M., Xue, J., Huang, W., Akesson, B., and Holmgren, A. (1994) *J Biol Chem* **269**, 29382-29384

130. Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., Motta, L., Richardson, A., and Prolla, T. A. (2003) *Free Radic Biol Med* **34**, 496-502

131. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) *Biochim Biophys Acta* **710**, 197-211

132. Roveri, A., Maiorino, M., Nisii, C., and Ursini, F. (1994) *Biochim Biophys Acta* **1208**, 211-221

133. Thomas, J. P., Geiger, P. G., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *Biochim Biophys Acta* **1045**, 252-260

134. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *J Biol Chem* **265**, 454-461

135. Arai, M., Imai, H., Koumura, T., Yoshida, M., Emoto, K., Umeda, M., Chiba, N., and Nakagawa, Y. (1999) *J Biol Chem* **274**, 4924-4933

136. Nomura, K., Imai, H., Koumura, T., Kobayashi, T., and Nakagawa, Y. (2000) *Biochem J* **351**, 183-193

137. Shi, Z. Z., Osei-Frimpong, J., Kala, G., Kala, S. V., Barrios, R. J., Habib, G. M., Lukin, D. J., Danney, C. M., Matzuk, M. M., and Lieberman, M. W. (2000) *Proc Natl Acad Sci U S A* **97**, 5101-5106

138. Jung, G., Yang, D. C., and Nakao, A. (1985) *Biochem Biophys Res Commun* **130**, 559-566

139. Schewe, T., Halangk, W., Hiebsch, C., and Rapoport, S. M. (1975) *FEBS Lett* **60**, 149-152

140. Chen, C. J., Huang, H. S., and Chang, W. C. (2003) *FASEB J* **17**, 1694-1696

141. Sakamoto, H., Imai, H., and Nakagawa, Y. (2000) *J Biol Chem* **275**, 40028-40035

142. Stafford, P. (1993)

143. Piomelli, D. (2003) *Nat Rev Neurosci* **4**, 873-884

144. Zias, J., Stark, H., Sellgman, J., Levy, R., Werker, E., Breuer, A., and Mechoulam, R. (1993) *Nature* **363**, 215

145. Gaoni, Y., and Mechoulam, R. (1964) *J Am Chem Soc* **86**, 1646-1647

146. Devane, W. A., Dysarz, F. A., 3rd, Johnson, M. R., Melvin, L. S., and Howlett, A. C. (1988) *Mol Pharmacol* **34**, 605-613

147. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) *Nature* **346**, 561-564

148. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) *Nature* **365**, 61-65

149. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258**, 1946-1949

150. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995) *Biochem Biophys Res Commun* **215**, 89-97

151. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994) *Nature* **372**, 686-691

152. Cadas, H., Gaillet, S., Beltramo, M., Venance, L., and Piomelli, D. (1996) *J Neurosci* **16**, 3934-3942

153. Hillard, C. J., and Jarrahian, A. (2000) *Chem Phys Lipids* **108**, 123-134

154. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997) *Science* **277**, 1094-1097

155. Schmid, P. C., Zuzarte-Augustin, M. L., and Schmid, H. H. (1985) *J Biol Chem* **260**, 14145-14149

156. Kozak, K. R., and Marnett, L. J. (2002) *Prostaglandins Leukot Essent Fatty Acids* **66**, 211-220

157. Yu, M., Ives, D., and Ramesha, C. S. (1997) *J Biol Chem* **272**, 21181-21186

158. Wang, L. H., Matijevic-Aleksic, N., Hsu, P. Y., Ruan, K. H., Wu, K. K., and Kulmacz, R. J. (1996) *J Biol Chem* **271**, 19970-19975

159. Stella, N., Schweitzer, P., and Piomelli, D. (1997) *Nature* **388**, 773-778

160. Bisogno, T., Berrendero, F., Ambrosino, G., Cebeira, M., Ramos, J. A., Fernandez-Ruiz, J. J., and Di Marzo, V. (1999) *Biochem Biophys Res Commun* **256**, 377-380

161. Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., and Lichtman, A. H. (2001) *Proc Natl Acad Sci U S A* **98**, 9371-9376

162. Farooqui, A. A., Rammohan, K. W., and Horrocks, L. A. (1989) *Ann N Y Acad Sci* **559**, 25-36

163. Higgs, H. N., and Glomset, J. A. (1994) *Proc Natl Acad Sci U S A* **91**, 9574-9578

164. Pete, M. J., Ross, A. H., and Exton, J. H. (1994) *J Biol Chem* **269**, 19494-19500

165. Goparaju, S. K., Ueda, N., Taniguchi, K., and Yamamoto, S. (1999) *Biochem Pharmacol* **57**, 417-423

166. Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., Kathuria, S., and Piomelli, D. (2002) *Proc Natl Acad Sci U S A* **99**, 10819-10824

167. Hoover, H. S., Blankman, J. L., Niessen, S., and Cravatt, B. F. (2008) *Bioorg Med Chem Lett* **18**, 5838-5841

168. Marrs, W. R., Blankman, J. L., Horne, E. A., Thomazeau, A., Lin, Y. H., Coy, J., Bodor, A. L., Muccioli, G. G., Hu, S. S., Woodruff, G., Fung, S., Lafourcade, M., Alexander, J. P., Long, J. Z., Li, W., Xu, C., Moller, T., Mackie, K., Manzoni, O. J., Cravatt, B. F., and Stella, N. (2010) *Nat Neurosci* **13**, 951-957

169. Marnett, L. J. (2009) *Nat Chem Biol* **5**, 8-9

170. Long, J. Z., Li, W., Booker, L., Burston, J. J., Kinsey, S. G., Schlosburg, J. E., Pavon, F. J., Serrano, A. M., Selley, D. E., Parsons, L. H., Lichtman, A. H., and Cravatt, B. F. (2009) *Nat Chem Biol* **5**, 37-44

171. Nomura, D. K., Dix, M. M., and Cravatt, B. F. (2010) *Nat Rev Cancer*

172. Nomura, D. K., Long, J. Z., Niessen, S., Hoover, H. S., Ng, S. W., and Cravatt, B. F. (2010) *Cell* **140**, 49-61

173. Kozak, K. R., Gupta, R. A., Moody, J. S., Ji, C., Boeglin, W. E., DuBois, R. N., Brash, A. R., and Marnett, L. J. (2002) *J Biol Chem* **277**, 23278-23286

174. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J Biol Chem* **275**, 33744-33749

175. Kozak, K. R., Crews, B. C., Morrow, J. D., Wang, L. H., Ma, Y. H., Weinander, R., Jakobsson, P. J., and Marnett, L. J. (2002) *J Biol Chem* **277**, 44877-44885

176. Rouzer, C. A., and Marnett, L. J. (2005) *J Biol Chem* **280**, 26690-26700

177. Kozak, K. R., Crews, B. C., Ray, J. L., Tai, H. H., Morrow, J. D., and Marnett, L. J. (2001) *J Biol Chem* **276**, 36993-36998

178. Nirodi, C. S., Crews, B. C., Kozak, K. R., Morrow, J. D., and Marnett, L. J. (2004) *Proc Natl Acad Sci U S A* **101**, 1840-1845

179. Hu, S. S., Bradshaw, H. B., Chen, J. S., Tan, B., and Walker, J. M. (2008) *Br J Pharmacol*

180. Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1990) *Proc Natl Acad Sci U S A* **87**, 1932-1936

181. Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J. F., Beslot, F., Bohme, G. A., Imperato, A., Pedrazzini, T., Roques, B. P., Vassart, G., Fratta, W., and Parmentier, M. (1999) *Science* **283**, 401-404

182. Di Marzo, V., Melck, D., De Petrocellis, L., and Bisogno, T. (2000) *Prostaglandins Other Lipid Mediat* **61**, 43-61

183. Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N., and Ishima, Y. (1999) *J Biol Chem* **274**, 2794-2801

184. Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., Suhara, Y., Takayama, H., and Waku, K. (2000) *J Biol Chem* **275**, 605-612

185. Burkey, T. H., Quock, R. M., Consroe, P., Ehlert, F. J., Hosohata, Y., Roeske, W. R., and Yamamura, H. I. (1997) *Eur J Pharmacol* **336**, 295-298

186. Kearn, C. S., Greenberg, M. J., DiCamelli, R., Kurzawa, K., and Hillard, C. J. (1999) *J Neurochem* **72**, 2379-2387

187. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., and Hogestatt, E. D. (1999) *Nature* **400**, 452-457

188. Sagar, D., Gaw, A. G., Okine, B., Woodhams, S., Wong, A., Kendall, D., and Chapman, V. (2009) *Molecular Pain* **5**, 59

189. Iversen, L., and Chapman, V. (2002) *Curr Opin Pharmacol* **2**, 50-55

190. Jennings, E. A., Vaughan, C. W., and Christie, M. J. (2001) *J Physiol* **534**, 805-812

191. Meng, I. D., Manning, B. H., Martin, W. J., and Fields, H. L. (1998) *Nature* **395**, 381-383

192. Lichtman, A. H., Cook, S. A., and Martin, B. R. (1996) *J Pharmacol Exp Ther* **276**, 585-593

193. Telleria-Diaz, A., Schmidt, M., Kreusch, S., Neubert, A. K., Schache, F., Vazquez, E., Vanegas, H., Schaible, H. G., and Ebersberger, A. (2010) *Pain* **148**, 26-35

194. Caille, S., Alvarez-Jaimes, L., Polis, I., Stouffer, D. G., and Parsons, L. H. (2007) *J Neurosci* **27**, 3695-3702

195. Alvarez-Jaimes, L., Stouffer, D. G., and Parsons, L. H. (2009) *J Neurochem* **111**, 37-48

196. Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T., and Kunos, G. (2001) *Nature* **410**, 822-825

197. Pi-Sunyer, F. X., Aronne, L. J., Heshmati, H. M., Devin, J., and Rosenstock, J. (2006) *Jama* **295**, 761-775

198. Scheen, A. J., Finer, N., Hollander, P., Jensen, M. D., and Van Gaal, L. F. (2006) *Lancet* **368**, 1660-1672

199. Topol, E. J., Bousser, M. G., Fox, K. A., Creager, M. A., Despres, J. P., Easton, J. D., Hamm, C. W., Montalescot, G., Steg, P. G., Pearson, T. A., Cohen, E., Gaudin, C., Job, B., Murphy, J. H., and Bhatt, D. L. (2010) *Lancet* **376**, 517-523

200. Boekholdt, S. M., and Peters, R. J. (2010) *Lancet* **376**, 489-490

201. Romero, J., Lastres-Becker, I., de Miguel, R., Berrendero, F., Ramos, J. A., and Fernandez-Ruiz, J. (2002) *Pharmacol Ther* **95**, 137-152

CHAPTER II

IN VITRO DETERMINANTS OF PGHS-2 MEDIATED OXYGENATION OF THE ENDOCANNABINOID 2-ARACHIDONOYL GLYCEROL

Introduction

Prostaglandin H Synthases (PGHSs) catalyze the first two steps in the biosynthesis of prostaglandins (PGs), a class of bioactive lipids that regulate a broad range of physiological responses. The cyclooxygenase activity oxygenates arachidonic acid (AA) (22) to the hydroperoxy endoperoxide, PGG_2, and the peroxidase activity reduces PGG_2 to PGH_2 (Figure 1).

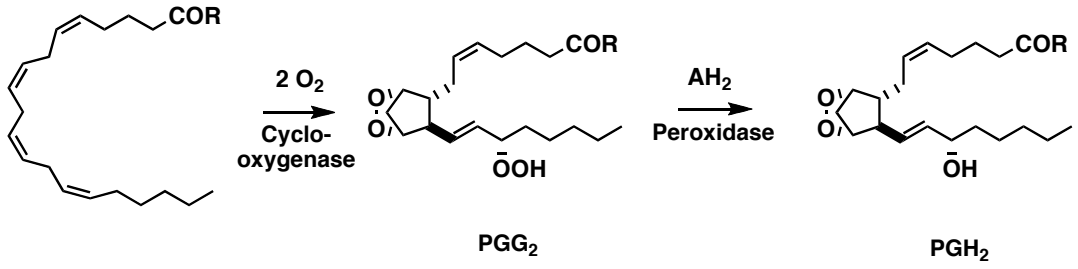


Figure 1. Fatty acyl substrate (FAH) oxygenation by PGHS-2

The two isoforms of PGHS - PGHS-1 and PGHS-2 - are distinguished by different patterns of expression. In general, PGHS-1 is constitutively expressed in most tissues suggesting a homeostatic role, whereas PGHS-2 is inducible by a variety of inflammatory and proliferative stimuli (cytokines, bacterial lipopolysaccharide, growth factors, and tumor promoters), consistent with a role for PGs in inflammation and carcinogenesis (27). In addition to differences in

regulation, PGHS enzymes exhibit biochemical differences related to their structures. These include the increased sensitivity of PGHS-2 to peroxide activation (35) and the presence of a sequence in PGHS-2 that renders it susceptible to cellular proteolysis (202). PGHS-2 also displays broader substrate specificity than PGHS-1. For example, it oxygenates ester and amide derivatives of AA much more efficiently than PGHS-1(202). 2-Arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA) are of particular interest as PGHS-2 substrates because they occur in human tissue and are endogenous ligands for the cannabinoid receptors (CB_1 and CB_2) (Figure 2)(149,159).

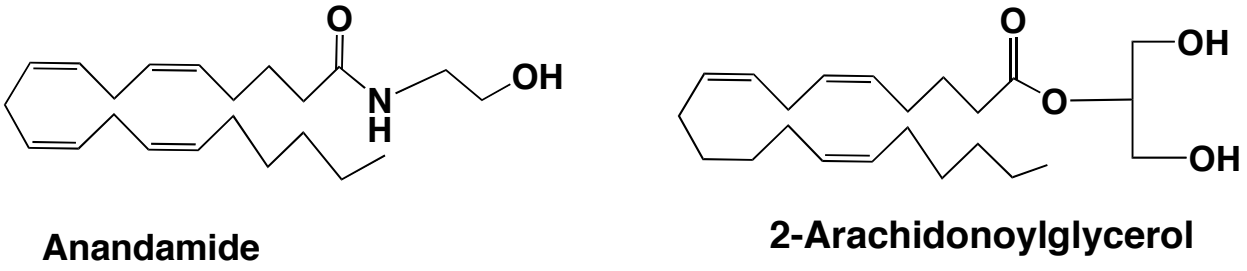


Figure 2. Structures of the endocannabinoids

PGHS-2-dependent oxygenation of 2-AG yields the glyceryl ester of PGH_2 (PGH_2-G). PGH_2-G is converted to a similar array of prostanoids as PGH_2 with the exception of its poor conversion to TxA_2-G (175). Murine resident peritoneal macrophages stimulated with an inflammatory agonist (zymosan) produce both PGE_2-G and PGI_2-G (203), and PGD_2-G formation is elicited from the RAW264.7 macrophage-like cell line upon pretreatment with LPS and IFNγ followed by ionomycin (175). PGE_2-G has been isolated from rat footpads in the presence and absence of an inflammatory stimulus (179). Thus, PG-Gs are produced by intact cells treated with a variety of stimuli and are present *in vivo*. The physiological relevance of PGHS-2-dependent 2-AG oxygenation is the subject of ongoing investigation. The k_{cat}s for

oxygenation of AA and 2-AG by hPGHS-2 are comparable, whereas the k_{cat} for oxygenation of AA by mPGHS-2 is approximately twice that of 2-AG (174). An important property of derivatives of 2-AG that has not been explored is the ability of glyceryl-hydroperoxides such as PGG_2-G, to interact with the peroxidase activity of PGHS, either as substrates for peroxidatic reduction or as activators of cyclooxygenase activity (Figure 3).

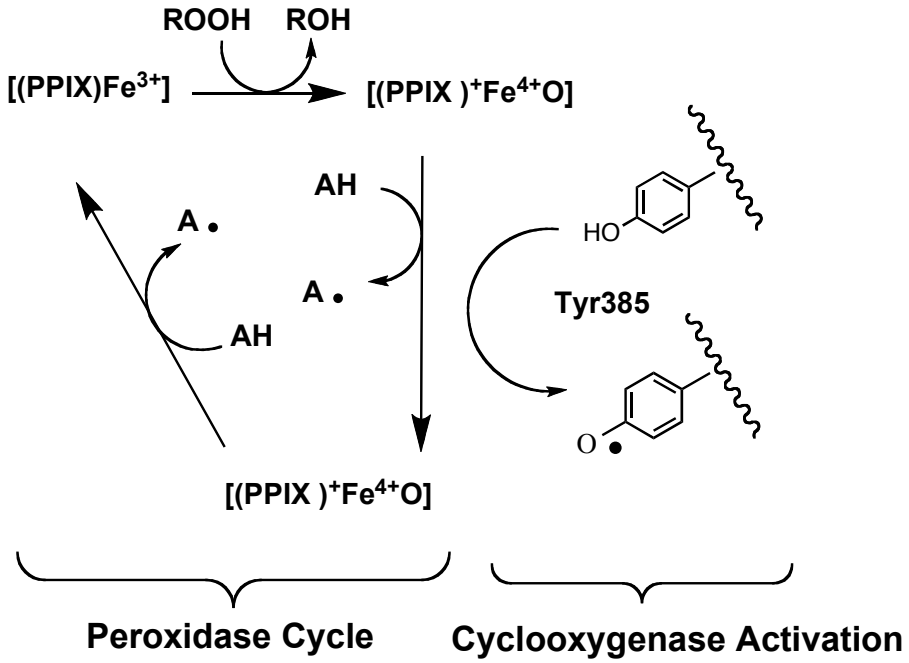


Figure 3. Interaction of the POX active site and the COX active site

Purified PGHS enzymes are inactive as oxygenases and require hydroperoxide-dependent activation to become functional. Fatty acyl hydroperoxides, PG hydroperoxides, or peroxynitrite react with the heme prosthetic group (Figure 3) to produce a ferryl-oxo complex that oxidizes Tyr-385 in the oxygenase active site to produce a tyrosyl radical (Figure 3); the tyrosyl radical oxidizes the fatty acyl substrate (27).

We have undertaken a study of the ability of glyceryl hydroperoxides compared to fatty acid hydroperoxides to serve as substrates for the peroxidase activity of PGHS-1 and PGHS-2, and to activate oxygenation by both enzymes. We also investigated the peroxide-dependent activation of AA and 2-AG oxygenation by PGHS-2, by conducting experiments that created decreased peroxide tone. The results reveal a major difference in the sensitivity of PGHS-2 with respect to the oxygenation of the two substrates i.e., the concentration of peroxide required to activate the cyclooxygenase functionality. This concentration, coined K_p (39), is 4-fold higher for 2-AG than AA with hPGHS-2. Altogether, these results indicate that the peroxidase active site may represent an important site of the regulation and pharmacologic modulation of PGHS-2 oxygenation of 2-AG. The development of POX inhibitors is described in Chapter IV.

Experimental Procedures

Materials - AA, 2-AG, and hematin were obtained from Nu Chek Prep (Elysian, MN), Cayman Chemicals (Ann Arbor, MI), and Frontier Scientific (Logan, UT), respectively. All other reagents were from Sigma-Aldrich (St. Louis, MO). Peroxide-free AA and 2-AG were prepared and confirmed to be free of peroxides as described (204,205).

Enzymes - Purified bovine erythrocyte glutathione peroxidase (GPx) and purified *S. cerevesiae* glutathione reductase (GSSG reductase) were obtained from Sigma-Aldrich (St. Louis, MO). Purified soybean 15-LOX was from Cayman Chemicals (Ann Arbor, MI). hPGHS-2 and mPGHS-2 were expressed and purified as described (206). Site directed mutagenesis to generate H388Y mPGHS-2 and subsequent expression and purification were performed as published (34). oPGHS-1 was purified from ram seminal vesicles (Oxford Biomedical Research, Oxford, MI), as previously described (207). The specific activities of hPGHS-2 and mPGHS-2

were both 62.5 μM AA per μM enzyme per minute; oPGHS-1 was 325 μM AA per μM enzyme per minute. H388Y mPGHS-2's specific activity was 31.3 μM AA per μM enzyme per minute.

Chemistry - 15-HpETE was generated with 40% yield as determined by UV spectroscopy (236 nm) using the molar absorptivity of conjugated dienes (ε_{236} = 29,500 $M^{-1}.cm^{-1}$) (208). 15-HpETE-G was generated as described (173) with minor modifications. Briefly, to 200 mL of oxygen-saturated 100 mM sodium borate buffer (pH 9.0) was added 750,000 U/mL purified soybean 15-lipoxygenase. 2-AG (25 mg in 2 mL acetonitrile) was added to the buffer with continued perfusion of O_2 over the surface of the buffer and stirring. An aliquot of the reaction mixture was monitored by UV spectroscopy (236 nm) until no further increase in absorbance was evident (~10 minutes). The reaction mixture was extracted twice with 200 mL of diethyl ether, and the combined organic layers were dried using $MgSO_4$, filtered, and concentrated under vacuum. The residue was resuspended in acetonitrile and filtered over glass wool. 15-HpETE-G was generated with 30% yield and was determined using UV spectroscopy (236 nm). Purity was determined by HPLC/UV to be ≥ 95%.

Hydroperoxide regiochemistry of 15-HpETE and 15-HpETE-G were confirmed by diagnostic fragmentation following collision-induced dissociation (CID) under the following liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) conditions: a Phenomenex Luna C18 column (100 mm × 2.0 mm, 3 μm) was held at 40°C and eluted with 40% A (5 mM NH_4OAc in H_2O): 60% B (5 mM NH_4OAc in 90% CH_3CN) at 0.3 mL/min. Mass spectra were obtained on a ThermoFinnigan Quantum triple-quadrupole instrument equipped with an electrospray source and operated in negative ion mode for 15-HpETE (ESI-MS *m/z* calculated for $C_{20}H_{32}O_4$ (M^-), 335.2; found 335.1) and in positive-ion mode for 15-HpETE-G (ESI-MS *m/z* calculated for $C_{23}H_{38}O_6$ ($M+NH_4^+$), 428.3; found 428.1)

(ThermoFinnigan, San Jose, CA). Hydroperoxide regiochemistry of 15-HpETE and 15-HpETE-G were also confirmed by ^{1}H NMR.

15-HpETE ^{1}H NMR at 500 MHz ($CDCl_3$)- δ 6.58-6.63 (dd, 1H, *J* = 11.1, 15.3 Hz CH), 5.98-6.02(t, 1H, *J* = 10.9 Hz CH), 5.56-5.61 (dd, 1H, *J* = 7.85, 15.3 Hz CH), 5.32-5.48 (m, 5H, 5 x CH), 4.39-4.43 (m, 1H, CH), 2.89-3.04 (m, 2H, CH_2), 2.77-2.86 (m, 2H, CH_2), 2.35-2.38 (m, 2H, CH_2), 2.10-2.15 (m, 2H, CH_2), 1.66-1.75 (m, 2H, CH_2), 1.46-1.53 (m, 2H, CH_2), 1.23-1.38 (m, 6H, 3 x CH_2), 0.85-0.88 (t, 3H, *J* = 6.85 Hz CH_3)

15-HpETE-G ^{1}H NMR at 500 MHz ($CDCl_3$)- δ 6.58-6.63 (dd, 1H, *J* = 11.1, 15.3 Hz CH), 5.98-6.02 (t, 1H, *J* = 10.9 Hz CH), 5.56-5.61(dd, 1H, *J* = 7.85, 15.3 Hz CH), 5.32-5.48 (m, 5H, 5 x CH), 4.38-4.42(m, 1H, CH), 4.28-4.32(t, 1H, *J* = 8.6 Hz CH), 4.09-4.26 (m, 1H, CH), 3.70-3.72 (m, 1H, CH), 3.60-3.63 (m, 1H, CH), 2.89-3.04 (m, 2H, CH_2), 2.77-2.86 (m, 2H, CH_2), 2.35-2.38 (m, 2H, CH_2), 2.10-2.15 (m, 2H, CH_2), 1.66-1.75 (m, 2H, CH_2), 1.46-1.53 (m, 2H, CH_2), 1.23-1.38 (m, 6H, 3 x CH_2), 0.85-0.88 (t, 3H, *J* = 6.85 Hz CH_3).

Detection of endoperoxide intermediates of PGHS synthase action - PGH_2, PGG_2, PGH_2-G and PGG_2-G levels were determined from reactions of hPGHS-2 as follows. hPGHS-2 (400 nM) was reconstituted with an equivalent of hematin and incubated in 600 μL of 100 mM Tris-HCl, pH 8.0 with 500 μM phenol at 25° C. Substrate (AA or 2-AG) was added to a final concentration of 50 μM. Reactions were quenched by addition of 600 μL of fresh anhydrous diethyl ether, vortexed for 10 seconds, and centrifuged at ~5000 RCF for 45 seconds. Ethereal fractions were removed and evaporated to dryness under a gentle stream of argon. Analytes were reconstituted in 300 μL acetonitrile:water (50:50 v:v) and analyzed immediately. LC/MS was

conducted as follows: Gradient elution [0% A (5 mM NH_4OAc in H_2O, pH 3.3) linearly to 60% B (5 mM NH_4OAc in 90% CH_3CN] at 0.3 mL/min was carried out on a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm). Mass spectra were obtained on a ThermoFinnigan Quantum triple-quadrupole instrument equipped with an electrospray source and operated in positive ion mode with selective reaction monitoring. Quadrupoles 1 and 3 were set to the same mass for each analyte (ESI-MS *m/z* for PGH_2 – 370.00 ($M+NH_4^+$), PGG_2 –386.00 ($M+NH_4^+$), PGH_2-G – 444.00 ($M+NH_4^+$) and PGG_2-G – 460.00 ($M+NH_4^+$).

O_2 uptake assay for the COX activity of PGHS - For determination of cyclooxygenase activity, pure PGHS protein (100 nM - 400 nM) was reconstituted with hematin and incubated in a 600 μL thermostatted cuvette, at 37°C, in 100 mM Tris-HCL, pH 8.0 with 500 μM phenol. Peroxide-free substrate was prepared and confirmed to be free of peroxides as described (204,205). Substrate was added to a final concentration of 50 μM. Activity was monitored using an FO125T Instech fiber optic oxygen probe, connected to an Instech Model 210 fiber optic oxygen monitor controlled with OOISensors software (Plymouth Meeting, PA). To test the effect of 15-HpETE and 15-HpETE-G in stimulating the turnover of 2-AG by H338Y mPGHS-2, either peroxide was added along with substrate to the final desired concentration.

LC/MS assay for COX activity and inhibition - Hematin-reconstituted PGHS-2 was incubated with inhibitor (0.25-50 μM) for 15 min at 37°C prior to the addition of 50 μM substrate (AA or 2-AG). The reaction was quenched after 30 seconds with ethyl acetate containing 0.5% acetic acid and deuterated internal standards. The organic layer was extracted and evaporated under nitrogen to near-dryness. The residue was resuspended in 50:50 methanol:water and product formation was monitored using LC-MS/MS. Products were separated on a Luna C18(2) column (5.0 cm x 0.2 cm, 3 μm) using an isocratic elution consisting

of 65% 5 mM ammonium acetate, pH 3.5 and 35% acetonitrile at a flow rate of 0.375 mL/min. The triple-quadrupole mass spectrometer was operated in positive ion mode using electrospray ionization using selective reaction monitoring to detect PGs and PG-Gs. The following transitions were monitored: for PGE_2/D_2 m/z ($M+NH_4^+$), 370→317, for PGE_2/D_2-d4 m/z ($M+NH_4^+$) 374→321, for PGE_2/D_2-G m/z ($M+NH_4^+$) 444→391 and for PGE_2/D_2-G-d5 m/z ($M+NH_4^+$) 449→396. Products are quantitated as the ratio of the area of the peak to its corresponding internal standard and normalized to a DMSO control.

Spectrophotometric assay for peroxidase activity of PGHS - oPGHS-1, hPGHS-2, or mPGHS-2 (46 nM) was reconstituted with a one-half equivalent of hematin in 80 mM Tris-HCL buffer (pH 8.0) containing 1 mM ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and incubated for 1 minute. The mixture was added to a quartz cuvette, and stirred continuously at 25° C. 15-HpETE or 15-HpETE-G in acetonitrile was added to desired concentrations (78 nM-50 μM). The concentration of acetonitrile was maintained constant for all samples during a given experiment and never exceeded 5% of the total reaction volume (2 mL). The reaction was monitored via UV spectroscopy (417 nm) for the formation of the $ABTS^{+\cdot}$ radical. The early linear portions of the reaction curves were used to determine initial reaction rates, at the various substrate concentrations (78 nM - 50 μM). Plots of initial rates versus substrate concentration were fit to the Michaelis-Menten equation by nonlinear regression analysis (Prism version 4.0).

GPx activity – GPx activity was determined as described (209). Briefly, varying concentrations of 15-HpETE or 15-HpETE-G (78 nM-50 μM) were added to a mixture of 0.25 units of GPx, 1 mM GSH, 1 unit GSSG reductase, and 250 μM NADPH in 100 mM sodium phosphate buffer (pH 8.0) at 37°C. The oxidation of NADPH was monitored via UV spectroscopy (340 nm). The early linear portions of the reaction curves were used to determine

initial reaction rates, and the data were fit to the Michaelis-Menten equation via non-linear regression analysis (Prism version 4.0). One unit of GPx catalyzes the oxidation by H_2O_2 of 1.0 μM of reduced glutathione (GSH) to oxidized glutathione (GSSG) per minute at pH 7.0 and 25 °C. One unit of GSSG reductase will reduce 1.0 μM of GSSG per minute at pH 7.6 at 25 °C.

Activation of cyanide-inhibited oPGHS-1 and mPGHS-2 by 15-HpETE and 15-HpETE-G – Activation of PGHS enzymes by hydroperoxides was determined through the ability of the hydroperoxide to reduce the NaCN-induced lag phase in the cyclooxygenase reaction (41). The standard PGHS activity assay above was modified to include 250 mM NaCN in the buffer. Peroxide-free AA along with varying concentrations of either 15-HpETE or 15-HpETE-G (15.6 nM- 1 μM) was added to the cuvette. Activity was monitored as above, with maximal rate determined by taking the first derivative of the reaction trajectory. Lag time (time to maximal rate) was determined for each concentration of peroxide (Prism version 4.0), for both oPGHS-1 and mPGHS-2, and plotted against the concentration of peroxide used. Statistical significance was determined using the Student's t-test (un-paired, two-tailed) using Prism (version 4.0) software.

GPx-mediated suppression of AA and 2-AG oxygenation by hPGHS-2 and mPGHS-2 - The standard PGHS activity assay was modified to include 1 mM GSH and varying concentrations of GPx (20 U- 160 U). Activity was monitored as above, with maximal rates achieved under each GPx concentration determined by taking the first derivative of the reaction trajectory. Maximal rates relative to the control (no GPx) were plotted against their respective concentration of GPx. Statistical significance was determined according to Student's t-test (un-paired, two-tailed) using Prism (version 4.0) software. Relative K_ps were determined by linear

regression of the plot of decay of cyclooxygenase activity and use of the K_m for 15-HpETE and 15-HpETE-G with each respective enzyme as previously published (39).

Results

Oxygenation of 2-AG by mCOX-2, hCOX-2, and oCOX-1 - Figure 4 displays the time courses of oxygenation of peroxide-free 2-AG and peroxide-free AA following incubation with purified recombinant mCOX-2. As reported earlier, the rate and extent of oxygenation were approximately 50% lower for 2-AG than AA (Figure 4A)(174). Similar results were obtained in incubations of peroxide-free 2-AG or peroxide-free AA with purified recombinant hCOX-2 (Figure 4B). The rate and extent of oxygenation of 2-AG was approximately 30% lower than for AA. Previous reports indicate that 2-AG and AA are equivalent substrates for COX-2 (174), but the data with peroxide-free substrates indicate a slight difference in efficiency. Consistent with prior findings, 2-AG was a poor substrate for oCOX-1 (174) (Figure 4C).

Glyceryl hydroperoxides as substrates for the peroxidase activities of mPGHS-2, hPGHS-2 and oPGHS-1 - To determine if the glyceryl ester affects the reduction efficiency of a substrate by the PGHS peroxidase, we determined the steady-state kinetic values for the reduction of 15-HpETE and 15-HpETE-G. 15-HpETE is comparable to PGG_2 as a peroxidase substrate and has been used as a model for PGG_2, which is unstable and difficult to prepare in highly purified form (28,210). By extension, we used 15-HpETE-G as a surrogate for PGG_2-G. Both hydroperoxides were prepared and tested as described in "Experimental Procedures". Heme-reconstituted mPGHS-2, hPGHS-2, or oPGHS1 was incubated with increasing concentrations of 15-HpETE or 15-HpETE-G and 1mM of the reducing substrate ABTS. Table I summarizes the k_{cat} and K_m values for the two substrates and these three enzymes.

Table I: Steady-state kinetic parameters for PGHS-1- and PGHS-2-mediated oxidation of ABTS by 15-HpETE and 15-HpETE-G

15-HpETE	**K_m (μM)**	**k_{cat} (S^{-1})**	**k_{cat}/K_m (S^{-1}. μM^{-1})**
oPGHS-1	4.9 ±1.3	34.0 ±3.0	6.9 ±2.3
mPGHS-2	1.0 ±0.2	6.6 ±0.3	6.4 ±1.5
hPGHS-2	3.0 ±0.5	9.3 ±0.4	3.2 ±0.8
15-HpETE-G	**K_m (μM)**	**k_{cat} (S^{-1})**	**k_{cat}/K_m (S^{-1}. μM^{-1})**
oPGHS-1	4.5 ±1.2	49.0 ±4.0	11.0 ±3.3
mPGHS-2	8.0 ±2.0	15.0 ±1.0	1.9 ±0.5
hPGHS-2	4.8 ±0.5	16.1 ±0.4	3.4 ±0.8

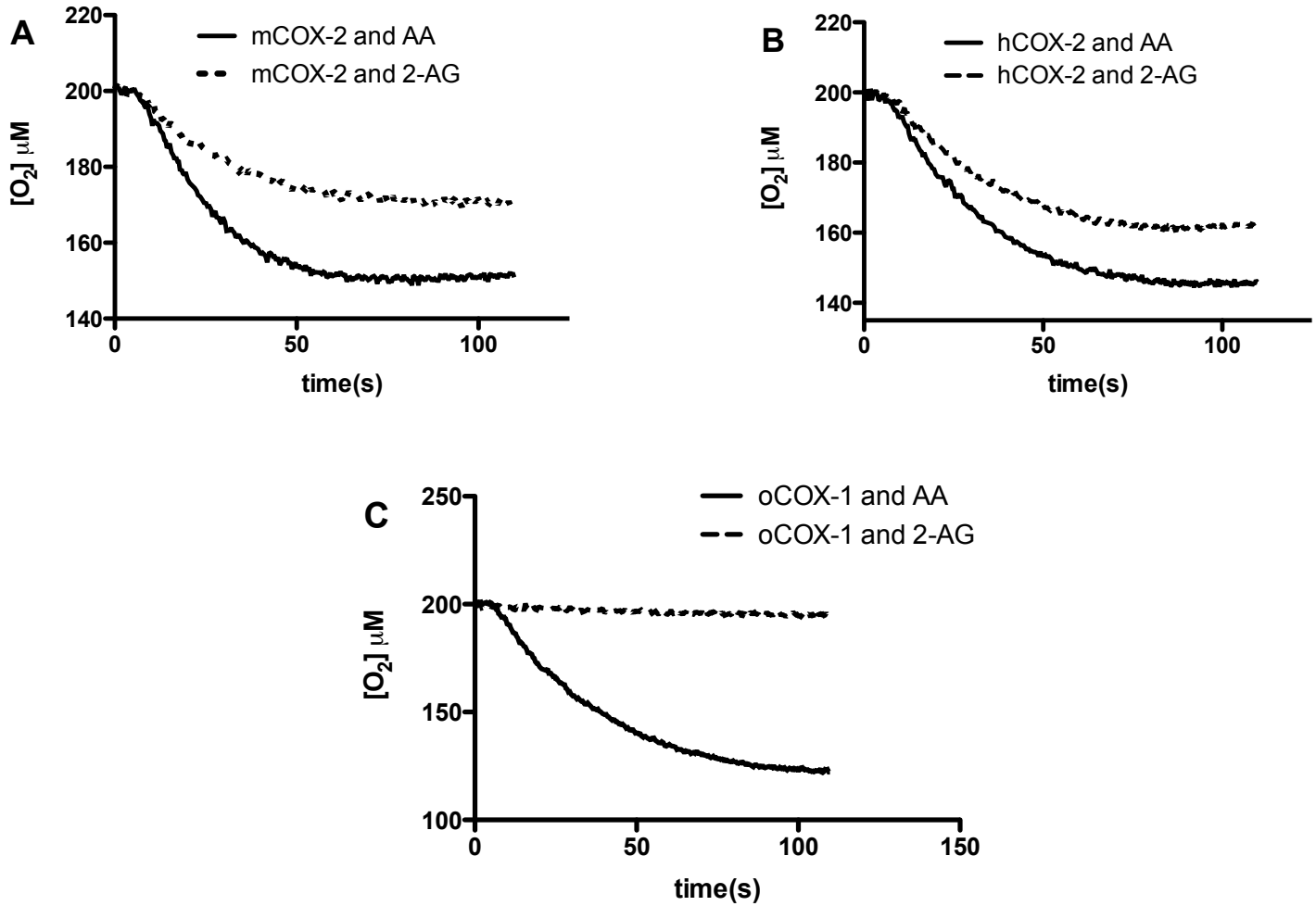


Figure 4. Time courses of the oxygenation of peroxide free AA and 2-AG by mCOX-2 (A) and hCOX-2 (B). Reaction conditions are described under "Experimental Procedures". Reactions contained purified recombinant mCOX-2 (400 nM)(A), purified recombinant hCOX-2 (400 nM)(B) and 50 μM of either peroxide-free AA or peroxide-free 2-AG.

The data revealed lower K_m values for mPGHS-2 and hPGHS-2 using 15-HpETE than 15-HpETE-G (Table I). mPGHS-2 and hPGHS2 also demonstrated lower k_{cat} values using 15-HpETE relative to 15-HpETE-G (Table I). The efficiency of 15-HpETE and 15-HpETE-G as peroxidase substrates for hPGHS-2 (k_{cat}/K_m), were very similar (Table I). In contrast, 15-HpETE-G was significantly less efficient than 15-HpETE as a peroxidase substrate for mPGHS-2 (Table I). This difference in k_{cat}/K_m stemmed solely from the higher K_m for 15-HpETE-G, despite the fact that its k_{cat} was actually higher than that of 15-HpETE (Table I). oPGHS-1 demonstrated k_{cat} and K_m values that did not differ significantly for either 15-HpETE and 15-HpETE-G (Table I). Thus, 15-HpETE and 15-HpETE-G are comparable substrates for the peroxidase activities of oPGHS-1 and hPGHS-2, suggesting that the presence of the glyceryl ester has minimal effect on the efficiency of peroxide reduction for these enzymes.

To qualitatively compare the ability of PGG_2 and PGG_2-G to serve as substrates for the POX activity of PGHS-2, we incubated hPGHS-2 with AA or 2-AG for 30 s, extracted the incubation mixtures with cold diethyl ether, and analyzed the extracts by LC/MS. Figure 5 compares the total ion chromatograms and selective ion monitoring profiles of extracts of AA and 2-AG incubated with Fe-hPGHS-2 or Mn-hPGHS-2. With Fe-hPGHS-2 and AA, a major peak was observed at 6.8 min with *m/z* 370, corresponding to PGH_2 and with Fe-hPGHS-2 and 2-AG, a major peak was observed at 6.1 min with *m/z* 444 corresponding to PGH_2-G. No peaks were observed in either incubation with *m/z* 386 or *m/z* 460, corresponding to PGG_2 or PGG_2-G, respectively (Figure 5). Thus, AA and 2-AG are oxygenated to PGG_2 and PGG_2-G, which are efficiently reduced to PGH_2 and PGH_2-G, respectively.

Parallel experiments were performed with Mn-PGHS-2, which exhibits POX activity levels that are <0.5% of those of Fe-PGHS-2 (Figure 5) (211). Incubations of AA and Mn-

hPGHS-2 generated LC/MS profiles with a major peak at 7.2 min and *m/z* 386, which co-eluted with a commercial sample of PGG_2. A small peak of PGH_2 was detected at 6.8 min, as were minor peaks at 5.4-5.9 min corresponding to PG's. Incubations of 2-AG and Mn-hPGHS-2 generated LC/MS profiles with a major peak at 6.6 min with an *m/z* 460 corresponding to PGG_2-G. A small peak of PGH_2-G was observed at 6.2 min as was a trace peak at 5.4 min corresponding to PG-G's. These experiments, although qualitative, indicate that PGG_2-G and PGH_2-G are detectable in incubations of 2-AG with hPGHS-2, and that PGG_2-G is efficiently reduced to PGH_2-G by the POX activity of hPGHS-2. The results support the conclusions summarized in Table 1 that the glyceryl esters of 15-HPETE and PGG_2 are comparable to the free acids as substrates for the POX activity of PGHS-2.

Activation of the oxygenase activity of oPGHS-1 and mPGHS-2 by 15-HpETE and 15-HpETE-G - After establishing the steady-state kinetics for the reduction of 15-HpETE and 15-HpETE-G by the peroxidase active sites of oPGHS-1, hPGHS-2, and mPGHS-2 (Table I) and qualitatively comparing the reduction of PGG2 and PGG2-G by hPGHS-2 (Figure 5); we evaluated the ability of 15-HpETE and 15-HpETE-G to activate oPGHS-1- and mPGHS-2-mediated oxygenation of AA in the presence of NaCN, as described in "Experimental Procedures". The time taken to achieve maximal rate was plotted against the concentration of exogenous peroxide added (Figure 6, A and B). 15-HpETE and 15-HpETE-G demonstrated a concentration-dependent diminution in the time taken to achieve maximal rate of turnover of AA with both oPGHS-1 (Figure 6A) and mPGHS-2 (Figure 6 B). These results are consistent with the peroxidase kinetics for oPGHS-1, where 15-HpETE and 15-HpETE-G did not differ in efficiency as peroxidase substrates (Table I). Likewise, there was no statistically significant difference in the ability of 15-HpETE and 15-HpETE-G to activate mPGHS-2 despite the

difference observed in the steady-state kinetics for reduction of 15-HpETE versus 15-HpETE-G (Table I).

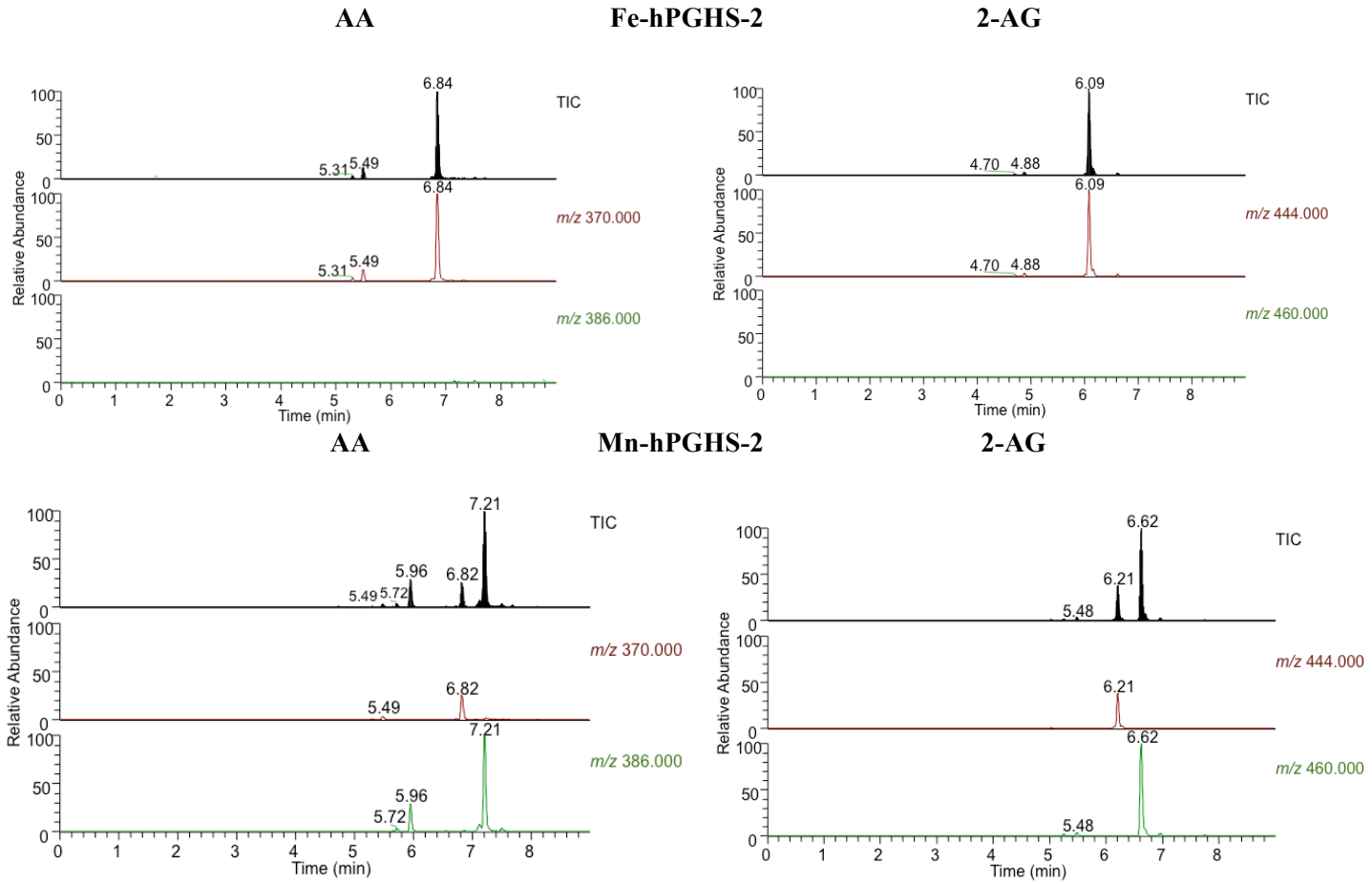


Figure 5. Comparison of the reduction of PGG_2 and PGG_2-G by the peroxidase activity of PGHS-2. The efficiencies of reduction of PGG_2 and PGG_2-G by hPGHS-2 were determined as described in "Experimental Procedures". The LC/MS chromatograms demonstrate that after 30 seconds, heme-reconstituted hPGHS-2 reduces *in situ* generated PGG_2 and *in situ* PGG_2-G completely to PGH_2 (*m/z* 370.00, RT 6.84) and PGH_2-G (*m/z* 444.00, RT 6.09) to the same extent, respectively. Use of Mn-hPGHS-2 demonstrates that our method was robust enough to detect PGH_2/PGG_2 and PGH_2-G/PGG_2-G, with high sensitivity (PGG_2 *m/z* 386.00, RT 7.21) and (PGG_2-G *m/z* 460.00, RT 6.62). The instability of the endoperoxides is highlighted by the formation of earlier eluting species with hPGHS-2 (PGE_2/D_2 → *m/z* 370.00, RT 5.31/5.49 and PGE_2-G /D_2-G→ *m/z* 444.00 RT 4.70/4.88) from PGH_2 and PGH_2-G respectively.

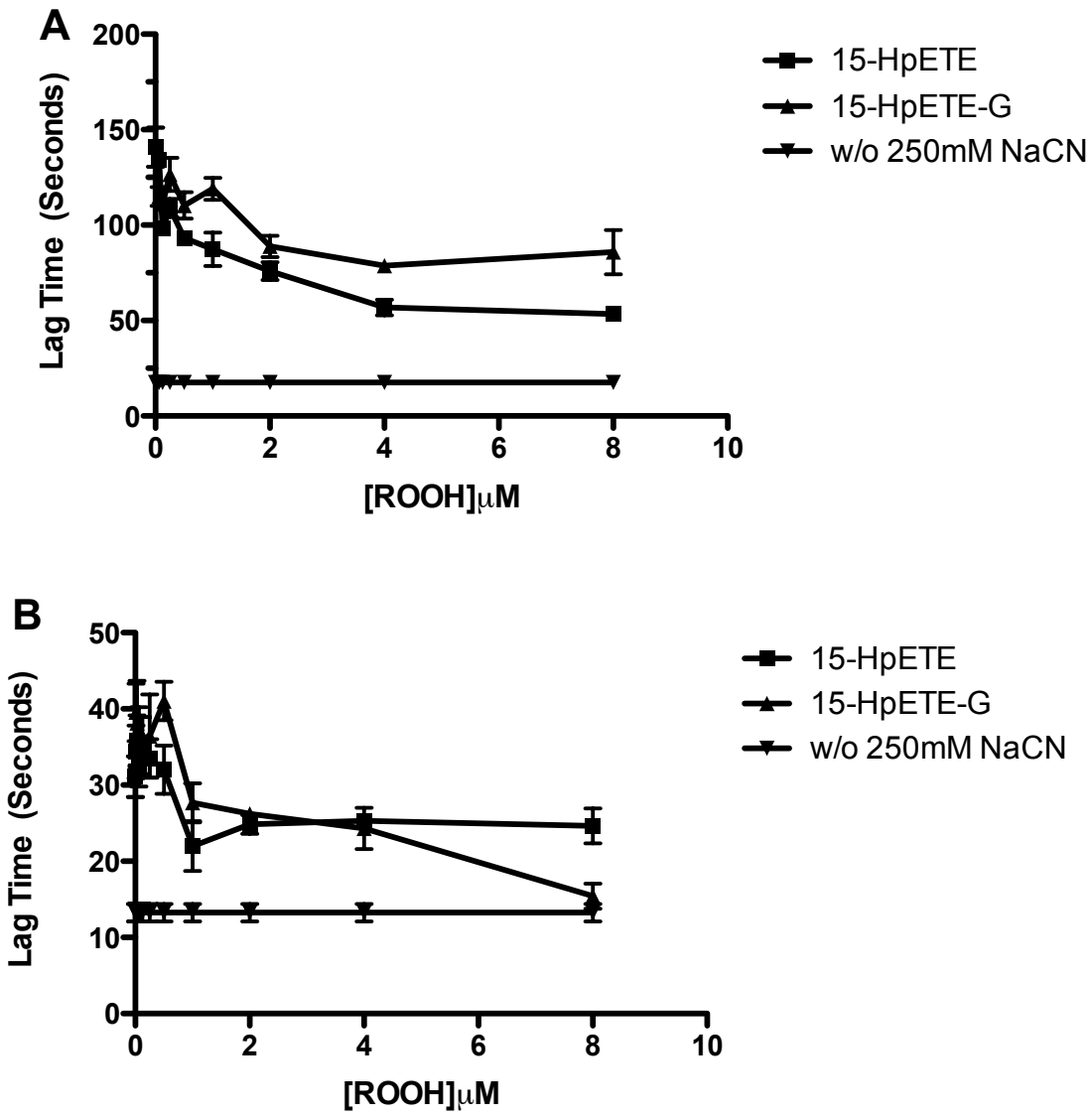


Figure 6. Determination of the activation of oxygenase activity in mPGHS-2 (A) and oPGHS-1 (B) by 15-HpETE and 15-HpETE-G. (A) A lag was induced in the turnover of AA by incubating either oPGHS-1 (60 nM) or (B) mPGHS-2 (120 nM) in standard assay buffer containing 250 mM NaCN. Lag time was defined as the time to maximal rate of oxygen consumption. Lag time was plotted against the final concentration of either 15-HpETE (■) or 15-HpETE-G (▲) in the cuvette. The lag time in the absence of 250 mM NaCN and exogenous peroxide (▼) was also plotted. The data are from triplicate determinations and are reported as mean ± S.E.

Reduction of 15-HpETE and 15-HpETE-G by GPx - We determined the ability of GPx to reduce 15-HpETE and 15-HpETE-G as described under "Experimental Procedures". While 15-HpETE qualitatively appears to be a better substrate for GPx as judged by the extent of GSH oxidation (Figure 7), kinetic parameters could not be determined because of the limit of the solubility of the substrates under the conditions used. In fact the critical micelle concentration for 15-HpETE-G is approximately 70 μM (data not shown).

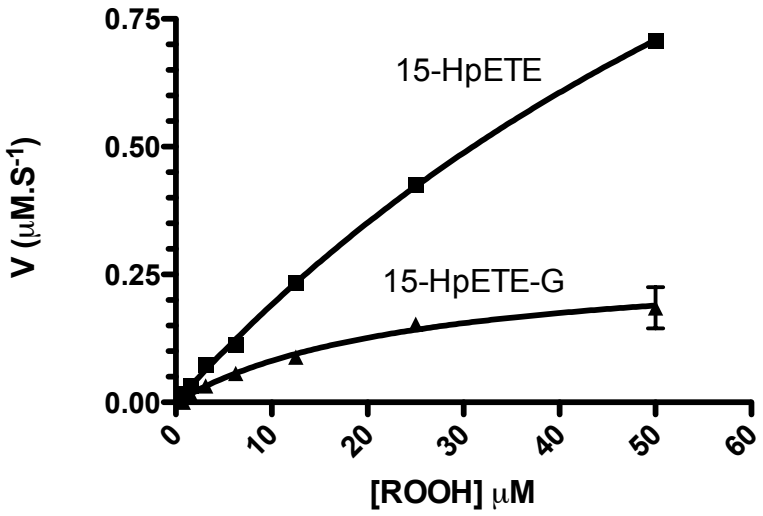


Figure 7. Determination of steady-state kinetic parameters for the reduction of 15-HpETE and 15-HpETE-G by purified bovine erythrocyte GPx. The ability of GPx to reduce 15-HpETE and 15-HpETE-G was determined using the spectrophotometric assay described under "Experimental Procedures". The initial maximal rates of reaction were obtained in duplicate over a range of substrate concentrations (78 nM to 50 μM). Data shown are from duplicate determinations, with the plots of mean ± S.E generated as described in "Experimental Procedures". While we were unable to obtain steady state kinetic parameters owing to the low solubility of 15-HpETE-G, here, we qualitatively demonstrate that 15-HpETE is a better substrate than 15-HpETE-G.

GPx-mediated suppression of the oxygenation of AA and 2-AG by mPGHS-2 and hPGHS-2- When hPGHS-2 or mPGHS-2 was incubated with increasing concentrations of GSH-Px in the presence of 1 mM GSH, the relative reduction in the rate of oxygenation of 2-AG was significantly greater than the relative reduction in rate of oxygenation of AA (Figure 8, A and B). Furthermore, the decrease in oxygenase activity was dependent on the concentration of GPx incubated with either hPGHS-2 or mPGHS-2 (Figure 8, A and B). These experiments demonstrate that the oxygenation of 2-AG, relative to AA, demonstrates greater sensitivity to suppression by GPx. Kulmacz and Lands demonstrated that the concentration of peroxide needed to activate the oxygenase activity of PGHS-1 was two orders of magnitude lower than the K_m of the peroxidase active site of PGHS-1 (39). Using the GPx-GSH system to scavenge peroxides from the peroxidase and cyclooxygenase reactions of PGHS-1, they determined that the peroxidase activity is 13-fold more sensitive to suppression under these conditions, than the cylooxygenase activity (39). From a linear fit of the plot of the decay of the cyclooxygenase activity due to suppression by GPx-GSH, they were able to calculate the concentration of peroxide required to activate the oxygenase for that particular substrate. This value was coined K_p, and incorporates the K_m of the peroxide intermediate from the substrate's oxygenation as follows: [K_mROOH/ sensitivity factor (POX vs. COX)= 13]* dY/dX decay of oxygenase activity under GSH-Px suppression. Km ROOH represents the Km of either 15-HpETE or 15-HpETE-G with either mPGHS-2 or hPGHS-2.

Here we have determined the relative K_ps for AA and 2-AG oxygenation with PGHS-2, from the data in Figure 7; these demonstrate that 2-AG requires greater than 3-fold and 40-fold more peroxide for the activation of its oxygenation by hPGHS-2 and mPGHS-2 respectively, relative to AA (Table II).

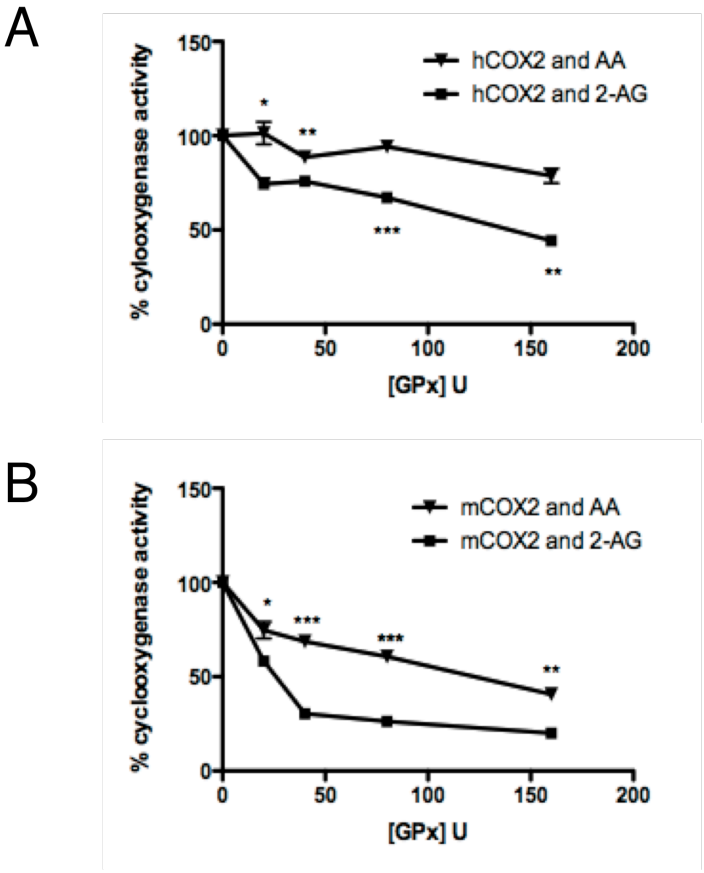


Figure 8. Determination of the ability of the GPx-GSH system to suppress the turnover of AA and 2-AG by hPGHS-2 (A) and mPGHS-2 (B). (A) hPGHS-2 (400 nM) or (B) mPGHS-2 (400 nM) was incubated with increasing concentrations of GPx (0-160 U) in standard assay buffer, and AA (▼) or 2-AG (■) was added to 50 μM as described in "Experimental Procedures". The maximal rate of oxygen consumption relative to the control (no GPx) was plotted against the concentration of GPx. Data are from triplicate determinations. Asterisks indicate statistically significant differences in the relative rate of

oxygen consumption between AA and 2-AG (Student's t-test, un-paired, two-tailed- * $p<0.05$, ** $p<0.001$, ***$p<0.0001$)

Table II. Cyclooxygenase activation parameters for PGHS-2 with AA and 2-AG

	AA K_p (μM)	**2-AG K_p (μM)**	**Relative K_p ($K_{p\ 2\text{-}AG}$/ $K_{p\ AA}$)**
mPGHS-2	0.08 ±0.03	3.1 ±1.5	39
hPGHS-2	0.2 ±0.1	0.8±0.2	4

The disparity in the relative K_ps of 2-AG with mPGHS-2 versus hPGHS-2 are somewhat in line with published k_{cat}/K_m values for 2-AG with both enzymes. mPGHS-2 oxygenates 2-AG with half the efficiency of hPGHS-2 (174). Furthermore, the decay in oxygenase activity with 2-AG for mPGHS-2 under suppression by GPx-GSH occurs faster, relative to hPGHS-2 (Figure 8); at 40 U GPx, the per cent cyclooxygenase activity of mPGHS-2 with 2-AG is ~3-fold lower than hPGHS-2 with 2-AG at 40 U GPx. This accelerated decay, coupled with an increased K_m for glyceryl peroxides with mPGHS-2 factor into yielding a calculated K_p higher than anticipated, and much higher than that obtained for hPGHS-2 with 2-AG.

Oxygenation of 2-AG by purified recombinant H388Y mPGHS-2 - Having established that the oxygenation of 2-AG appears more sensitive to peroxide tone than the oxygenation of AA, we investigated 2-AG oxygenation by an mPGHS-2 mutant (H388Y) that has greatly diminished peroxidase activity (34). Figure 9 displays the oxygenation of AA and 2-AG by purified recombinant wild-type mPGHS-2 and purified recombinant H388Y mPGHS-2.

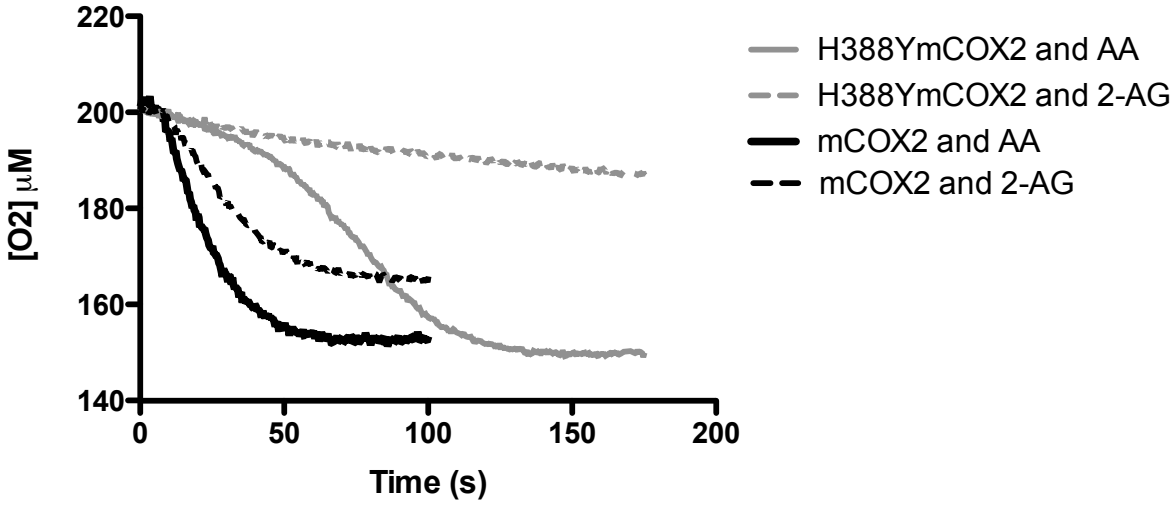


Figure 9. Oxygenation of AA and 2-AG by H388Y mPGHS-2. Wild-type mPGHS2 (400 nM) was incubated with 50 μM of either AA (──) or 2-AG (- - -), in standard assay buffer, as described in "Experimental Procedures". For comparison, H388Y mPGHS2 (400 nM) was incubated with 50 μM of either AA (──) or 2-AG (- - -), in standard assay buffer as described in "Experimental Procedures". Traces are representative of triplicate determinations.

The turnover of AA by H338Y mPGHS-2 displays a characteristic lag in its initial trajectory relative to that of the wild-type mPGHS-2 (Figure 9). This lag is effected by H388Y mPGH2's diminished peroxidase activity and subsequent lack of activation of oxygenase activity (34). Despite the lag, the extent of oxygenation of AA by H388Y mPGHS-2 is the same as that by wild-type mPGHS-2. Interestingly, the turnover of 2-AG by H388Y is severely diminished relative to the turnover of 2-AG by wild-type mPGHS-2 (Figure 9), lacking the distinguishable lag and accelerative phases in its trajectory. This result, along with the finding that 2-AG turnover was more sensitive to GPx suppression, indicated that 2-AG oxygenation is more sensitive to peroxide activation than AA

oxygenation. These results are supported by LC/MS experiments that qualitatively compared the levels of PGG_2-G to the levels of PGG_2 in reactions mixtures of H388Y mPGHS-2 with 2-AG and AA, respectively, quenched after identical time points (Figure 10). In incubations of AA and H388Y, a peak at 7.2 min with *m/z* 386 corresponding to PGG_2 was seen to increase with time over the entire 120 sec incubation period. In contrast, in incubations of 2-AG with the enzyme, the peak at 6.6 min with *m/z* 460, which corresponds to PGG_2-G, was present in very low abundance and scarcely increased with time (Figure 10). This indicates that PGG_2-G levels never approach those of PGG_2, so levels necessary to initiate the accelerative phase of the cyclooxygenase reaction are not attained. This leads to the prolonged lag for 2-AG observed with H388Y mPGHS-2 (Figure 9).

Stimulation of 2-AG oxygenation by purified recombinant H388Y mPGHS-2 via 15-HpETE and 15-HpETE-G - In light of the discovery that the turnover of 2-AG was severely impaired in H388Y mPGHS-2 and that PGG_2-G levels remain extremely low relative to PGG_2 over the course of 2-AG oxygenation, we incubated H388Y mPGHS-2 with increasing concentrations of either 15-HpETE or 15-HpETE-G. Figure 11A demonstrates that incubation with either 32 μM 15-HpETE or 32 μM 15-HpETE-G, led to the recovery of levels of 2-AG oxygenation equal to those observed with wild-type enzyme. The recovery of 2-AG oxygenation by H388Y mPGHS-2 was dependent on the concentration of 15-HpETE and 15-HpETE-G, with both demonstrating comparable capacity to overcome the lack of 2-AG turnover (Figure 11B). These data provide further validation that 15-HpETE and 15-HpETE-G are equivalent activators of oxygenase activity.

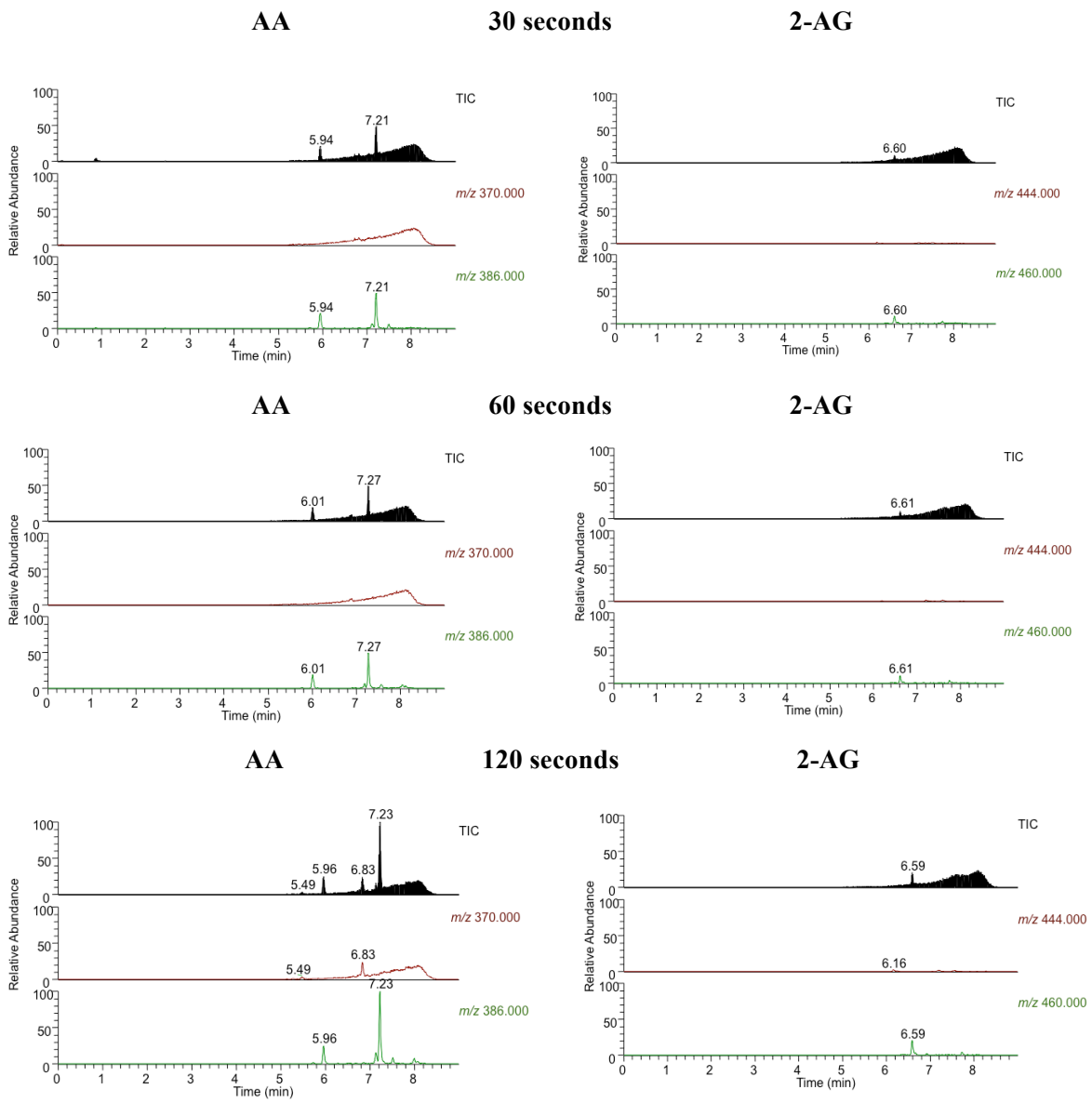


Figure 10. Comparison of the levels of PGG_2 and PGG_2-G over the course of AA and 2-AG oxygenation by H388Y mPGHS-2. The levels of PGG_2 and PGG_2-G produced by the oxygenation of AA and 2-AG over the course of oxygenation by H388Y mPGHS-2 were determined as described in "Experimental Procudures". The LC/MS chromatograms demonstrate that after 30 seconds robust levels of PGG_2 (*m/z* 386.00, RT 7.21) are readily detectable, while PGG_2-G (*m/z* 460.00, RT 6.61) is barely detectable. Robust levels of PGG_2 are detectable at 60 and 120 seconds while PGG_2-G remains barely detectable at 60 and 120 seconds. These data indicate that PGG_2-G levels from 2-AG oxygenation by H388Y mPGHS-2 never approach those of PGG_2 with AA and H388Y mPGHS-2.

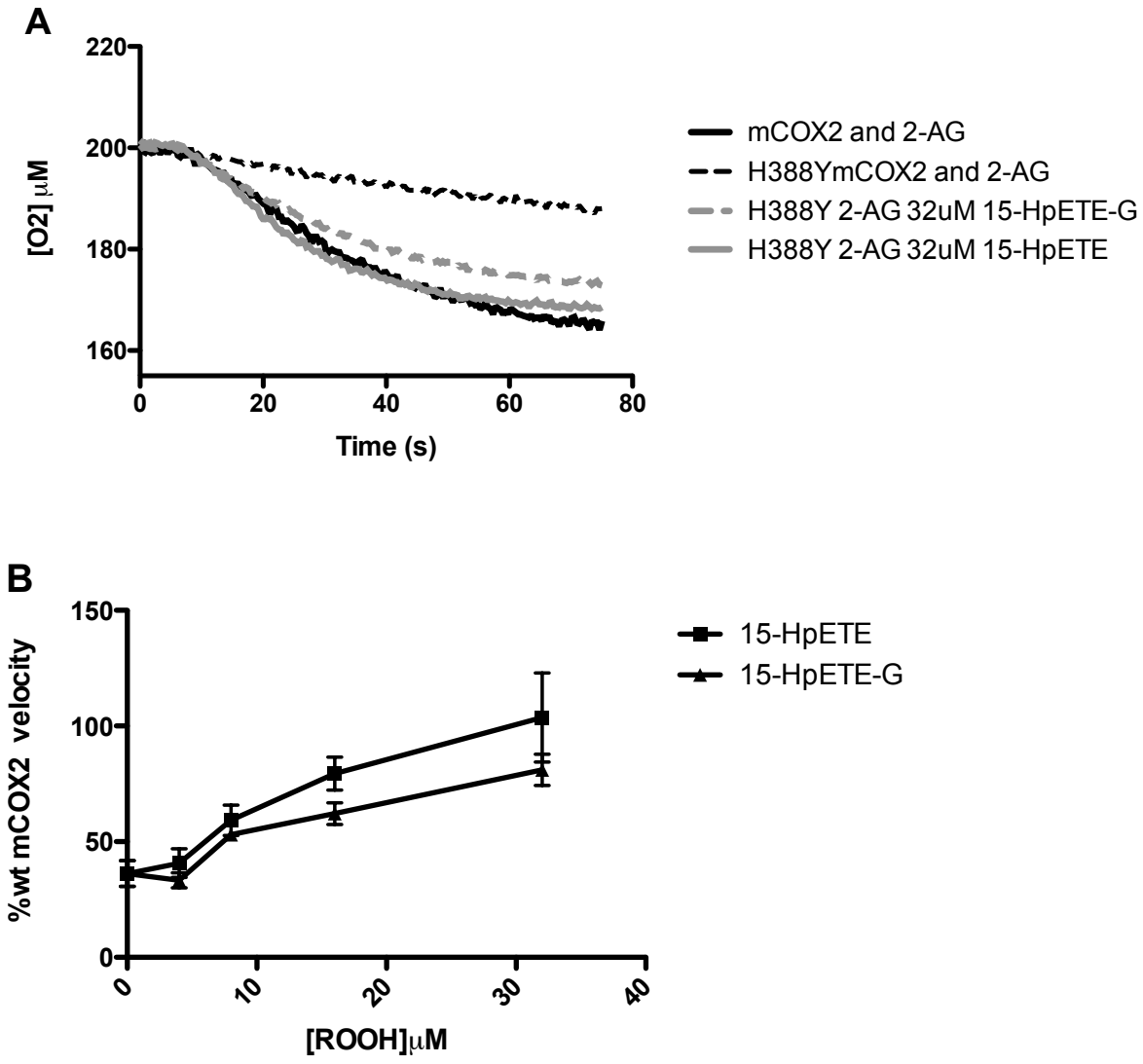


Figure 11. Stimulation of oxygenation of 2-AG by H388Y mPGHS-2 via 15-HpETE and 15-HpETE-G. Wild-type mPGHS2 (400 nM) was incubated with 50 μM of 2-AG (—), in standard assay buffer, as described in "Experimental Procedures". **(A)** For comparison, H388Y mPGHS2 was incubated with 50 μM 2-AG alone (**---**), or with either 32 μM 15-HpETE (—) or 32 μM 15-HpETE-G (---) in standard assay buffer as described in "Experimental Procedures". **(B)** A plot of the rate of His338Tyr mPGHS-2-mediated 2-AG turnover, relative to that of wild-type mPGHS-2, was generated over a range of concentrations (4 μM- 32 μM) of either 15-HpETE (■) or 15-HpETE-G (▲). Data are from triplicate determinations.

Discussion

The present study compared the ability of hydroperoxide derivatives of AA and 2-AG to serve as substrates for the peroxidase activities of PGHS-1 and PGHS-2 and as activators of the cyclooxygenase activities of both enzymes. The results clearly show that the 15-hydroperoxy derivatives of both molecules are similar in their efficiencies as substrates for the PGHS-1 and PGHS-2 peroxidases, and that they activate the cyclooxygenase activities of the two enzymes equivalently with AA as the substrate (Table I, Figure 6). The k_{cat}/K_ms for oxidation of ABTS by the peroxidase activity of PGHS-1 and PGHS-2 are similar for 15-HpETE and 15-HpETE-G although there are some small differences (Table I). oPGHS-1 exhibits a k_{cat}/K_m for 15-HpETE-G that is approximately 2-fold higher than for 15-HpETE, whereas mPGHS-2 exhibits a k_{cat}/K_m for 15-HpETE that is approximately 3-fold higher than for 15-HpETE-G. The ability of 15-HPETE-G to serve as a peroxidase substrate and to activate the cyclooxygenase activity of oCOX-1 also suggests that the reduced ability of 2-AG to serve as a substrate for COX-1 (~20-fold) (Figure 4) (174) is not due to an inability of hydroperoxides derived from 2-AG (e.g., 15-HpETE-G or PGG_2-G) to support peroxidase-dependent activation.

Although hydroperoxy derivatives of 2-AG and AA are equivalent in their ability to activate PGHS-1 and PGHS-2, we discovered significant differences between 2-AG and AA in their sensitivity to oxidation by PGHS-2 which were exhibited under conditions of limiting peroxidase-dependent activation. This was unanticipated because 2-AG and AA have similar k_{cat}/K_ms for oxidation by human or mouse PGHS-2 (174). However, reduction of peroxidase-dependent cyclooxygenase activation by hydroperoxide scavenging with GSH and GPx or utilization of an mPGHS-2 mutant (H388Y) with significantly reduced peroxidase activity,

disproportionately affects 2-AG oxidation compared to AA. This is especially dramatic in experiments with the H388Y mutant (Figure 9). As reported previously, oxidation of AA by this mutant occurs with a pronounced lag phase but eventually an accelerative phase of oxidation occurs and the extent of oxidation equals that observed with AA oxidation by wild-type PGHS-2 (34). In contrast, the lag phase for oxidation of 2-AG by H388Y is so pronounced that an accelerative phase of oxidation is not observed, and the enzyme never attains a robust rate of 2-AG oxidation (Figure 9).

The inability of H388Y to oxidize 2-AG, which is due to a rate of hydroperoxide generation from 2-AG insufficient to rapidly activate other inactive PGHS-2 molecules (Figure 9), is overcome by the addition of hydroperoxide (Figure 10). Thus, despite the similarity of the k_{cat}/K_ms of AA and 2-AG, there are differences in their oxidation that are only unmasked under conditions of peroxidase deficiency. The concentration of peroxide required for activation of cyclooxygenase activity, K_p, is a significantly lower concentration of peroxide than the concentration of peroxide required for half-maximal activity of the peroxidase active site (K_m), and can be measured under conditions of peroxide depletion by GSH and GPx as previously reported (39). We were able to determine the relative K_ps of AA and 2-AG from our experiments with both hPGHS-2 and mPGHS-2 with GPx, finding that 2-AG's K_ps differ by 4-fold and ~40-fold, respectively, relative to those of AA (Table II). This inherent difference in the requirement for sustained activation of oxygenation with 2-AG relative to AA, explains why 2-AG oxygenation demonstrates sensitivity to decreased peroxide tone. The 10-fold difference in the K_ps of 2-AG with mPGHS-2 and hPGHS-2 are somewhat in line with the k_{cat}/K_m of 2-AG with both enzymes. hPGHS-2 oxygenates 2-AG twice as efficiently as mPGHS-2 (174). Furthermore the decay of 2-AG oxygenase activity under suppression by GPx-GSH is 3-fold faster than for mPGHS-2 than for hPGHS-2 (Figure

7). Also, the K_m for 15-HpETE-G is 8-fold higher than for 15-HpETE, which when factored into the calculation of the K_p, leads to a higher than anticipated K_p for 2-AG with mPGHS-2. The increased sensitivity of 2-AG relative to AA to reduced peroxide tone is reminiscent of the behavior of EPA with PGHS-1. EPA is a poor substrate for oxygenation by PGHS-1 but its oxidation is stimulated by exogenous peroxide (212). Examination of the crystal structure of EPA with PGHS-1 demonstrates a shift in the conformation of Tyr-385 from its critical position near the 13-*pro-(S)*-hydrogen, leading to poor initiation of EPA oxygenation relative to AA (213). Interestingly, EPA is also oxygenated poorly by H388Y mCOX-2 (Supplemental Figure 1). Oxygenation can be stimulated by exogenous peroxide but cannot reach wild-type mCOX-2 levels of EPA oxygenation (Supplemental Figure 2). Furthermore, EPA demonstrates a K_p that is 20-fold greater than that for AA (Supplemental Figure 3 and Supplemental Table I), indicating that altered positioning of Tyr-385 near the 13-*pro-S*-hydrogen can impact the K_p of a substrate. Although our laboratory has proposed a model for the binding of 2-AG in the PGHS-2 active site that is similar to the conformation of AA in the PGHS-1-AA crystal structure (214), this structure has not been confirmed by crystallography.

Taken together, these findings indicate that the oxygenation of 2-AG by PGHS-2 is more sensitive to peroxide tone than is the oxygenation of AA. We propose that this phenomenon may be exploited pharmacologically, to prevent the oxygenation of 2-AG by PGHS-2. Recent studies in pain models indicate that increases in endocannabinoid tone lead to analgesia (193), so it would be interesting to see whether pharmacological agents that can selective block 2-AG oxygenation by PGHS-2, can effect analgesia. The development of these agents will be discussed in Chapter IV.

REFERENCES:

1. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and Van Dorp, D. A. (1977) *Biochim Biophys Acta* **487**, 315-331

2. Rouzer, C. A., and Marnett, L. J. (2003) *Chem Rev* **103**, 2239-2304

3. Kulmacz, R. J., and Wang, L. H. (1995) *J Biol Chem* **270**, 24019-24023

4. Rouzer, C. A., and Marnett, L. J. (2008) *J Biol Chem* **283**, 8065-8069

5. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258**, 1946-1949

6. Stella, N., Schweitzer, P., and Piomelli, D. (1997) *Nature* **388**, 773-778

7. Kozak, K. R., Crews, B. C., Morrow, J. D., Wang, L. H., Ma, Y. H., Weinander, R., Jakobsson, P. J., and Marnett, L. J. (2002) *J Biol Chem* **277**, 44877-44885

8. Rouzer, C. A., Tranguch, S., Wang, H., Zhang, H., Dey, S. K., and Marnett, L. J. (2006) *Biochem J* **399**, 91-99

9. Hu, S. S., Bradshaw, H. B., Chen, J. S., Tan, B., and Walker, J. M. (2008) *Br J Pharmacol*

10. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J Biol Chem* **275**, 33744-33749

11. Kulmacz, R. J., and Lands, W. E. (1983) *Prostaglandins* **25**, 531-540

12. Kulmacz, R. J., Miller, J. F., Jr., Pendleton, R. B., and Lands, W. E. (1990) *Methods Enzymol* **186**, 431-438

13. Jiang, Z. Y., Woollard, A. C., and Wolff, S. P. (1991) *Lipids* **26**, 853-856

14. Rowlinson, S. W., Crews, B. C., Lanzo, C. A., and Marnett, L. J. (1999) *J Biol Chem* **274**, 23305-23310

15. Goodwin, D. C., Rowlinson, S. W., and Marnett, L. J. (2000) *Biochemistry* **39**, 5422-5432

16. Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. R., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., and Eling, T. E. (1984) *Mol Pharmacol* **26**, 328-335

17. Funk, M. O., Isacc, R., and Porter, N. A. (1976) *Lipids* **11**, 113-117

18. Kozak, K. R., Gupta, R. A., Moody, J. S., Ji, C., Boeglin, W. E., DuBois, R. N., Brash, A. R., and Marnett, L. J. (2002) *J Biol Chem* **277**, 23278-23286

19. Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996) *Proc Natl Acad Sci U S A* **93**, 15069-15074

20. Hemler, M. E., and Lands, W. E. (1980) *J Biol Chem* **255**, 6253-6261

21. Dietz, R., Nastainczyk, W., and Ruf, H. H. (1988) *Eur J Biochem* **171**, 321-328

22. Tsai, A., Wei, C., Baek, H. K., Kulmacz, R. J., and Van Wart, H. E. (1997) *J Biol Chem* **272**, 8885-8894

23. Strieder, S., Schaible, K., Scherer, H. J., Dietz, R., and Ruf, H. H. (1992) *J Biol Chem* **267**, 13870-13878

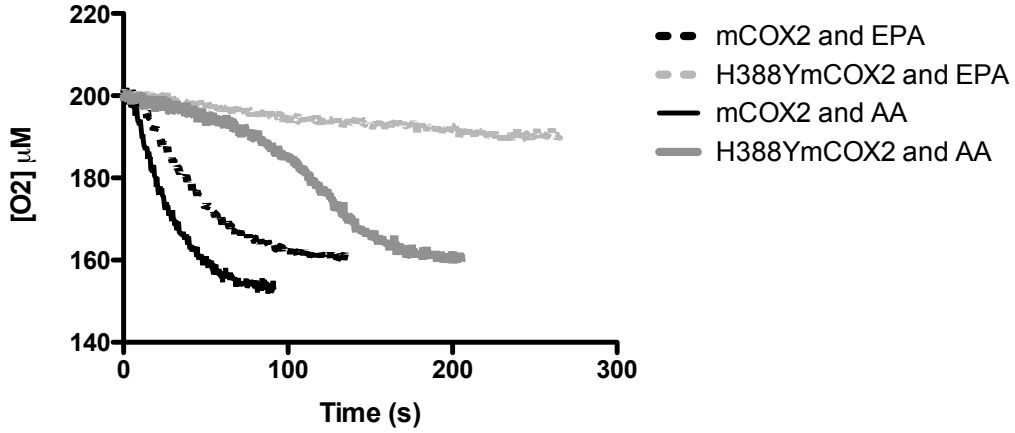
24. Wada, M., DeLong, C. J., Hong, Y. H., Rieke, C. J., Song, I., Sidhu, R. S., Yuan, C., Warnock, M., Schmaier, A. H., Yokoyama, C., Smyth, E. M., Wilson, S. J., FitzGerald, G. A., Garavito, R. M., Sui de, X., Regan, J. W., and Smith, W. L. (2007) *J Biol Chem* **282**, 22254-22266

25. Malkowski, M. G., Thuresson, E. D., Lakkides, K. M., Rieke, C. J., Micielli, R., Smith, W. L., and Garavito, R. M. (2001) *J Biol Chem* **276**, 37547-37555

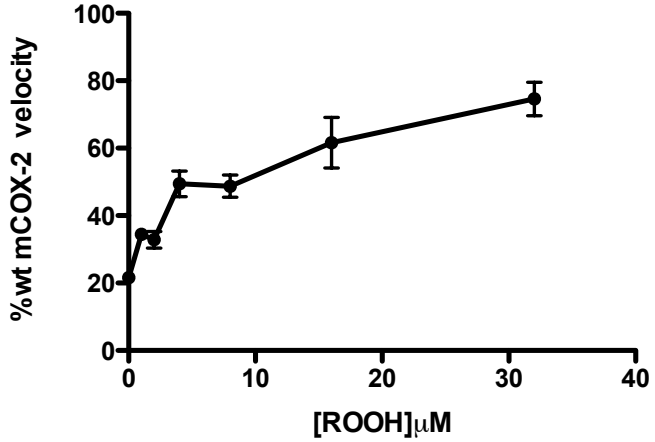
26. Kozak, K. R., Prusakiewicz, J. J., Rowlinson, S. W., Schneider, C., and Marnett, L. J. (2001) *J Biol Chem* **276**, 30072-30077

27. Telleria-Diaz, A., Schmidt, M., Kreusch, S., Neubert, A. K., Schache, F., Vazquez, E., Vanegas, H., Schaible, H. G., and Ebersberger, A. (2010) *Pain* **148**, 26-35

Supplementary Material

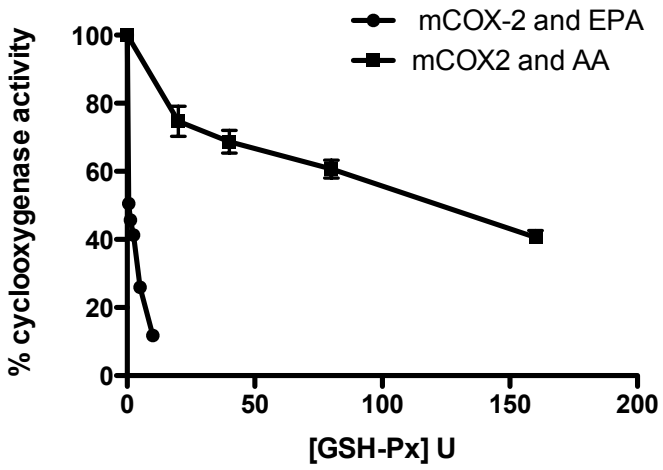


Supplemental Figure 1. Oxygenation of AA and EPA by H388Y mCOX-2. 400 nM of wt mCOX2 was incubated with 50 μM of either AA (—) or EPA(- - -), in standard assay buffer, as described in "Experimental Procedures". For comparison, H388Y mCOX2 was incubated with 50 μM of either AA (—) or EPA (- - -), in standard assay buffer as described in "Experimental Procedures". Traces are representative of triplicate determinations.



Supplemental Figure 2. Stimulation of oxygenation of EPA by H388Y mCOX-2 via 15-HpETE. 400 nM of H388Y mCOX2 was incubated with 50 μM of EPA (—), in standard assay buffer, as described in "Experimental Procedures" with increasing concentrations of 15-HpETE. A plot of the rate of His338Tyr mCOX-2-mediated EPA turnover, relative to that

of wild-type mCOX-2, was generated over a range of concentrations (1 μM- 32 μM) of 15-HpETE. Data are from triplicate determinations.



Supplemental Figure 3. Determination of the ability of the GSH-Px-GSH system to suppress the turnover of EPA by mCOX-2 mCOX-2 (400 nM) was incubated with increasing concentrations of GSH-Px (0-20 U) in standard assay buffer, and AA (■) or EPA (●) was added to 50 μM as described in "Experimental Procedures". The maximal rate of oxygen consumption relative to the control (no GSH-Px) is plotted against the concentration of GSH-Px. Data are from triplicate determinations.

Supplemental Table I. Cyclooxygenase activation parameters for mCOX-2 with AA and EPA

	AA K_p (μM)	**EPA K_p (μM)**	**Relative K_p ($K_{p\ EPA}$/ $K_{p\ AA}$)**
mCOX-2	0.08 ±0.03	1.6 ±0.6	20

CHAPTER III

DETERMINANTION OF THE *EX VIVO* ROLE OF THE GPX SYSTEM IN THE PGHS-2-MEDIATED OXYGENATION OF THE ENDOCANNABINOID 2-AG

Introduction

Prostaglandin H Synthase-2 (PGHS-2) has been shown to oxygenate the endocannabinoid 2- arachidonoyl glycerol (2-AG) *in vivo* and *ex vivo*, leading to the formation of prostaglandin glyceryl esters (PG-Gs) (Figure 1)(174-176,179). The oxygenation of 2-AG leads to a termination of analgesic endocannabinoid signaling, which has been proposed to lead to pain (179,193). The actions of PG-Gs are an area of intense investigation. PGE_2-G has been shown to stimulate Ca^{2+} mobilization in RAW264.7 macrophages via inositol *tris*-phosphate (IP_3) and to induce translocation and activation of protein kinase C (PKC) in a concentration-dependent manner independent of its hydrolysis to PGE_2 (178). Both PGE_2-G and $PGF_{2\alpha}$-G also stimulate Ca^{2+} mobilization in H1819 non-small cell lung carcinoma cells with EC_{50}s of ~ 0.6 pM and ~ 0.5 pM respectively (215). Both $PGF_{2\alpha}$-G and PGE_2-G has been shown to bind at unique, yet to be identified G-protein-coupled receptors (GPCR) (178,179,215).

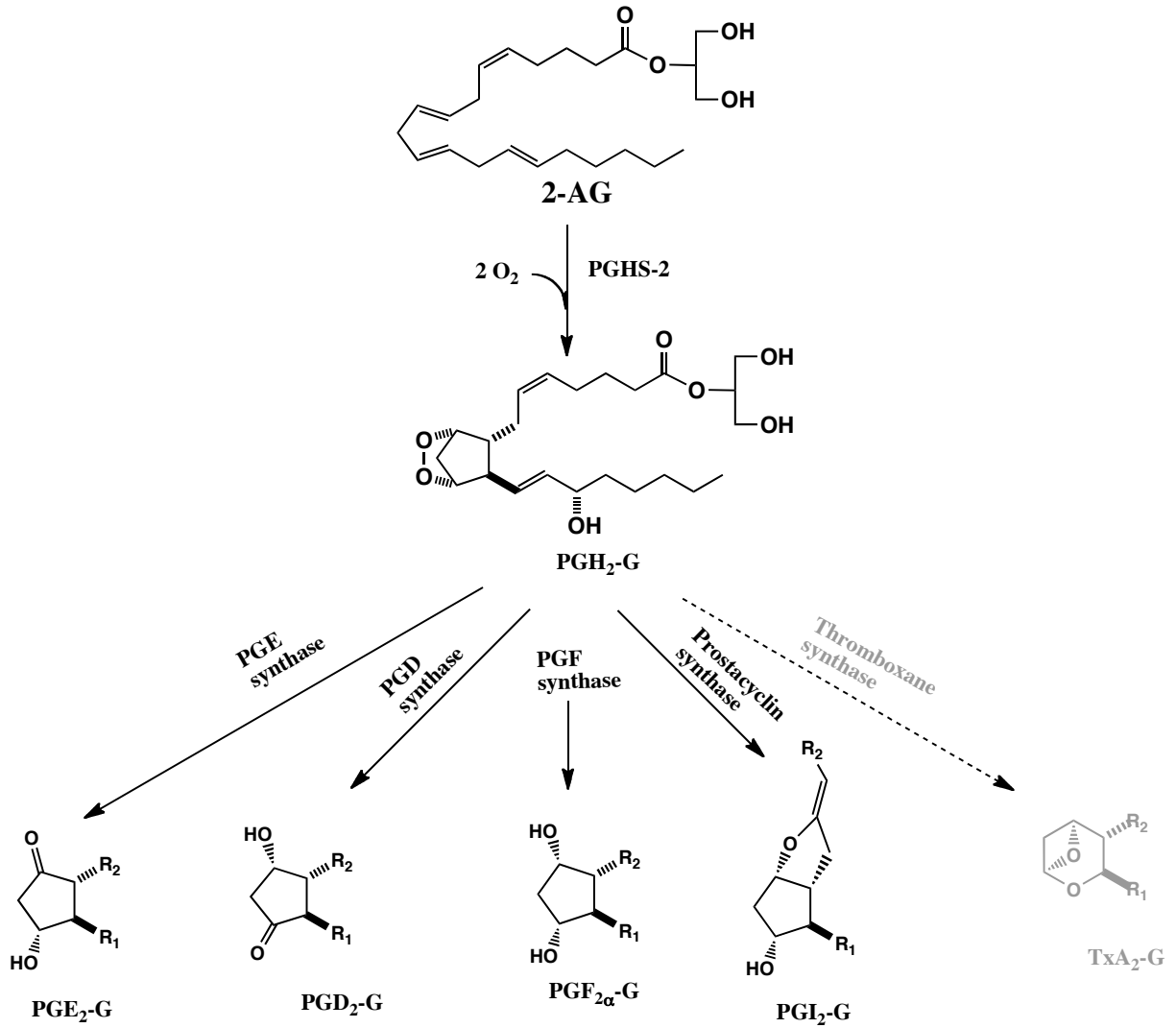


Figure 1. Oxidative Metabolism of 2-AG by PGHS-2- 2-AG is efficiently oxygenated by PGHS-2 to PGH_2-G and PGH_2-G is efficiently isomerized to prostaglandins analogs to those derived from AA, with the exception of thromboxane synthase, for which PGH_2-G is a poor substrate (175).

The possibility that PGHS-2-dependent metabolism of analgesic 2-AG may play a role in the mediation of pain has become an attractive hypothesis. Indeed, PGE_2-G has been shown to cause an increase in the gain in the pain system leading to an exaggerated pain response (hyperalgesia) and pain in response to non-pain evoking stimuli (allodynia) (179). While the steady state kinetic parameters of the oxygenation of 2-AG and AA by PGHS-2 *in vitro* are virtually identical (174), examination of the formation of PG-Gs *ex vivo* demonstrates that they are detected at levels 1000-fold lower than those of AA derived prostaglandins (PGs), upon stimulation of murine resident peritoneal macrophages with an inflammatory stimulus (176). While the levels of 2-AG released upon stimulation by inflammatory stimuli are only 10-fold lower than the levels of AA, there is incongruity between the levels of 2-AG and PG-Gs realized (176). This is further confounded by the fact that 2-AG is rapidly hydrolyzed by monoacylglycerol lipase (MAGL) to AA, and PG-Gs are also subject to hydrolysis to PGs by an active, unique, and yet to be identified serine hydrolase (177).

Despite all of these factors, it is now apparent that the oxygenation of 2-AG is exquisitely sensitive to peroxide-mediated activation of PGHS-2 (Chapter II). Peroxide tone in the cell has been shown to be critical for PGHS-2 activation and PG formation (140,141). Peroxide tone is modulated by the glutathione peroxidase-glutathione system (GPx-GSH) which reduced fatty acid hydroperoxides to alcohols (216). There are four known mammalian GPxs: GPx1-4. The brunt of oxidant homeostasis is borne by GPx1, however, GPx4 is the only isoform that can reduce phospholipid bound hydroperoxides (216). In fact, knock-out of GPx4 in mouse embryonic fibroblasts has been shown to cause embryonic lethality by overt increases in lipid peroxidation (110). Interestingly,

over-expression of GPx4 has been shown to mute PGD_2 synthesis in activated mast cells (141), while siRNA knock-down of GPx4 has been shown to double the formation of PGs in human epidermoid carcinoma (140). This has led us to hypothesize that increasing peroxide tone by impairment of the endogenous peroxide detoxification mechanism could lead to increased 2-AG oxygenation by PGHS-2.

I report that stable silencing of GPx4 using shRNA in murine NIH/3T3 fibroblasts leads to an increase in lipid peroxidation and oxidant stress relative to GPx1-silenced cells and scrambled shRNA controls. Furthermore, silencing of GPx4 in these cells leads to a doubling of PG-G production following stimulation. These observations extend my *in vitro* findings discussed in Chapter II, and demonstrate that PG-G formation in the cell is sensitive to peroxide tone. Also, the detection of PG-Gs in a widely used mammalian cell line gives credence to the relevance of PGHS-2-mediated, 2-AG oxygenation.

Experimental Procedures

RNA interference - Low-passage murine NIH/3T3 fibroblasts were cultured in DMEM (10% heat-inactivated FBS) and plated in 100 mm dishes at 75% confluence. Cells were cultured at 37°C and 5% CO_2 (v/v). shRNA plasmids (scrambled-negative control, GFP positive control, Gpx1, and Gpx4) were obtained from Santa Cruz (Santa Cruz, CA). Transfection was carried out following the manufacturer's instructions, with selection of successfully transfected cells achieved by including 10 μg/mL puromycin (Sigma-Aldrich) in the growth medium. Selection was carried out for two weeks.

Immunoblotting for GPx protein expression - For the immunoblotting of GPx, monolayers of each shRNA-treated NIH/3T3 cell line from 35 mm dishes were lysed in

200 μl M-PER lysis buffer (Thermo) containing a cocktail of mammalian protease inhibitors (Sigma). Cell lysates were mixed by vortexing and placed on ice for 30 min. Cellular debris was then removed by centrifugation for 10 min at 16,000 g. Samples were stored at -80 °C until analyses could be completed. Equal quantities of protein (~ 20 μg) were resolved by gradient (2-12%) SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were blocked (20 mM Tris, pH 7.6, 140 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) prior to incubation with antibodies. The primary antibodies, αGPx1 and αGPx4 (R&D systems), were used at 1:1000 and the secondary antibodies at 1:5000 (R&D systems) dilution. Luminol-based detection was performed using SuperSignal West Pico reagents (Thermo Scientific).

Stimulation of PG and PG-G synthesis in shRNA-treated cell-lines - Murine NIH/3T3 fibroblasts were plated at 1,000,000 cells per well, in 6-well dishes in media with 10 μg/mL puromycin and allowed to attach overnight. PG and PG-G production was stimulated by incubation with 12-O-tetradecanoylphorbol-13-acetate (TPA)/ionomycin (0.08 μM/2 μM) for 4 hours.

Assay for production of PGs, PG-Gs by cultured cells - Media from cell cultures were subjected to liquid-liquid extraction as developed by Kingsley et al. (217) with some modifications as follows. Briefly, internal standards [(100 pmol of the following: PGD_2-d4, PGE_2-d4, $PGF_{2\alpha}$-d4, and 6-keto-$PGF_{1\alpha}$-d4, and 10 pmol of the following: PGD_2-G-d5, PGE_2-G-d5, $PGF_{2\alpha}$-G -d5, 6-keto-$PGF_{1\alpha}$-G-d5)] were added to medium (2 mL). The medium was acidified by the addition of glacial acetic acid to 1% (volume), followed by 2 mL of ethanol and 2 mL of hexanes:ethyl acetate (20:80 v:v). Samples

were vortexed and centrifuged at 3000 RPM for 20 minutes. The organic layer was retrieved and evaporated to dryness under a stream of nitrogen. Samples were resuspended in 300 μL of methanol:water (50:50 v:v) and analyzed as previously described (217).

Assay for lipid peroxidation and oxidant stress- Murine NIH/3T3 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and plated in 100 mm dishes at 75% confluence. Cells were incubated with (2 μM) boron-dipyrromethene (BODIPY) 581/591 C11, (Invitrogen, USA) overnight for flow cytometry analysis. BODIPY when oxidized exhibits a shift in fluorescence from red to green (110). Cells were washed once with cold-Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), trypsinized, and resuspended in 10% FBS DMEM. Cells were loaded onto a 5-laser BD LSRII flow cytometer, equipped with a 535 nm laser. 10,000 cells were analyzed for each sample, with scrambled shRNA-treated NIH/3T3 as the baseline for peroxidation. A histrogram of each cell line was generated, with a threshold set as P1 for cells exhibiting shifts from red to green fluorescence. Analysis for oxidative-stress marker, 8-isoprostane (8-epi $PGF_{2\alpha}$) was carried out by the eicosanoid core (Ginger Milne) as previously published (218).

Results

RNA interference- shRNA-mediated GPx depletion was determined by immunblot for each respective GPx. Relative to the scrambled control, a knockdown of ~50% of GPx1 and ~75% of GPx4 was achieved (Figure 2).

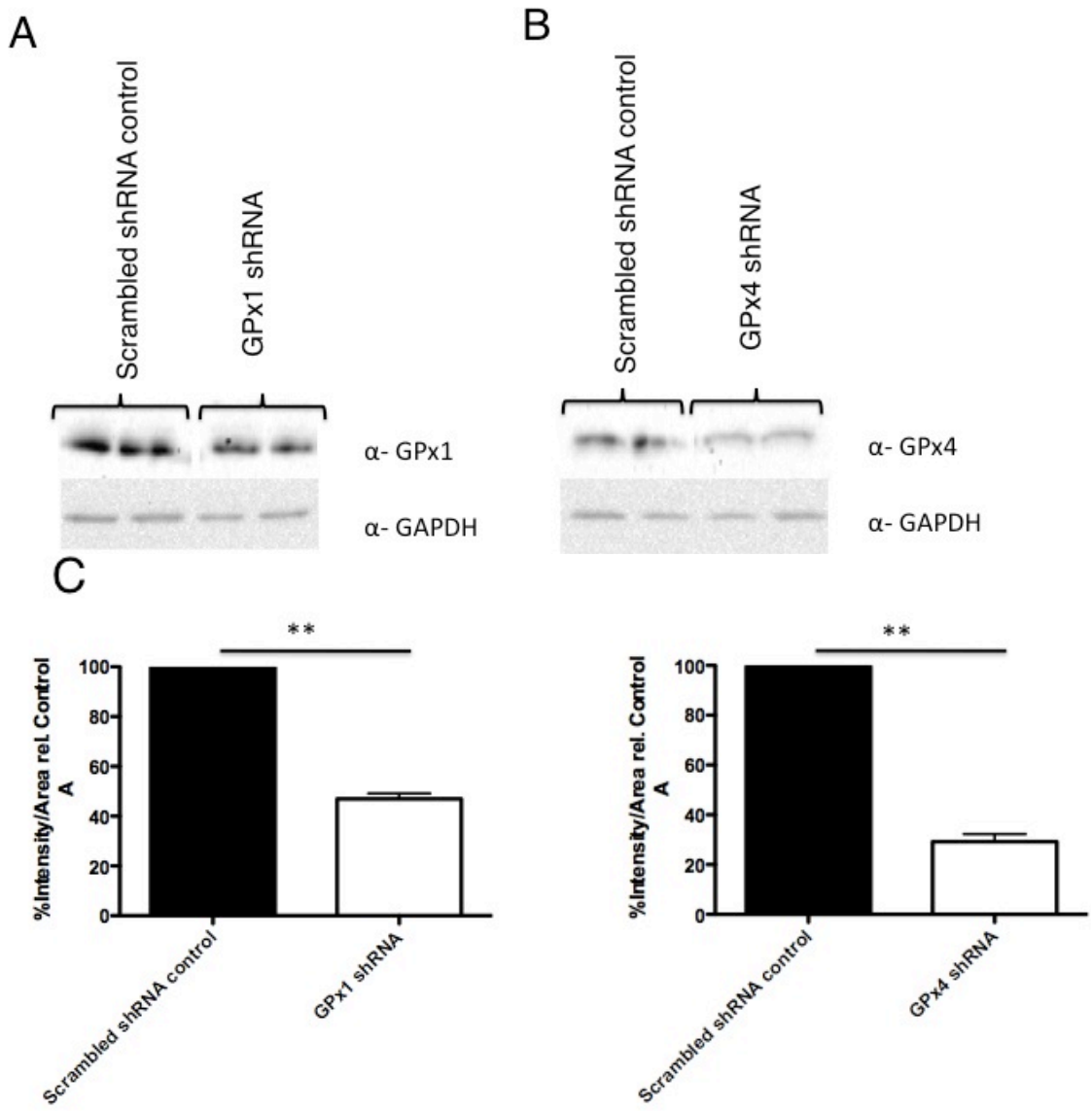
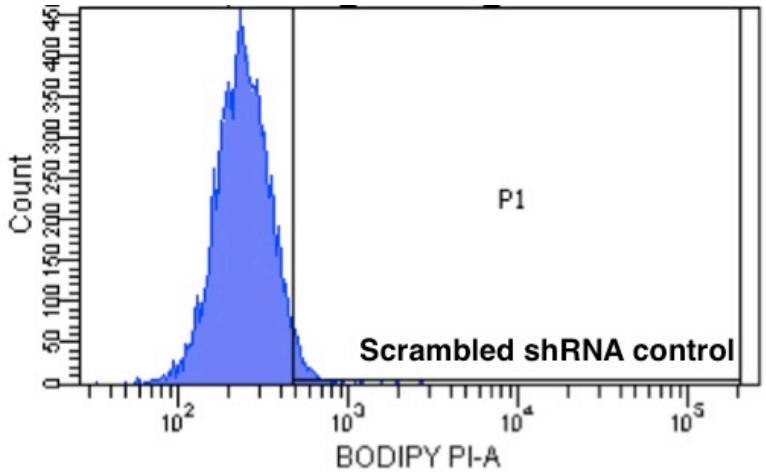
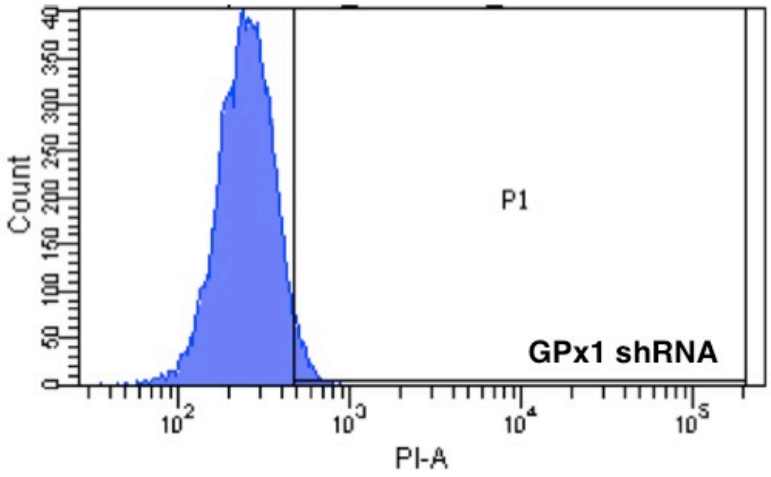
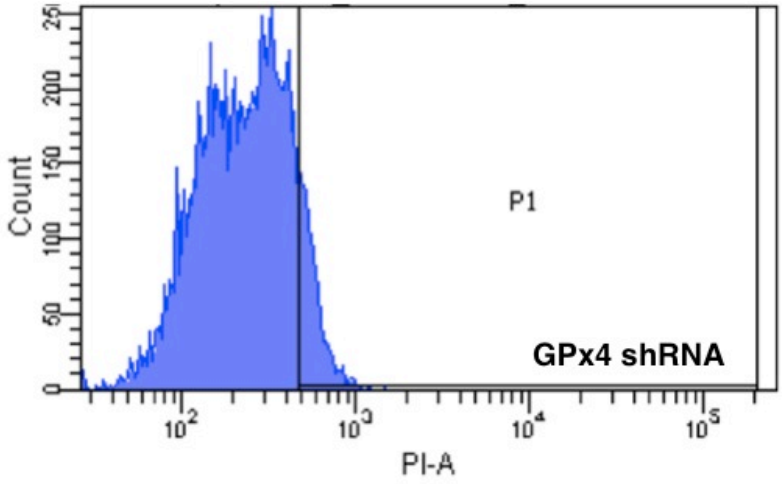


Figure 2. Determination of the levels GPx1 and GPx4 following shRNA-mediated silencing (A) Immunoblot for GPx1 and GPx4 in NIH/3T3 fibroblasts transfected with shRNA plasmids to knock-down GPx1 and GPx4 as described in "Experimental Procedures". (B) GPx1 is depleted to ~50% below control shRNA and GPx4 to ~75% below control shRNA. Asterisks indicate statistically significant differences in the levels of GPx1 and GPx4 in GPx1 and GPx4 shRNA transfected NIH/3T3 fibroblasts, relative to a scrambled shRNA control (Student's t-test, un-paired, two-tailed- ** $p<0.001$).

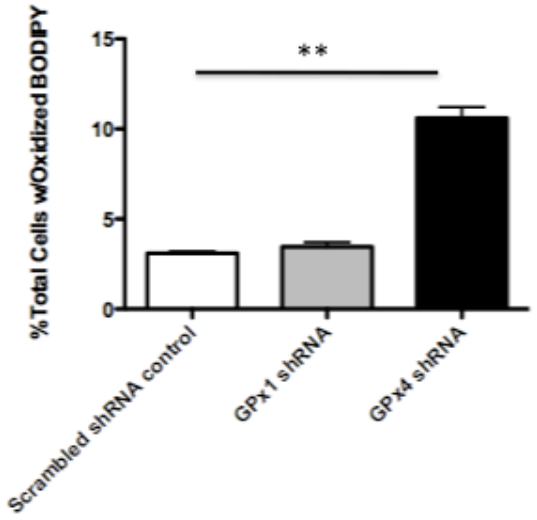
A


Count
P1
Scrambled shRNA control
BODIPY PI-A


Count
P1
GPx1 shRNA
PI-A


Count
P1
GPx4 shRNA
PI-A

B



C

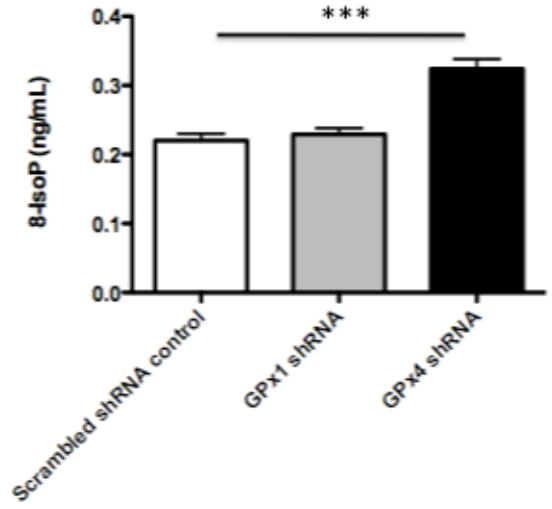


Figure 3. Determination of the impact of shRNA-mediated silencing of the GPx system on peroxide tone in murine NIH/3T3 fibroblasts. Lipid peroxidation was assayed using the membrane-specific dye BODIPY and flow cytometry as described in "Experimental Procedures". (**A**) Histograms of 10,000 cell counts for each shRNA-treated cell line. A larger proportion of anti-GPx4 shRNA-treated 3T3 fibroblasts

demonstrates a green fluorescence shift from red as BODIPY is oxidized. (**B**) Quantitation of the proportions of cells shifting fluorescence past the pre-set threshold (P1) revealed that anti-GPx4 shRNA-treated 3T3 fibroblasts demonstrate a greater than 3-fold increase in lipid peroxidation over control shRNA- and anti-GPx1 shRNA-treated 3T3 fibroblasts.

Determination of the effect of GPx1 and GPx4 knockdown on peroxide tone and oxidant stress - Following the determination that adequate depletion of GPx1 and GPx4 was achieved, we evaluated the levels of lipid peroxidation in each cell line as described in "Experimental Procedures". Flow cytometric determination demonstrates that anti-GPx4 shRNA-treated fibroblasts demonstrate 3-fold more lipid peroxidation relative to scrambled control shRNA-treated fibroblasts. Anti-GPx1 shRNA-treated fibroblasts did not differ from control (Figure 3A and B). Interestingly, examination of the histograms from the BODIPY assay indicate that the anti-GPx4 shRNA-treated fibroblasts display a bi-modal distribution in its population. Part of this population extends past the threshold P1, indicating that a significant proportion of these cells are shifting their fluorescence away from baseline indicative of increased BODIPY oxidation. Furthermore measurement of levels of oxidant-stress biomarker, 8-isoprostane, revealed that anti-GPx4 shRNA-treated fibroblasts demonstrate statistically significant higher levels of this marker, relative to scrambled control shRNA-treated fibroblasts (Figure 3C).

Determination of the effect of GPx1 and GPx4 knockdown on PG and PG-G Synthesis - Following the demonstration that depletion of GPx4 could lead to increased lipid peroxidation, we determined the effect of increased peroxide tone on PG and PG-G synthesis. Following agonist treatment, control, anti-GPx1 and anti-GPx4 shRNA-treated cells all demonstrated similar levels of PG synthesis (Figure 4). Interestingly, the two

major species of PG-G generated by fibroblasts, PGE_2-G and $PGF_{2\alpha}$-G, were elevated 2- and 4-fold, respectively, in anti-GPx4 shRNA-treated fibroblasts, relative to control. Anti-GPx1 shRNA-treated cells did not differ from control.

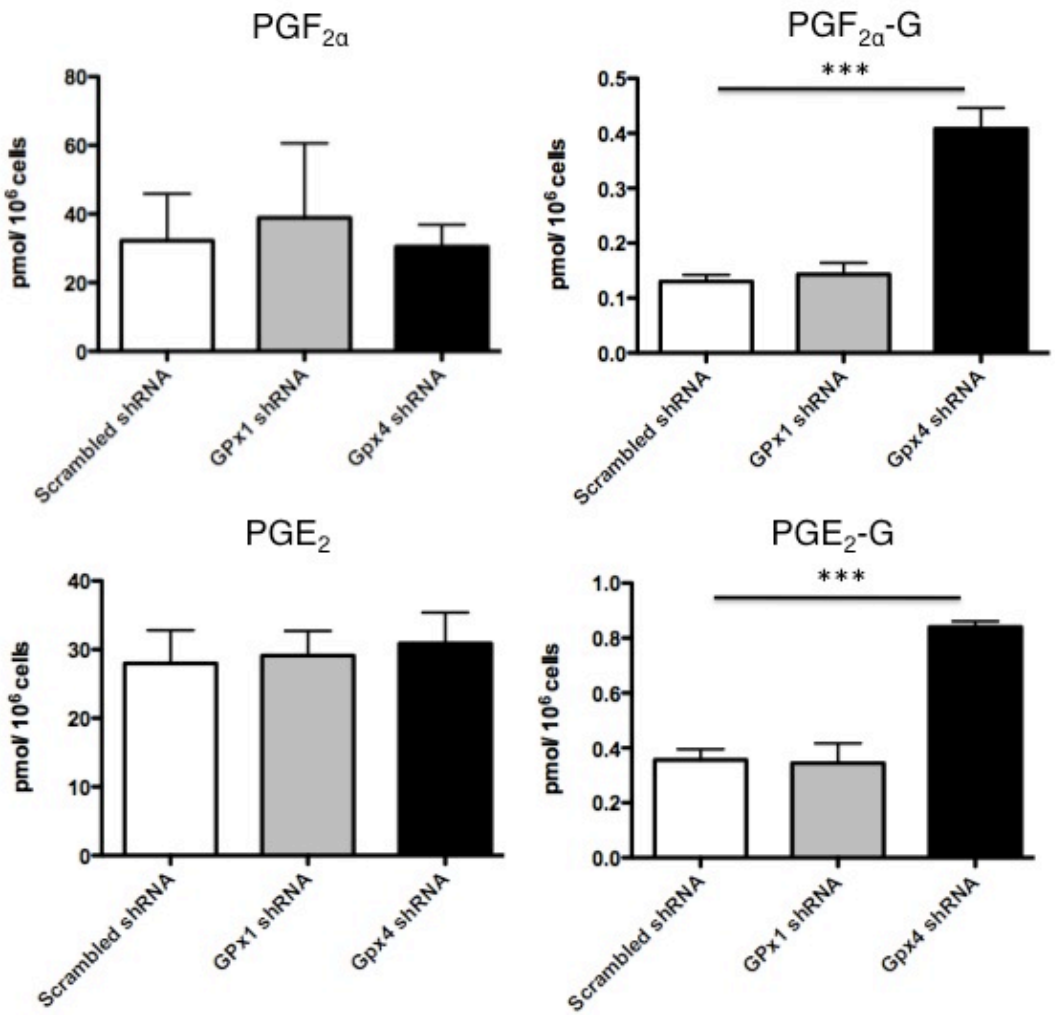


Figure 4. Determination of the impact of shRNA-mediated silencing of the GPx system on PG and PG-G production by murine NIH/3T3 fibroblasts. Anti-GPx4 shRNA-treated 3T3 fibroblasts demonstrate 2-4 fold increases in PG-G production over control shRNA- and anti-GPx1 shRNA-treated 3T3 fibroblasts. PG production is not significantly different across the three cell-lines.

Discussion

This study extends previous findings from resident peritoneal macrophages and RAW264.7 macrophages, where treatment with inflammatory agonists and/or Ca^{2+} ionophores leads to the formation of PG-Gs from 2-AG. Here we demonstrate that a commonly used fibroblast cell line, murine NIH/3T3 fibroblasts, also generate appreciable amounts of PG-Gs following an inflammatory stimulus following treatment with a Ca^{2+} ionophore. Furthermore, this study is an extension of the work discussed in Chapter II, where we demonstrated that PGHS-2-mediated 2-AG oxygenation was sensitive to peroxide tone. Here we establish a role for the phospholipid hydroperoxide glutathione peroxidase (GPx4) in the modulation of peroxide tone in murine NIH/3T3 fibroblasts. Specifically, shRNA-mediated depletion (~75%) of GPx4 leads to a 3-fold increase in lipid peroxidation (Figure 2) and is consistent with literature reports of this enzyme's role in redox homeostasis at the lipid bilayer (131-134). Interestingly, while shRNA-mediated depletion of GPx1 (~50%) was statistically different from control shRNA fibroblasts (Figure 2), there was no effect on lipid peroxidation noted for this level of knock-down (Figure 3). It is possible that the levels of depletion achieved under these conditions were not sufficient to observe any effects. GPx-1 knock-out mice do not have an overt phenotype, developing normally and only succumbing to exceedingly high levels of oxidant stress (113,114). Therefore, with the depletion demonstrated here (~50%), it is not surprising that there is no overt phenotype. However, these data do not exclude the possibility that further depletion of GPx1 may lead to similar effects as observed with Gpx4 depletion.

As discussed before, peroxide tone is more critical for the initiation of 2-AG oxygenation *in vitro* (Chapter II) than for the initiation of AA oxygenation. Here, we demonstrate that increasing peroxide tone in NIH/3T3 fibroblasts leads to 2- and 4-fold increases in the oxygenation of 2-AG to PGE_2-G and $PGF_{2\alpha}$-G, respectively (Figure 4). Interestingly, PGE_2 and $PGF_{2\alpha}$ production did not change significantly under these conditions, and it appears that PG-Gs are produced at levels 100-fold lower than PGs, making changes in PG-G production easier to discern (Figure 4). The fact that AA derived PGs do not increase under these conditions may indicate that AA and 2-AG are oxygenated in separate cellular compartments, since increased peroxide tone would stimulate AA oxygenation as well as 2-AG oxygenation by PGHS.

These results are the first indication that increased peroxide tone leads to increased 2-AG oxygenation by PGHS-2 and are congruent with previous reports on the role of peroxide tone in AA oxygenation by PGHS (140,141). Inflammatory states are associated with increases in redox stress and the relevance of 2-AG oxygenation by PGHS could play a role under these conditions, leading to increased conversion of the analgesic 2-AG, to a pro-inflammatory and hyperalgesic PGE_2-G. These findings provide further credence to the PGHS-2/2-AG axis in the mediation of pain and demonstrate that under increased cellular oxidant stress, PGHS-2-mediated oxygenation of 2-AG may be augmented.

References.

1. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J Biol Chem* **275**, 33744-33749

2. Kozak, K. R., Crews, B. C., Morrow, J. D., Wang, L. H., Ma, Y. H., Weinander, R., Jakobsson, P. J., and Marnett, L. J. (2002) *J Biol Chem* **277**, 44877-44885

3. Rouzer, C. A., and Marnett, L. J. (2005) *J Biol Chem* **280**, 26690-26700

4. Hu, S. S., Bradshaw, H. B., Chen, J. S., Tan, B., and Walker, J. M. (2008) *Br J Pharmacol*

5. Telleria-Diaz, A., Schmidt, M., Kreusch, S., Neubert, A. K., Schache, F., Vazquez, E., Vanegas, H., Schaible, H. G., and Ebersberger, A. (2010) *Pain* **148**, 26-35

6. Nirodi, C. S., Crews, B. C., Kozak, K. R., Morrow, J. D., and Marnett, L. J. (2004) *Proc Natl Acad Sci U S A* **101**, 1840-1845

7. Richie-Jannetta, R., Nirodi, C. S., Crews, B. C., Woodward, D. F., Wang, J. W., Duff, P. T., and Marnett, L. J. (2010) *Prostaglandins Other Lipid Mediat* **92**, 19-24

8. Kozak, K. R., Crews, B. C., Ray, J. L., Tai, H. H., Morrow, J. D., and Marnett, L. J. (2001) *J Biol Chem* **276**, 36993-36998

9. Chen, C. J., Huang, H. S., and Chang, W. C. (2003) *FASEB J* **17**, 1694-1696

10. Sakamoto, H., Imai, H., and Nakagawa, Y. (2000) *J Biol Chem* **275**, 40028-40035

11. Brigelius-Flohe, R., and Kipp, A. (2009) *Biochim Biophys Acta* **1790**, 1555-1568

12. Seiler, A., Schneider, M., Forster, H., Roth, S., Wirth, E. K., Culmsee, C., Plesnila, N., Kremmer, E., Radmark, O., Wurst, W., Bornkamm, G. W., Schweizer, U., and Conrad, M. (2008) *Cell Metab* **8**, 237-248

13. Kingsley, P. J., Rouzer, C. A., Saleh, S., and Marnett, L. J. (2005) *Anal Biochem* **343**, 203-211

14. Milne, G. L., Sanchez, S. C., Musiek, E. S., and Morrow, J. D. (2007) *Nat Protoc* **2**, 221-226

15. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) *Biochim Biophys Acta* **710**, 197-211

16. Roveri, A., Maiorino, M., Nisii, C., and Ursini, F. (1994) *Biochim Biophys Acta* **1208**, 211-221

17. Thomas, J. P., Geiger, P. G., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *Biochim Biophys Acta* **1045**, 252-260

18. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *J Biol Chem* **265**, 454-461

19. Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M., and Funk, C. D. (1997) *J Biol Chem* **272**, 16644-16651

20. de Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998) *J Biol Chem* **273**, 22528-22536

CHAPTER IV

TARGETTING THE POX ACTIVE SITE OF PGHS-2 FOR THE SELECTIVE INHIBITION OF PGHS-2 MEDIATED OXYGENATION OF THE ENDOCANNABINOID 2-AG

Introduction

Prostaglandin H Synthase-2 (PGHS-2) has been shown to oxygenate the endocannabinoid 2- arachidonoyl glycerol (2-AG) *in vivo* and *ex vivo*, leading to the formation of prostaglandin glyceryl esters (PG-Gs) (174-176,179). The relevance of these findings has been questioned since PG-Gs are detected at low levels relative to AA-derived prostaglandins (PGs) (176,179). This is partly due to the hydrolysis of 2-AG to AA by an active and specific serine hydrolase, monoacyl glycerol lipase (MAGL) (144), reducing the amounts of 2-AG available for oxygenation by PGHS-2 (176). Furthermore, PG-Gs are rapidly hydrolyzed *in vivo*, by an unidentified serine hydrolase (177). Effectively, these data indicate that a proportion of PGs detected *in vivo* may be 2-AG-derived, either from hydrolysis of 2-AG or PG-Gs. Indeed, co-incubation of 2-AG with murine peritoneal macrophages effectively leads to both increased PG and PG-G generation (176). It has therefore been difficult to demonstrate specifically the contribution of 2-AG metabolism by PGHS-2, and its relevance in diminution of analgesic endocannabinoid tone.

Non-steroidal anti-inflammatory drugs (NSAIDs), are inhibitors of fatty acyl substrate (FAH) oxygenation by PGHS and do so by competing with the substrate for

binding in the oxygenase active site of PGHS. Functional PGHS is made up of two homodimers, and binding of substrate or FAH in one of these monomers is thought to cause a conformational change in the other, rendering it incapable of oxygenation of FAH. In fact titrations of three different NSAIDS, indomethacin, flurbiprofen and meclofenamic acid, against functional PGHS homodimers demonstrated that it took one molecule of NSAID per homodimer to effect inhibition of AA oxygenation (219). This phenomenon has been dubbed half-sites reactivity. Recent work has demonstrated that weak inhibitors of AA oxygenation by PGHS-2 are about 100-fold more potent as inhibitors of 2-AG oxygenation (220). This phenomenon has been dubbed substrate-selective inhibition and has been proposed to be related to the half-sites reactivity of PGHS. Prusakiewicz et al. proposed that when weak reversible NSAIDs bind one monomer, they can effect a conformational change that reduced the affinity of the other monomer for 2-AG, which leads to a dimunition of 2-AG oxygenation (220). The inhibition of AA oxygenation by PGHS-2, however, requires the binding of these weak NSAIDs in both monomers of the PGHS homodimer (220). This finding could explain the analgesic effects of weak NSAIDs, which are not fully explained by the inhibition of AA oxygenation (221).

To further this concept of selectively inhibiting the oxygenation of 2-AG over that of AA, we targeted the peroxidase active site of PGHS-2. The findings reported in the preceding two chapters (Chapter II and Chapter III) indicate that the oxygenation of 2-AG by PGHS-2 is sensitive to peroxide tone. Increasing concentrations of GPx lead to a selective suppression of 2-AG oxygenation by PGHS-2 relative to AA. Furthermore, mutation of the heme-binding residue in the POX active site (His388Tyr), leads to ~ 300-

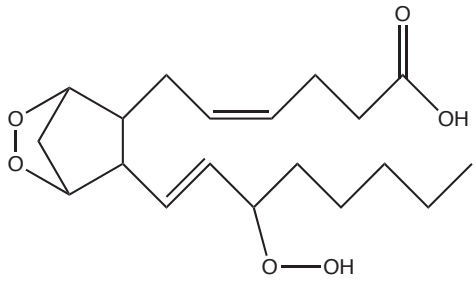
fold reduction in peroxidase activity (34). H388Y PGHS-2 retains its ability to oxygenate AA, but does not appreciably oxygenate 2-AG (Chapter II). This defect was restored to wild-type levels with exogenous peroxide in a concentration-dependent manner (Chapter II). The sum of all of these data indicate that competitive inhibition of the peroxidase activity of PGHS could lead to reduced peroxide-mediated activation of oxygenase activity, selectively inhibiting the oxygenation of 2-AG.

While a variety of structurally and chemically distinct agents have been successfully generated to target the COX active site of PGHS, none has been reported for the POX active site to date. The difficulty in designing specific ligands to target the POX active site is a testament to the relative promiscuity of this site for substrates, relative to the specific determinants for binding in the COX active site. While the POX active site is largely solvent accessible and relatively open to its environment, lipophilic hydroperoxides such as 15-hydroperoxyeicosatetraenoic acid (15-HpETE) have been shown to be reduced more efficiently than smaller peroxides such as hydrogen peroxide (H_2O_2) (34,209). Peroxynitrite ($ONOO^-$) is perhaps the single exception to this rule as it is reduced at a rate that is comparable to those of PGG_2 and 15-HpETE (27).

Chubb et al. have modeled PGG_2 (Figure 1A) in the POX active site of ovine PGHS-1 and demonstrated that the majority of the interactions of PGG_2 with the POX active site are hydrophobic (222). Their best conformer aligns the 15-hydroperoxide on PGG_2 with the Fe^{3+} in the heme of PGHS-1, forming the sixth heme ligand. This alignment, coupled with the aforementioned hydrophobic interactions, forms a productive complex revealing how the POX active site binds and reduces PGG_2. To design an agent that could putatively fulfill the majority of these interactions, we designed a PGG_2 mimic,

2-methyl-4,5-dihexylimidazole, putatively fulfilling the hydrophobic interactions and possibly forming the sixth heme ligand (Figure 1B). Here we report that this agent is a selective inhibitor of 2-AG oxygenation with little to no impact on AA oxygenation over the concentrations tested. Furthermore, 2-methyl-4,5-dihexylimidazole is a potent selective inhibitor of 2-AG oxygenation in agonist-treated RAW264.7 macrophages, effectively preventing the conversion of 2-AG to PG-Gs. This represents the first ligand to inhibit the POX active site of PGHS and presents a substrate-selective inhibitor of 2-AG oxygenation by PGHS-2.

A



B

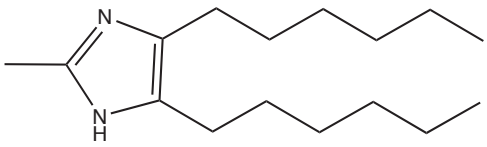
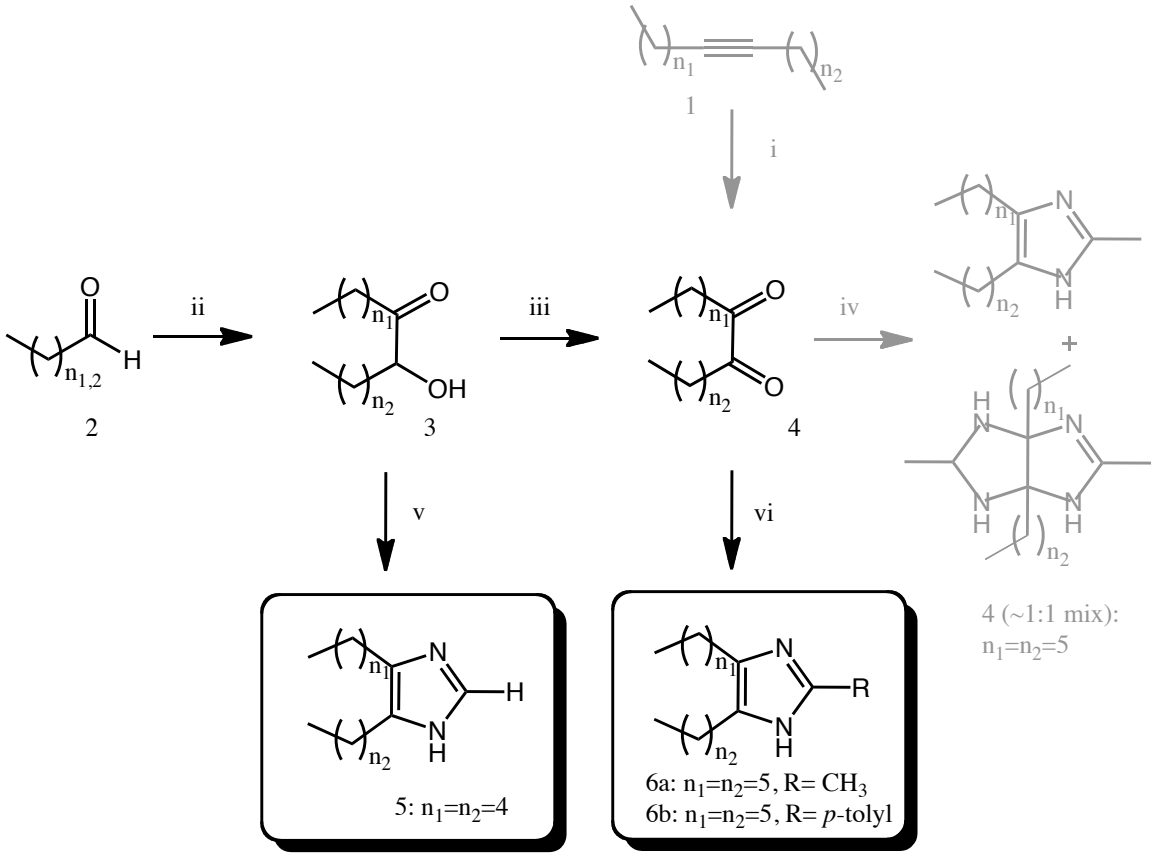


Figure 1. Structure of PGG_2 (A) and 2-methyl-4,5-dihexyl imidazole (B). 2-methyl-4,5-dihexyl imidazole was designed to mimic the reported binding parameters of PGG_2 in the POX active site of PGHS(222).

Experimental Procedures

Chemistry- 2-Methyl 4,5- dihexylimidazole and its analogs, were generated by Andy Liedtke as follows:

Reaction Scheme:



Reagents and Conditions: (i)(223) $KMnO_4$, $NaHCO_3$, $MgSO_4$, acetone, H_2O, rt, 4h (ii)(224) 1,3-dimethylbenzimidazolium iodide, DBU, dioxane, reflux, 3 h, (iii)(225) NBS, CCl_4, pyridine, 80-85 ˚C, 1.5 h, (iv) NH_4OAc, acetaldehyde, $InCl_3$ x 4 H_2O, MeOH, rt, 16h (v)(226,227) formamide, AcOH, μwave radiation, 180 ˚C, 5 min, (vi)(227) aliphatic/aromatic aldehyde, NH_4-acetate, AcOH, μwave radiation, 180 ˚C, 5 min.

Enzymes - hPGHS-2 and mPGHS-2 were expressed and purified as described (206). oPGHS-1 was purified from ram seminal vesicles (Oxford Biomedical Research, Oxford, MI), as previously described (207). The specific activities of hPGHS-2 and

mPGHS-2 were both 62.5 μM AA per μM enzyme; oPGHS-1 was 325 μM AA per μM enzyme. H388Y mPGHS-2's specific activity was 31.3 μM AA per μM enzyme

PPHP reduction assay for peroxidase activity - Determination of the inhibition of the peroxidase active site of PGHS were carried out by monitoring the oxidation of ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) with 5-phenyl-4-pentenyl hydroperoxide (PPHP) as a peroxidase substrate, as previously published (228).

LC/MS assay for in vitro COX activity and inhibition - Hematin-reconstituted PGHS-2 was incubated with inhibitor (0.25 - 50 μM) for 15 min at 37°C prior to the addition of 50 mM substrate (AA or 2-AG). The reaction was quenched after 30 seconds with ethyl acetate containing 0.5% acetic acid and deuterated internal standards. The organic layer was extracted and evaporated under nitrogen to near-dryness. The residue was resuspended in 50:50 methanol:water, and product formation was monitored using LC-MS/MS. Products were separated on a Luna C18(2) column (5.0 cm x 0.2 cm, 3 μm) using an isocratic method consisting of 65% 5 mM ammonium acetate, pH 3.5 and 35% acetonitrile (v/v) at a flow rate of 0.375 mL/min. The triple-quadrupole mass spectrometer was operated in positive ion mode using electrospray ionization and selective reaction monitoring (SRM) to detect PGs and PG-Gs. The following transitions were monitored: for PGE_2/D_2 m/z ($M+NH_4^+$), 370→317, for PGE_2/D_2-d4 m/z ($M+NH_4^+$) 374→321, for PGE_2/D_2-G m/z ($M+NH_4^+$) 444→391 and for PGE_2/D_2-G-d5 m/z ($M+NH_4^+$) 449→396. Products are quantitated as a ratio of the area of the peak to its corresponding internal standard and normalized to a DMSO control.

Thin Layer Chromatography assay for in vitro COX activity and inhibition- Hematin-reconstituted enzyme and inhibitor were pre-incubated for 17 min at room

temperature followed by a 3 min incubation at 37 °C prior to addition of 50 μM [1-^{14}C]-AA for 30 sec at 37 °C. The reactions were then terminated by extraction with diethyl ether/methanol/citrate (30:4:1) and analyzed for substrate consumption by thin-layer chromatography as previously described (229). All inhibitor concentrations for 50% enzyme activity (IC_{50}) were determined by nonlinear regression analysis using Graphpad Prism software and are the average of multiple determinations of duplicate analyses. Inhibitors were prepared as stock solutions in dimethyl sulfoxide (DMSO), and diluted into reaction buffer so that the final DMSO concentration was 2.5%. Reactions were run with hematin-reconstituted proteins at final enzyme concentrations adjusted to give approximately 30- 35% substrate consumption (mCOX-2 = 154 nM). AA was prepared as a stock solution in 0.1 N NaOH.

LC/MS assay for ex vivo COX activity and inhibition -RAW 264.7 macrophages were plated at 3 million cells/dish in 3 ml Dubelcco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (HI-FBS). After 4 hours, the medium was replaced, and 20 ng/mL mouse GM-CSF was added. Following overnight incubation, the medium was replaced with DMEM with no FBS, and the cells were treated with 100 ng/mL LPS, 20 units/ml IFN γ, and 10 μM 15(S)-HETE. Treatment with DMSO vehicle or drug was performed at either 0 or 2 hours, and the medium was collected with 2 equivalents of ethyl acetate plus 0.1% glacial acetic acid spiked with deuterated internal standards. The solution was vortexed for 1 minute and then frozen overnight. The organic layer was extracted, evaporated to dryness under a stream of nitrogen gas, and reconstituted in 150 μl water:methanol (1:1 v:v). The samples were then analyzed by reverse-phase liquid chromatography using an Ascentis C18 (5 cm x 2.1 mm with 3 μm

particle size) column and mobile phases of 5 mM ammonium acetate in water, pH of 3.4 and acetonitrile :water (94:6 v:v). Mass spectral analysis was conducted on a Thermofinnigan triple quadrupole mass spectrometer in positive ion mode using SRM. The product amounts were calculated by integrating the peak areas of the samples and comparing them to the area of the spiked deuterated standard peaks. The product identities were confirmed by comparing the retention times and collision-induced dissociation spectra to standards. The following transitions were monitored: for PGE_2/D_2 m/z ($M+NH_4^+$), 370→317, for PGE_2/D_2-d4 m/z ($M+NH_4^+$) 374→321, for PGE_2/D_2-G m/z ($M+NH_4^+$) 444→391 and for PGE_2/D_2-G-d5 m/z ($M+NH_4^+$) 449→396. The data were analyzed using Xcalibur and then graphed and analyzed in GraphPad Prism.

Results

Assay for 2-methyl 4,5 dihexylimidazole as a Peroxidase Inhibitor - Determination of the inhibition of the peroxidase active site of PGHS were carried out by monitoring the oxidation of ABTS as the reducing co-substrate with PPHP as a peroxidase substrate, as previously published (228). 2-Methyl 4,5 dihexylimidazole modest inhibitor of the POX activity of PGHS-2 (Figure 2), never achieving greater than 50% inhibition of the reduction of PPHP. 2-Methyl 4,5 dihexyl imidazole did not inhibit the peroxidase activity of PGHS-1 (data not shown).

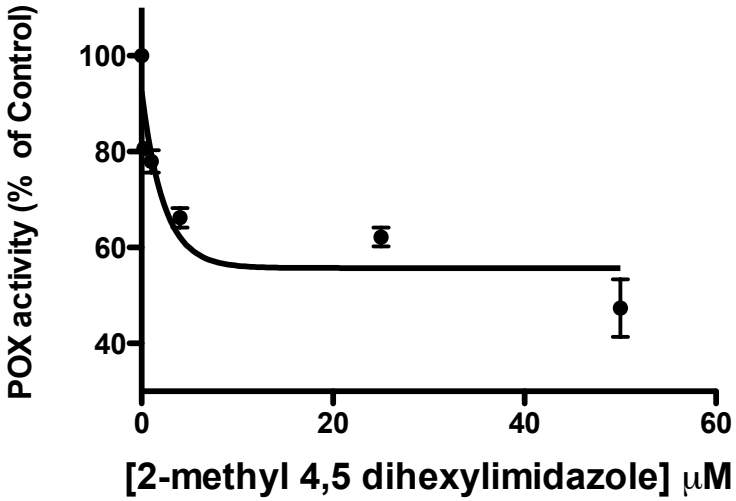


Figure 2. Inhibition of the peroxidase active site of PGHS-2. 2-Methyl-4,5-dihexyl imidazole was tested against the peroxidase active sites of PGHS-2 in inhibiting the reduction of PPHP in a spectrophotometric assay monitoring the oxidation of ABTS as the reducing co-substrate as previously published (230).

Assay for 2-methyl-4,5-dihexylimidazole as an inhibitor of AA and 2-AG oxygenation – To determine if the inhibition of the peroxidase active site by 2-methyl-4,5-dihexyl imidazole could inhibit the oxygenation of either AA or 2-AG, we tested it as described in "Experimental Procedures". 2-Methyl-4,5-dihexyl imidazole is a selective inhibitor of 2-AG oxygenation, with an IC_{50} of ~ 8.3 μM, with no impact on AA oxygenation (Figure 3).

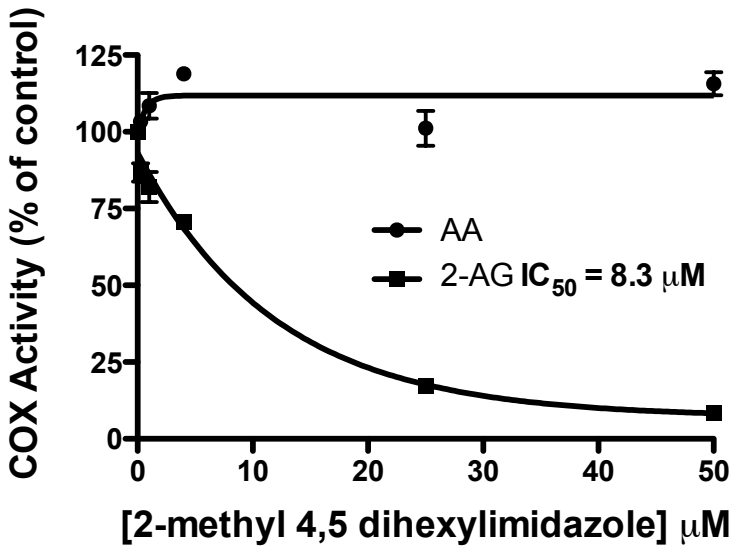


Figure 3. Selective inhibition of PGHS-2 oxygenation of 2-AG by 2-methyl-4,5-dihexyl imidazole, *in vitro*. 2-Methyl-4,5-dihexyl imidazole was tested for its ability to inhibit the oxygenation of AA and 2-AG by PGHS-2. 2-Methyl-4,5-dihexyl imidazole demonstrated an IC_{50} of 8.3 μM against 2-AG, with no inhibition of AA oxygenation.

Assay for 2-methyl-4,5-dihexylimidazole as competitive COX inhibitor- Since weak COX inhibitors have been shown to be potent selective 2-AG inhibitors in vitro (220), we determined the ability of 2-methyl-4,5-dihexylimidazole to bind at the COX active site. We determined the IC_{50} of a weak reversible inhibitor, ibuprofen, alone, or in the presence of 25 μM of 2-methyl-4,5-dihexylimidazole. As shown in figure 4, testing of ibuprofen in the presence of 2-methyl-4,5-dihexylimidazole slightly potentiates the inhibitory actions of ibuprofen, demonstrating a slight leftward shift in the IC_{50} curve. This indicates that under these conditions, 2-methyl-4,5-dihexylimidazole does not interact with ibuprofen at the COX active site. Had the IC_{50} curve had demonstrated a

rightward shift, this would been an indication 2-methyl-4,5-dihexylimidazole can actively bind at the COX active site and possibly effect inhibition.

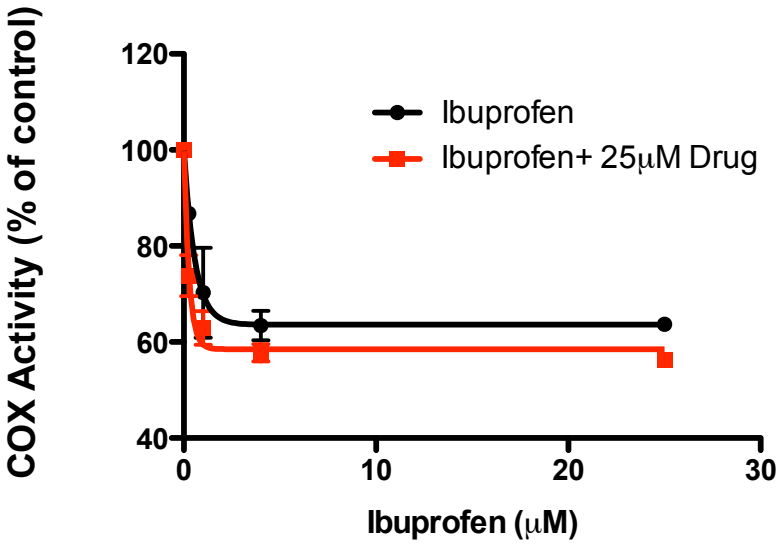


Figure 4. Determination of the ability of 2-methyl-4,5-dihexyl imidazole to bind at the COX active site of PGHS-2- The IC_{50} of ibuprofen was determined either alone, or in the presence of 25 μM 2-methyl-4,5-dihexyl imidazole (Drug). The potency of ibuprofen is not affected by the presence of 25 μM 2-methyl-4,5-dihexyl imidazole indicating that 2-methyl-4,5-dihexyl imidazole does not effectively bind at the COX active site.

Assay for 2-methyl-4,5-dihexylimidazole as an inhibitor of AA and 2-AG oxygenation in activated RAW264.7 macrophages– Following the demonstration that 2-methyl-4,5-dihexyl imidazole was a selective inhibitor of 2-AG oxygenation *in vitro*, we used RAW264.7 macrophages which have been shown to generate robust amounts of PGs and PG-Gs in response to physiologic inflammatory stimulus and Ca^{2+} ionophore (174). This was carried out as described in "Experimental Procedures". Interestingly, 2-

methyl-4,5-dihexyl imidazole inhibited AA oxygenation, with an IC_{50} of ~ 14 μM and 2-AG oxygenation with and IC_{50} ~ 25 nM.

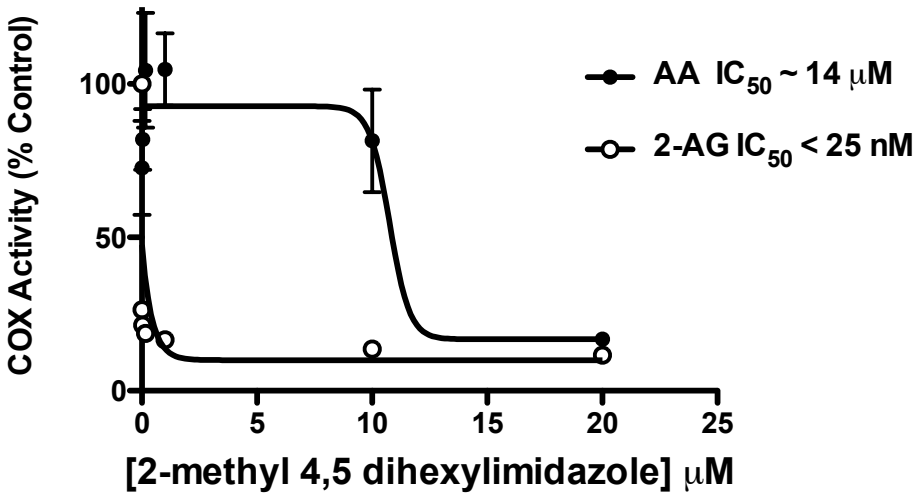


Figure 5. Selective inhibition of oxygenation of 2-AG by 2-methyl-4,5-dihexyl imidazole in activated murine RAW264.7 macrophages. 2-Methyl-4,5-dihexyl imidazole was tested for its ability to inhibit the oxygenation of AA and 2-AG in RAW264.7 macrophages as described in "Experimental Procedures". 2-Methyl-4,5-dihexyl imidazole demonstrated an IC_{50} of < 14 nM against 2-AG, and 14 μM against AA oxygenation.

Discussion

The present study reports that a modest peroxidase inhibitor of PGHS has been successfully generated, and that it is a selective inhibitor of the oxygenation of 2-AG both *in vitro* and *ex vivo*. 2-Methyl-4,5-dihexyl imidazole, while a modest inhibitor of the peroxidase active site, is a potent inhibitor of 2-AG oxygenation against purified enzyme and in RAW264.7 macrophages treated with inflammatory stimuli. These findings are in

agreement with previous findings that demonstrate that 2-AG oxygenation is sensitive to peroxide depletion *in vitro* and *ex vivo* (Chapter II and Chapter III). Here we have further confirmation of this finding through the selective attenuation of 2-AG oxygenation by a peroxidase active site inhibitor. While this represents the first peroxidase inhibitor of PGHS, a report of a similar compound, 2-methyl imidazole, has been shown to interact with the peroxidase active site of a mutant PGHS-2 (231). 2-Methyl imidazole was able to restore full peroxidase activity in a peroxidase active site mutant, His193Ala PGHS-2, that exhibited ~200-fold diminished POX activity relative to wild-type PGHS-2 (231). While the action of 2-methyl-4,5-dihexyl imidazole has the opposite effect on wild-type PGHS-2, the fact that these ligands share close structural similarity and interact with the peroxidase active site might indicate that imidazole-based compounds could form an appropriate scaffold to explore the structure activity of future inhibitors based on 2-methyl-4,5-dihexyl imidazole.

The development of a potent substrate-selective inhibitor of 2-AG oxygenation by PGHS-2 could be very useful in the determination of the contribution of PGHS-2 in the mediation of pain. While some weak NSAIDs have also been shown to be very potent selective inhibitors of PGHS-2-mediated 2-AG oxygenation (220), their use as experimental probes is confounded by the fact that many of these same compounds have also been shown to inhibit fatty acid amide hydrolase (FAAH) (232). FAAH is the chief enzyme that metabolizes the endocannabinoid anandamide (AEA) hydrolyzing it to AA. The use of these weak NSAIDs to dissect the exact contribution PGHS-2-mediated oxygenation of 2-AG to pain control could be complicated by the preservation of AEA levels from FAAH inhibition (232).

These results along with those from the preceding chapters indicate that the oft-ignored peroxidase active site of PGHS is a veritable pharmacological target. Future work should be geared toward the generation of analogs of 2-methyl-4,5-dihexyl imidazole, which could also be used to probe the molecular determinants of the interations of peroxidase active site inhibitors with the POX active site of PGHS. Furthermore testing of promising analogs of 2-methyl-4,5-dihexyl imidazole in an *ex vivo* model of pain such as will be discussed in Chapter V or even in a mammalian model for pain (193), could lead to an appreciation for the role of PGHS-2 mediated 2-AG metabolism, in mediating pain.

References

1. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J Biol Chem* **275**, 33744-33749

2. Kozak, K. R., Crews, B. C., Morrow, J. D., Wang, L. H., Ma, Y. H., Weinander, R., Jakobsson, P. J., and Marnett, L. J. (2002) *J Biol Chem* **277**, 44877-44885

3. Rouzer, C. A., and Marnett, L. J. (2005) *J Biol Chem* **280**, 26690-26700

4. Hu, S. S., Bradshaw, H. B., Chen, J. S., Tan, B., and Walker, J. M. (2008) *Br J Pharmacol*

5. Piomelli, D. (2003) *Nat Rev Neurosci* **4**, 873-884

6. Kozak, K. R., Crews, B. C., Ray, J. L., Tai, H. H., Morrow, J. D., and Marnett, L. J. (2001) *J Biol Chem* **276**, 36993-36998

7. Kulmacz, R. J., and Lands, W. E. (1985) *J Biol Chem* **260**, 12572-12578

8. Prusakiewicz, J. J., Duggan, K. C., Rouzer, C. A., and Marnett, L. J. (2009) *Biochemistry* **48**, 7353-7355

9. Vasquez, E., Bar, K. J., Ebersberger, A., Klein, B., Vanegas, H., and Schaible, H. G. (2001) *J Neurosci* **21**, 9001-9008

10. Goodwin, D. C., Rowlinson, S. W., and Marnett, L. J. (2000) *Biochemistry* **39**, 5422-5432

11. Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996) *Proc Natl Acad Sci U S A* **93**, 15069-15074

12. Rouzer, C. A., and Marnett, L. J. (2003) *Chem Rev* **103**, 2239-2304

13. Chubb, A. J., Fitzgerald, D. J., Nolan, K. B., and Moman, E. (2006) *Biochemistry* **45**, 811-820

14. Srinivasan, N. S., and Lee Donald, G. (1979) *Journal of Organic Chemistry* **44**, 1574

15. Miyashita, A., Suzuki, Y., Iwamoto, K.-i., and Higashino, T. (1994) *Chem. Pharm. Bull.* **42**, 2633-2635

16. Khurana, J. M., and Kandpal, B. M. (2003) *Tetrahedron Letters* **44**, 4909-4912

17. Bredereck, H. G., R.; Schuh, H. G. V.; Theilig, G. (1959) *Angewandte Chemie* **71**, 753-774

18. Wolkenberg Scott, E., Wisnoski David, D., Leister William, H., Wang, Y., Zhao, Z., and Lindsley Craig, W. (2004) *Org Lett* **6**, 1453-1456

19. Rowlinson, S. W., Crews, B. C., Lanzo, C. A., and Marnett, L. J. (1999) *J Biol Chem* **274**, 23305-23310

20. Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. R., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., and Eling, T. E. (1984) *Mol Pharmacol* **26**, 328-335

21. Duggan, K. C., Musee, J., and Marnett, L. J. (2010) *Methods Mol Biol* **644**, 55-65

22. Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K., and Marnett, L. J. (1998) *Science* **280**, 1268-1270

23. Duggan, K. C., Musee, J and L. J. Marnett. (2010) *Methods Molecular Biology* **644**, 55-67

24. Landino, L. M., Crews, B. C., Gierse, J. K., Hauser, S. D., and Marnett, L. J. (1997) *J Biol Chem* **272**, 21565-21574

25. Staniaszek, L. E., Norris, L. M., Kendall, D. A., Barrett, D. A., and Chapman, V. (2010) *Br J Pharmacol* **160**, 669-676

26. Telleria-Diaz, A., Schmidt, M., Kreusch, S., Neubert, A. K., Schache, F., Vazquez, E., Vanegas, H., Schaible, H. G., and Ebersberger, A. (2010) *Pain* **148**, 26-35

CHAPTER V

EVALUATION OF PGHS-2 MEDIATED OXYGENATION OF THE ENDOCANNABINOID 2-AG IN THE NEURONAL MILLEU

Introduction

The endocannabinoid system was discovered when an endogenously produced lipid was found to agonize the G-protein-coupled cannabinoid receptor (CB_1) (149). This lipid was identified as an amide of AA with ethanolamine (arachidonoyl ethanolamine, AEA) (Figure 1). Soon thereafter, another endogenously produced lipid CB ligand was discovered. It was a glyceryl ester of AA, 2-arachidonoyl glycerol (2-AG) (Figure 1) (150).

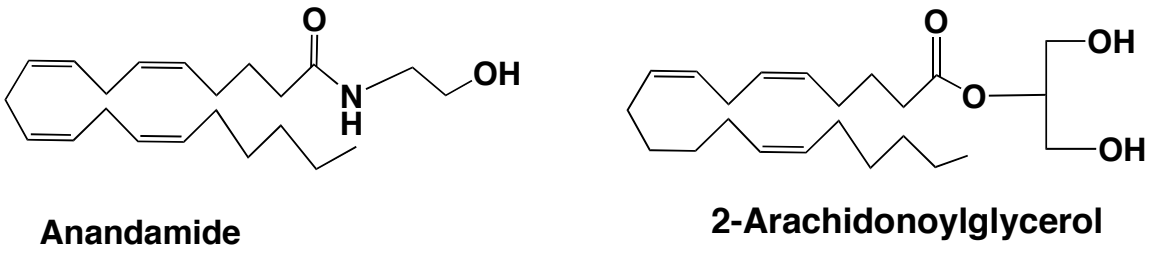


Figure 1. Structures of the endocannabinoids

Although a number of other lipids produced *in vivo* have been shown to agonise CB receptors, AEA and 2-AG remain the best understood and most physiologically relevant (144). The CB_1 receptor is the most abundant GPCR in the mammalian brain,

hightlighting the importance of endocannabinoid signalling. The pertussis toxin (PTX) sensitive $G_{i/o}$ CB_1 receptor is expressed primarily in the hippocampus, basal ganglia, cerebellum, brain stem, spinal cord and dorsal root ganglia (180). Endocannabinoids have been shown to tonically modulate neuronal transmission in the rostral vental medulla (RVM), the periaqueductal grey and the spinal trigeminal nucleus, areas critically involved in the promotion of central analgesia (190-192). Supporting the importance of these areas in the modulation of pain, is the fact that treatment of these areas with a CB_1 receptor antagonist leads to the induction of hyperalgesia, in a opioid–independent manner (233,234). Noxious and painful stimuli from the periphery create neuronal hyperexcitablity in the dorsal horn of the spinal cord; this hyperexcitablity leads to increased glutaminergic signaling and excitotoxicity that induces the expression of Prostaglandin H Synthase-2 (PGHS-2) via production of inflammatory cytokines, such as IL-1β (67,235).

PGHS-2 has been shown to oxygenate the endocannabinoid 2- arachidonoyl glycerol (2-AG) *in vivo* and *ex vivo*, leading to the formation of prostaglandin glyceryl esters (PG-Gs). Since 2-AG is also subject to hydrolysis to arachidonic acid (AA) by monoacylglycerol lipase (MAGL), the relevance of 2-AG metabolism by PGHS-2 has been called into question. Infinitesimal amounts of PG-Gs have been detected in both inflamed and non-inflamed rat footpad, probably owing to their rapid hydrolysis to traditional AA-derived prostaglandins (PGs) by an unidentified specific serine hydrolase (177,179). Furthermore, while the steady state kinetic parameters of the oxygenation of 2-AG and AA by PGHS-2 *in vitro* are virtually identical (174), examination of the formation of PG-Gs *ex vivo* upon stimulation of murine resident peritoneal macrophages

with an inflammatory stimulus demonstrates that they are detected at levels 1000-fold lower than those of AA-derived prostaglandins (PGs) (176). While the levels of 2-AG released upon stimulation by an inflammatory stimulus are only 10-fold lower than the levels of AA, there is incongruity between the levels of 2-AG and PG-Gs realized (176). That said, PGE_2-G has been shown to effect Ca^{2+} mobilization in RAW264.7 macrophages and H1819 non-small cell lung carcinoma cells at sub-picomolar concentrations, indicating that PG-Gs may be active at very low concentrations (178,215). PGE_2-G also induces hyperalgesia (exaggerated pain sensation) and mechanical allodynia (nociception in response to non-nociceptive stimuli) in rats, acting through a unique, unidentified receptor (179). Therefore, the oxygenation of 2-AG by PGHS-2 not only terminates analgesic endocannabinoid signaling, but also creates novel molecules that bear unique nociceptive activities.

To date, there has been no demonstration of the formation of PG-Gs from the neuronal milieu. Pivotal to making a case for the role of PGHS-2 in the metabolism of 2-AG *in vivo*, we have taken two approaches to determine the PG-G generating capacity in the neuronal environment. The first is directly assessing the production of PGs and PG-G in cerebrospinal fluid (CSF) in the rat carrageenan–footpad pain model. Peripheral pain has been shown to induce the expression of PGHS-2 in dorsal root ganglia and in the sensory dorsal horn via the production of inflammatory cytokines such as IL-1β (64,236,237). This phenomenon is what leads to central sensitization in response to peripheral pain, leading to an amplification of pain. CSF bathes the entire central nervous system, meaning products from oxidative metabolism of AA and 2-AG should be

detectable therein (64,236,237). Furthermore, extraction of CSF from rats has been used to detect neuropeptides and hormones by a previously published technique (238).

The second approach to determining neuronal capacity to generate PG-Gs is in primary cultures of murine dorsal root ganglia (DRG), which are collections of neurons that feed information from the periphery into the sensory horn of the spinal cord. Inflammatory stimuli have been shown to induce PGHS-2 in DRGs, which leads to a hyperexcitability that is transmitted into the sensory dorsal horn of the spinal cord (235). Hyperexcitability is associated with hyperalgesia and allodynia due to a large increase in the gain in the sensory system. Using both of these approaches, we find that while we cannot detect PG-Gs in CSF, PGs were detected and associated with carrageenan-induced footpad inflammation, consistent with prior reports (64,236,237). Primary DRG cell cultures demonstrate robust PG and PG-G generation in response to various physiological inflammatory stimuli along with Ca^{2+} ionophores. This is the first demonstration that there are robust levels of PG-Gs formed in neuronal tissues in response to inflammatory stimuli. Furthermore, while PGE_2-G is produced by inflamed DRG cultures, it is rapidly degraded in DRGs. This adds to increasing evidence that PG-Gs are rapidly degraded in the *ex vivo* setting, adding difficulty to their detection and demonstration of relevance. Since DRG primary cultures primarily consist of microglia and neurons, we have determined that the source of PG-Gs is the microglia and not the neurons.

Beyond these findings, we have established the DRG primary cell culture as a suitable platform for screening for agents that selectively inhibit the oxygenation of 2-AG by PGHS-2. As a proof of concept, here we demonstrate that an inactive enantiomer of a

commonly used NSAID *R*-flurbiprofen is a potent inhibitor of 2-AG and AEA oxygenation in DRG primary cultures.

Experimental Procedures

Induction of carrageenan-induced footpad inflammation in the rat - Carrageenan (100 μL, 1% (w/v), Fluka, dissolved in sterile physiological saline) was injecected subcutaneously into the plantar side of the right hind paw of adult male Sprague-Dawley rats while under light isoflurane anesthesia. Local inflammation was assessed by measurement of paw thickness using a plethysmometer. Sham-treated rats were injected with 100 μL of saline. Inflammation was allowed to develop for 2 hours.

Assay for production of PGs and PG-Gs from rat CSF - Adult male Sprague-Dawley rats were euthanized using isoflurane. CSF was extracted using 25-gauge insulin needles inserted into the cisterna magna. The position of the cisterna magna was determined by palpitation along the nuchal line on the rat's occiput ending at C1. The syringe was inserted ~5mm into the space between cervical vertebrae C1 and C2, and CSF was aspirated. CSF (50 μL- 150 μL) was spiked with 20 μL of a solution containing deuterated standards as follows : 100 pmol of the following: PGD_2-d4, PGE_2-d4, $PGF_{2\alpha}$-d4, 6-keto-$PGF_{1\alpha}$-d4, and 10 pmol of the following: PGD_2-G-d5, PGE_2-d5 $PGF_{2\alpha}$-d5, 6-keto-$PGF_{1\alpha}$-G-d5, and 150 μL methanol:water (50:50 v:v). Samples were stored at -80° C until LC/MS analysis as previously published (217).

Isolation of embryonic dorsal root ganglia - Pregnant (E14-15) mice were euthanized by CO_2 asyphyxiation. Embryos were surgically removed from the uterus of the euthanized mouse, and had their entire spinal column dissected and dorsal root ganglia (DRGs) collected. DRGs were washed in PBS and dissociated by incubation in 0.001% collagenase/DNase and 0.15% trypsin at 37°C for one hour. Cells (80,000) were plated onto acid-treated, collagen-coated coverslips in 35 mm dishes and incubated in 3 mL of UltraCulture Medium (10% Hyclone FBS, 1 mM L-glutamine, 1% Pen-Strep and 50 ng/mL nerve growth factor). Cells were cultured at 37 °C and 5% CO_2.

Generation of pure neuronal DRG Cultures - Following the isolation of DRG described above, cultures were allowed to acclimatize for 48 hours at 37 °C and 5% CO_2. Cells were then treated with 5 μM cytosine arabinoside (AraC) for 7 days, and confirmed to be pure neuronal cells by microscopic inspection.

Induction of inflammation in DRG cultures and immunoblotting for PGHS-2 expression – Following the 48 hour acclimation period, DRG cultures were treated with vehicle (sterile phosphate-buffered saline with 0.1% bovine serum albumin) or 20,000 U/mL IFNγ (R&D systems) or 50 ng/mL IL-1β (R&D systems) for 24 hours. The medium was collected and frozen at -80°C until extraction. Cells were incubated in fresh medium with 2 μM ionomycin for 4 hours and the medium was collected and stored at -80°C until extraction for LC/MS/MS analysis. Cells were scraped into lysis buffer and immunoblotted for PGHS-2 expression as previously described (239), with some modifications as follows: cells were lysed in 200 μl M-PER lysis buffer (Thermo) containing a cocktail of mammalian protease inhibitors (Sigma). Cell lysates were mixed by vortexing and placed on ice for 30 min. Cellular debris was then removed by

centrifugation for 10 min at 16,000 *g*. Samples were stored at -80 °C until analyses could be completed. Equal quantities of protein (~ 20 μg) were resolved by gradient (2 - 12%) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were blocked (20 mM Tris, pH 7.6, 140 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) prior to incubation with antibodies. Both primary antibodies (α-actin and α-PGHS-2, Cayman Chemical, USA) were used at 1:1000 dilution and the secondary antibody (Cayman Chemical, USA) at 1:5000 dilution.

Assay for Production of PGs and PG-Gs from Cell Culture- Medium from cell culture was subjected to liquid-liquid extraction as developed by Philip Kingsley with some modifications. Briefly, internal standards (100 pmol of the following: PGD_2-d4, PGE_2-d4, $PGF_{2\alpha}$-d4, 6-keto-$PGF_{1\alpha}$-d4, and 10 pmol of the following: PGD_2-G-d5, PGE_2-d5 $PGF_{2\alpha}$-d5, 6-keto-$PGF_{1\alpha}$-G-d5 2 mL media was acidified by the addition of 1% glacial acetic acid, followed by 2 mL of ETOH and 2 mL Hexanes:Ethyl Acetate (20:80). Samples were vortexed and centrifuged at 3000 RPM for 20 minutes. The organic layer was retrieved and dried down under a stream of nitrogen. Samples were resuspended in 300μL Methanol:Water (50:50) and analyzed as previously described (217).

Results

Assay of PG and PG-G Formation in CSF in Response to Peripheral Inflammation- Comparative LC/MS analysis of CSF from saline-treated rats and rats with carrageenan-induced footpad inflammation revealed that PG-Gs could not be detected under either of these conditions (data not shown). Even though PG-Gs have been shown

to be stable in CSF (177), it is possible that they may be rapidly hydrolyzed within the ventricular system, by serine hydrolase(s) expressed in cells within the ventricular system. Further analysis revealed that the CSF from saline-treated control rats did not contain PGs, but CSF from rats with carrageenan-induced inflammation of the foot contained significant quantities of pro-inflammatory PGE_2 (Figure 2 and 3). This is in line with recent work demonstrating that PGE_2 is detectable in CSF of rats with carrageenan-induced footpad inflammation (240).

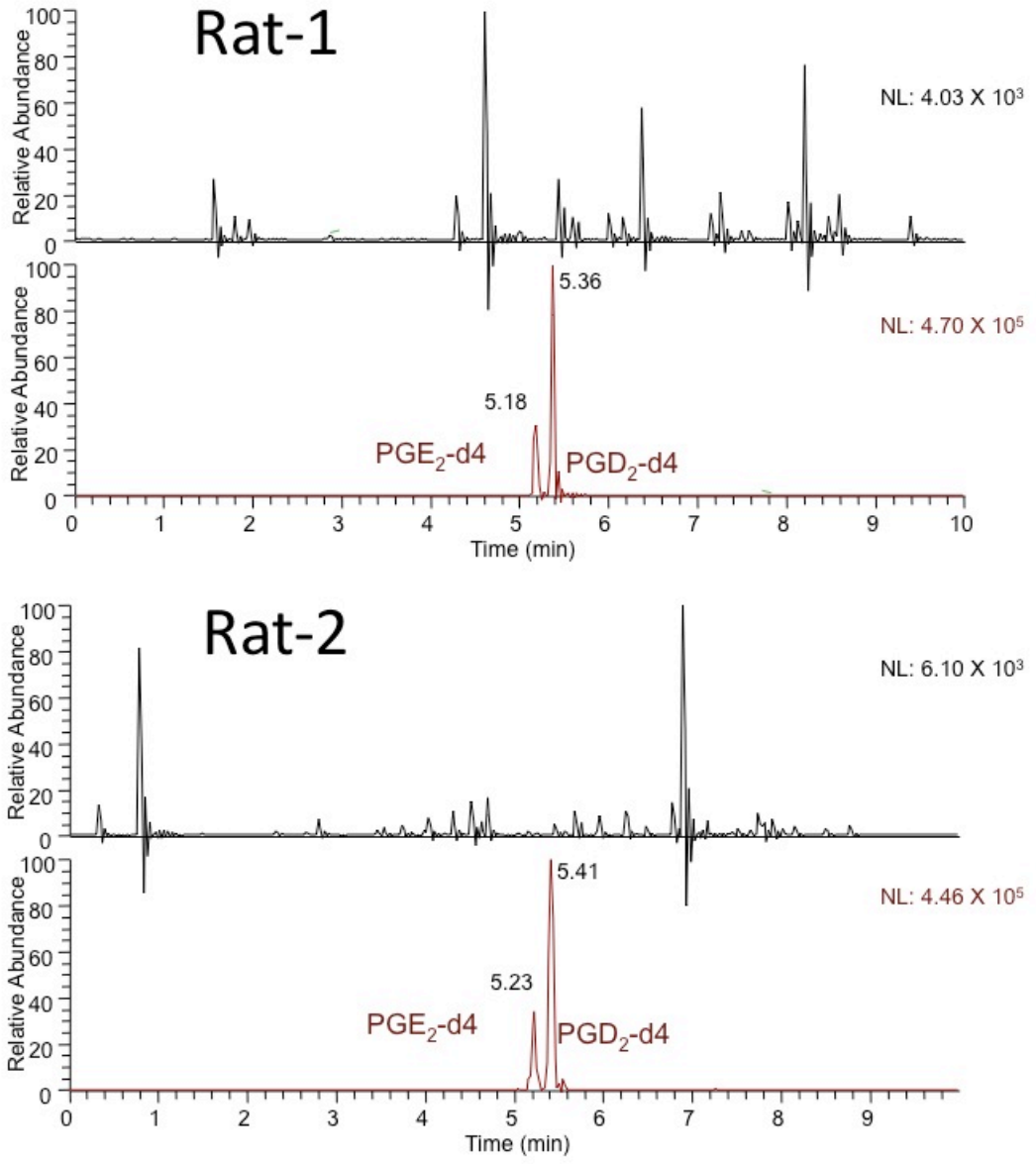


Figure 2. Assay of PGs in rat CSF <u>WITHOUT</u> inflammation –Rat 1 and Rat 2 were sham treated (saline) as controls to assay baseline PG formation. PGs and PG-Gs (not shown) were undetectable in the CSF of these rats.

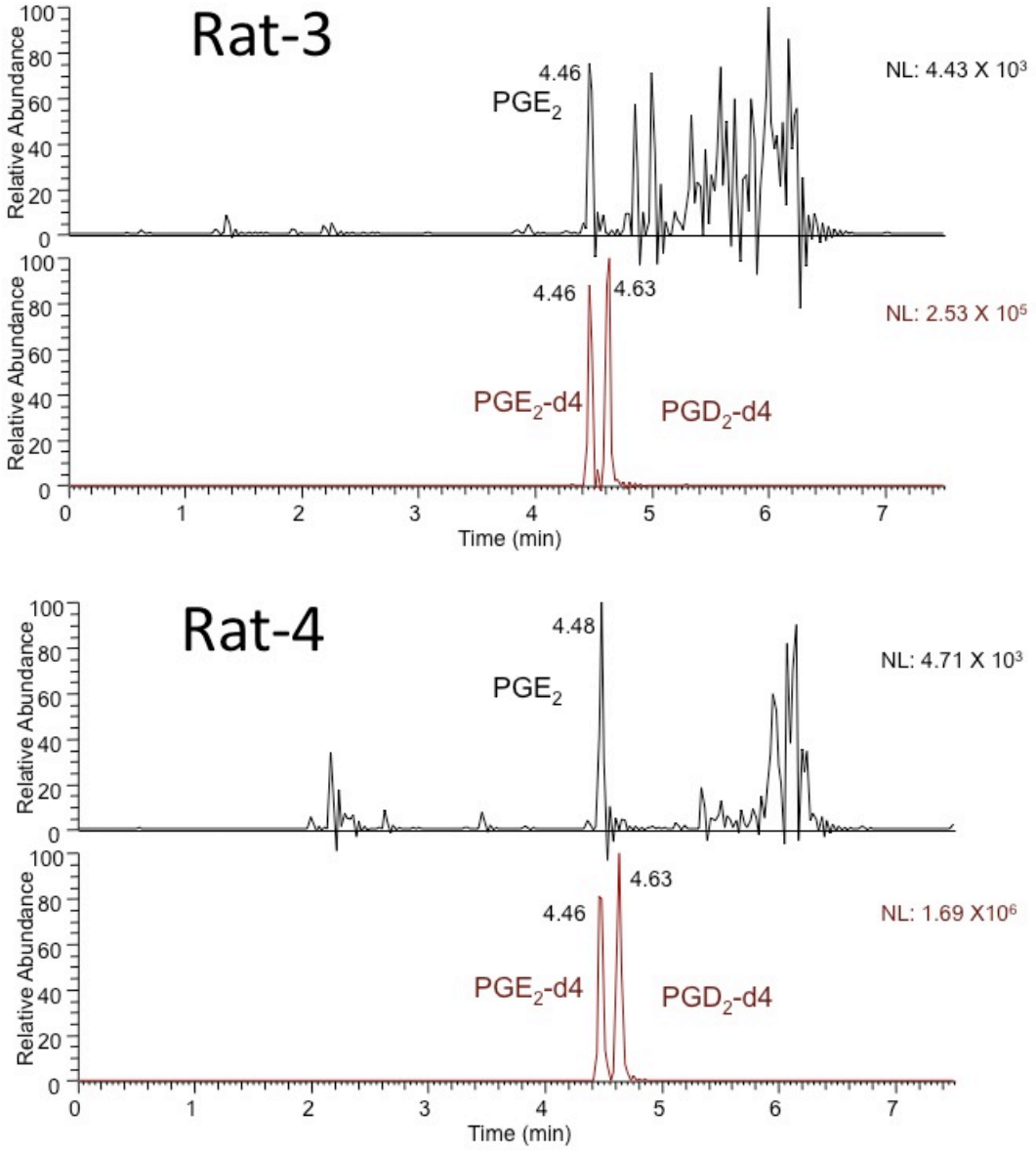


Figure 3. Assay of PGs in rat CSF <u>WITH</u> carrageenan-induced rat footpad inflammation –CSF from Rat 3 and Rat 4 revealed that PGE_2 is generated in the spinal cord in response to peripheral pain and inflammation. This is in line with recent reports demonstrating that peripheral inflammation can lead to PGE_2 production in the spinal cord. PG-Gs were undetectable in these rats (not shown).

Assay of PG-G formation in DRG Cultures in Response to Inflammation- LC/MS analysis of medium from cells treated as described in "Experimental Procedures" indicated that under control conditions, modest amounts of PGs are formed (Figure 4). PG generation was increased in DRG cultures pre-treated with the physiologic inflammatory agonists, IFNγ and Il-1β, consistent with literature findings that these two cytokines induce PG synthesis in DRG cultures by upregulating PGHS-2 expression (65,66). Immunoblotting for PGHS-2 demonstrates that both IFNγ and Il-1β induce PGHS-2 (Figure 5). Interestingly, the stable PGI_2-G metabolite, 6-keto-$PGF_{1\alpha}$-G was only found in cultures treated with either IFNγ or Il-1β, followed by ionomycin (Figure 4). Ionomycin on its own did not lead to the generation of any PG-Gs. This is the first demonstration of the formation of a PG-G in primary neuronal cultures. A time course of the production of PG-Gs was conducted and the data were plotted (Figure 6). Here we expand the initial observation and demonstrate that PGE_2/D_2-G and $PGF_{2\alpha}$-G are generated in addition to the previously detected PGI_2-G metabolite (6-keto-$PGF_{1\alpha}$-G), and at levels that are 10-fold higher than 6-keto-$PGF_{1\alpha}$-G (Figure 6). A time course of PG-G production reveal that PGE_2-G levels peak at 2 hours, and drop precipitously by 4 hours, indicating that PGE_2-G is unstable under these conditions (Figure 7). This is probably due its hydrolysis to PGE_2 by a yet to be identified esterase (177). Surprisingly, $PGF_{2\alpha}$-G levels remain high through out the time course, and seemingly not as unstable as PGE_2-G (Figure 7). This is the first time PG-Gs have been demonstrated in primary neuronal culture.

Assay of PG and PG-G formation in pure neuronal DRG cultures in response to inflammation - Pure neuronal DRG cultures were prepared as described in "Experimental Procedures". The pure population of neurons did not generate any detectable amounts of PG-Gs (data not shown), and PG production was diminished relative to mixed cultures containing microglia. In fact the only PG generated under these conditions is PGE_2 (Figure 8). This indicates that the microglia are the chief source of PG-Gs in these cell culture systems. Also, to determine if the hydrolysis of 2-AG prevented the oxygenation of 2-AG released from the neurons, we co-incubated a potent irreversible MAGL inhibitor, JZL-184 (170) at 1μM for 30 minutes before the beginning of the experiment. However, as stated earlier, pure neuronal cultures of DRGs did not produce any PG-Gs. Infact, inhibition of MAGL seemed to affect PGE_2 generation (Figure 8).

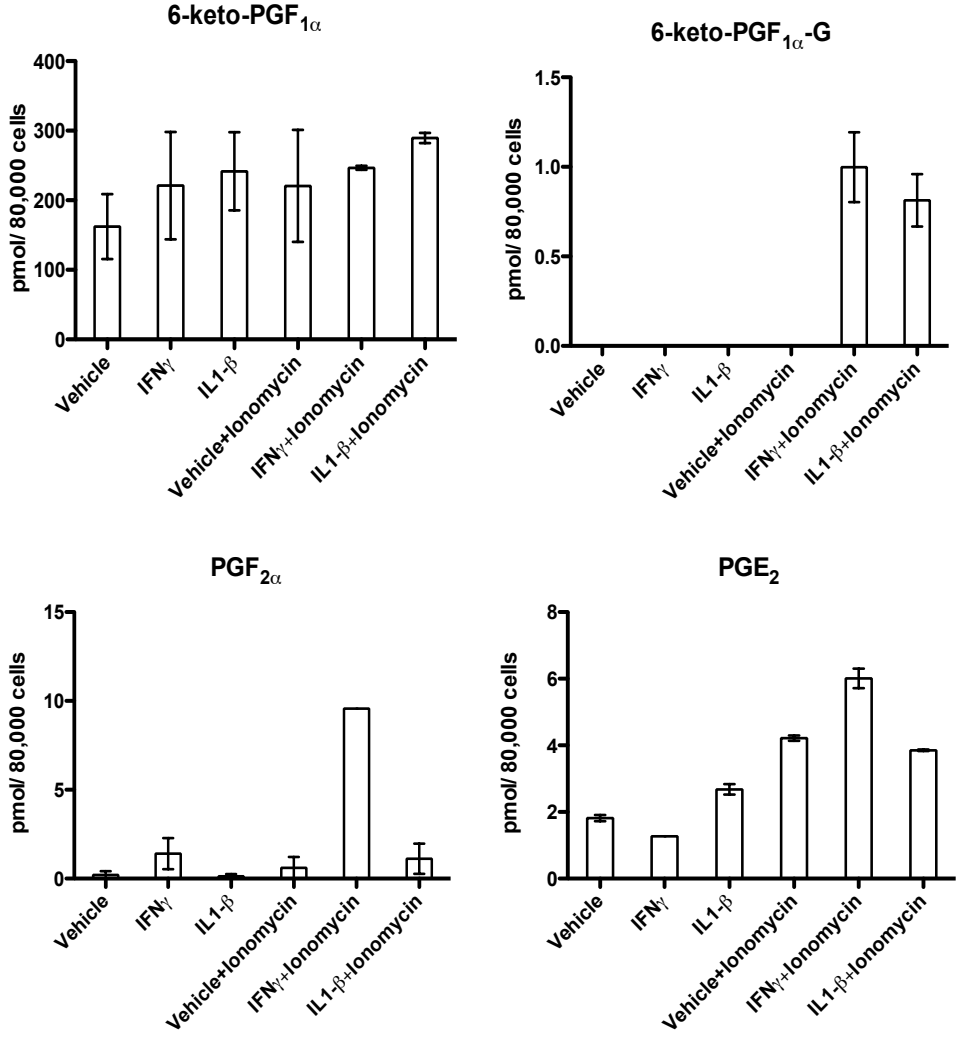


Figure 4. Assay of PG-G formation in DRG Cultures in Response to Inflammation –Primary DRG cultures were pre-treated for 24hours with IFNγ and IL-1β. and PG and PG-G formation was evoked by treating these cultures with ionomycin for 4 hours.

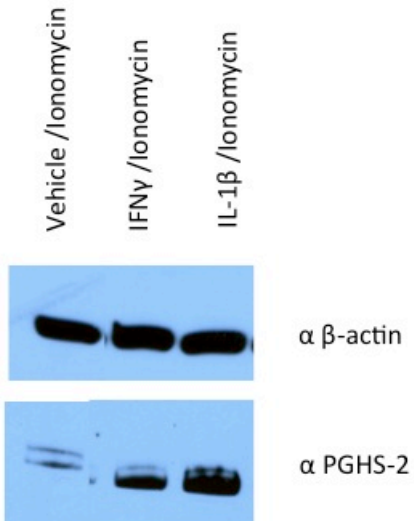


Figure 5. Determination of PGHS-2 levels in DRG cultures treated with IFNγ and IL-1β. (A) Immunoblot for PGHS-2 was conducted as described in "Experimental Procedures". PGHS-2 levels are almost undetectable in unstimulated cells, whereas either IFNγ or IL-1β treatment leads to a large upregulation of PGHS-2.

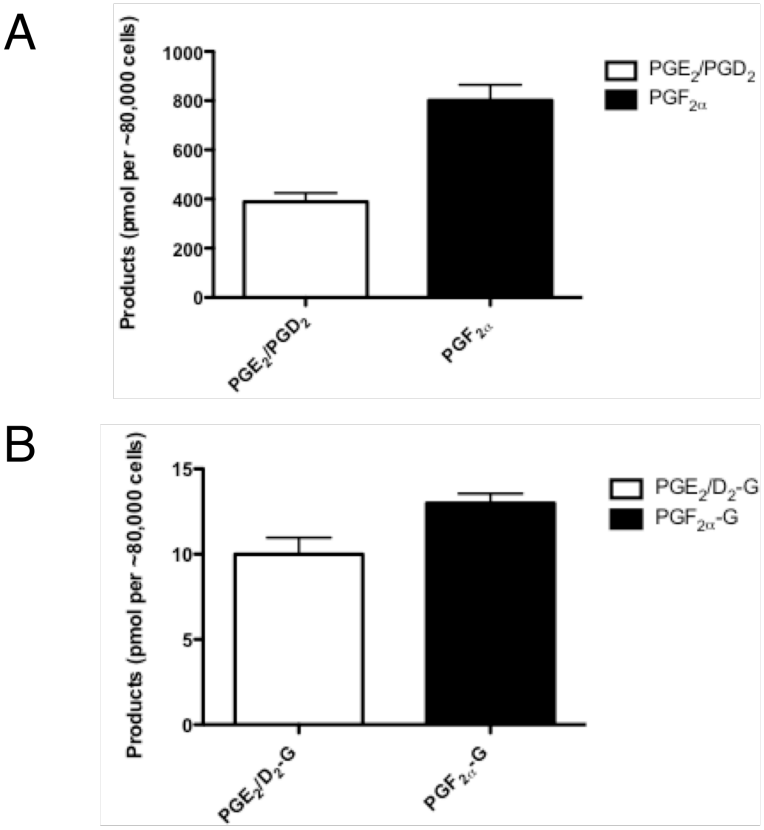


Figure 6. Assay of PG and PG-G formation in DRG cultures in response to inflammation –Primary DRG cultures were pre-treated for 24hours with IFNγ and IL-1β. and PG (**A**) and PG-G (**B**)formation was evoked by treating these cultures with ionomycin for 2 hours as opposed to the 4 hour time treatment described initially.

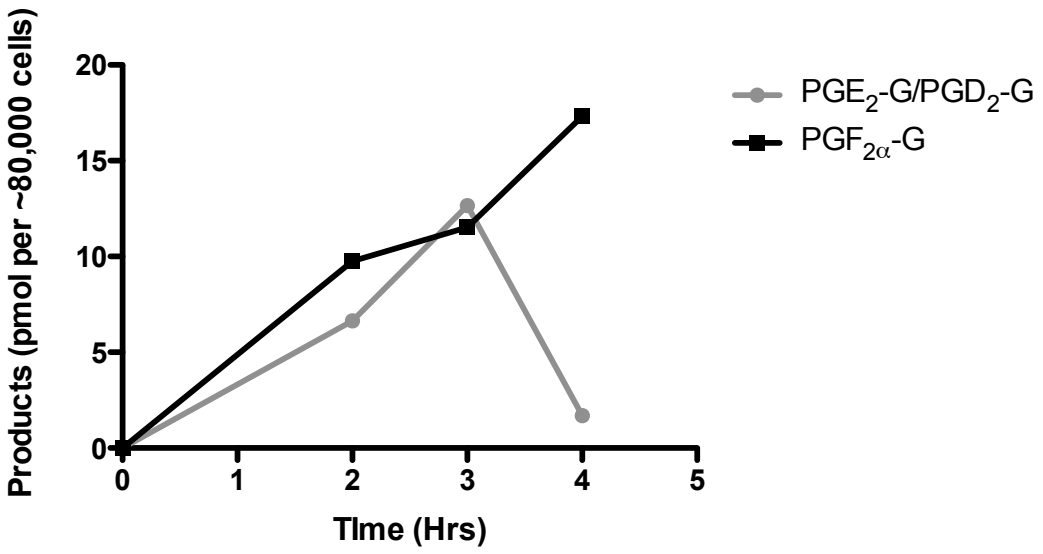


Figure 7. Time course of PG-G formation in DRG cultures in response to inflammatory agonist, IFNγ – Primary DRG cultures were pre-treated for 24 hours with IFNγ. PG-G formation was evoked by treating these cultures with ionomycin for 2, 3, and 4 hours. Results were from single determination.

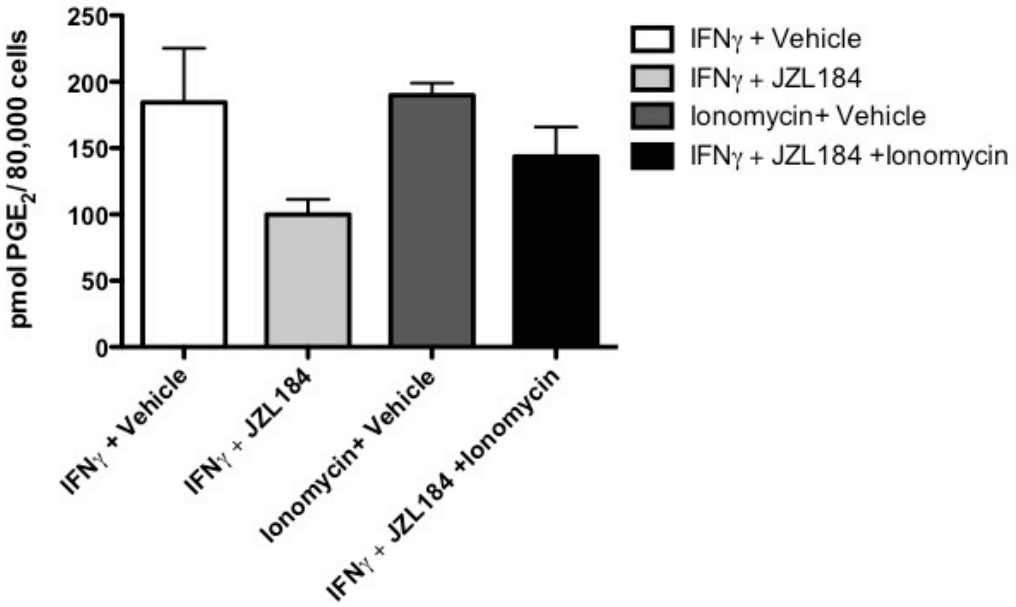


Figure 8. The Determination of the Source of PG-Gs in DRGs in response to IFNγ– To determine the source of the PG-Gs produced in response to the inflammatory agonist

IFN, pure neuronal cultures were generated as described in "Experimental Procedures". Pure neuronal cultures did not produce any detectable PG-Gs (not shown).

Assay of inhibition of PG and PG-G formation in DRG Cultures in Response to Inflammation - Following the determination that DRG cultures produced robust levels of PG-Gs in response to a physiologic inflammatory agonist, we determined the capacity of the poorly active enantiomer of flurbiprofen, *R*-flurbiprofen, to inhibit the oxygenation of 2-AG in inflamed DRG cultures. *R*-Flurbiprofen has been shown to be a potent selective inhibitor of 2-AG oxygenation *in vitro* (Kelsey Duggan, personal communication). Here we show that *R*-flurbiprofen is a potent selective inhibitor of 2-AG and AEA oxygenation in inflamed DRG cultures (Figure 9). We have also expanded our analysis to AEA-derived products, upon the demonstration that these cultures also release appreciable amounts of AEA upon stimulation with Ca^{2+} ionophore (Daniel Hermanson, personal communication).

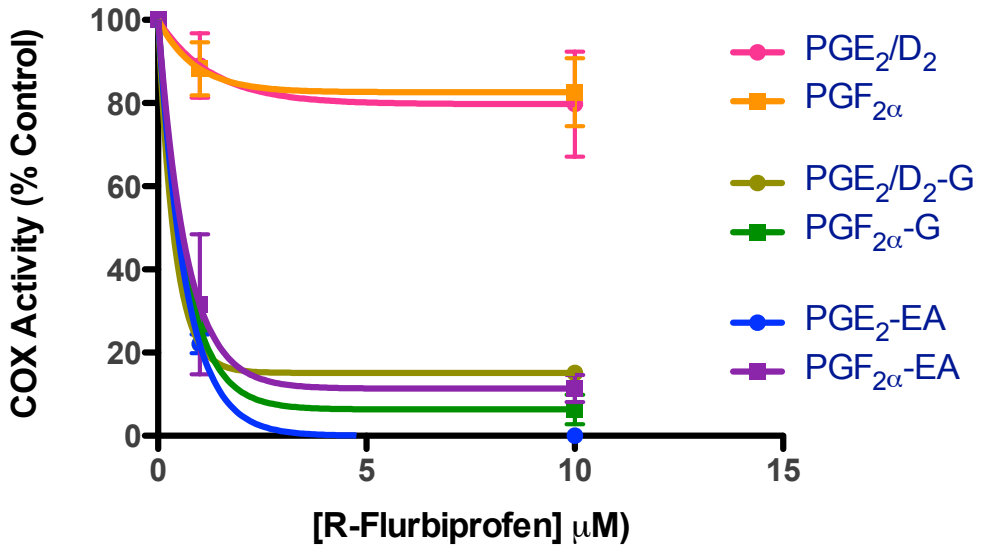


Figure 9. Assay for *R*-flurbiprofen-mediated inhibition of PG, PG-EA, and PG-G formation in DRG cultures in response to an inflammatory agonist - Primary DRG cultures were pre-treated for 24 hours with IFNγ and then pre-incubated with increasing concentrations of *R*-flurbiprofen for 2 hours. Product formation was evoked by treating these cultures with ionomycin for 2 hours.

Discussion

The present study establishes that the inflammatory prostaglandin PGE_2 is present in the CSF of rats with carrageenan-induced pain and inflammation. While the chief source of PGE_2 is AA oxygenation by PGHS, the lack of detection of its 2-AG-derived congener might imply that PGE_2-G is rapidly hydrolyzed to PGE_2. While PGE_2-G is chemically stable in CSF, it is not implausible that serine hydrolases in the ventricular system that the CSF bathes could metabolize PGE_2-G to PGE_2. Our study also highlights a recently reported phenomenon where peripheral pain induces central sensitization leading to PGHS-2 induction and PG production (240). This study and the present work indicate that PGE_2 could be a useful pain biomarker, as it is unequivocally associated

with pain in relatively linear manner (64). Partly due to the prevalence of pain medicine abuse and our poor understanding of the pathology of pain, pain is overwhelmingly undertreated. Having a biomarker that can be rapidly and reliably assayed for efficacy of treatment would be indispensable.

While the present study failed to detect PG-Gs in CSF, we demonstrate for the first time that 2-AG is oxygenated by PGHS in primary neuronal cultures, in response to two physiologically relevant inflammatory agonists. The importance of the inflammatory response in the generation of PG-Gs is seen when the neuronal population of macrophages (microglia) are eliminated; PG-G generation is completely abrogated (Figure 6). Microglia respond to IFNγ and IL-1β by upregulating PGHS-2 (65,66). This possibly implies that the neurons could be the source of 2-AG that is subsequently oxygenated by the PGHS-2 expressed in microglia. Further work to elaborate this connection is needed. As with previous work, we also demonstrate that PGE_2-G is rapidly degraded in primary DRG cultures, even though $PGF_{2\alpha}$-G seems particularly stable. Determination of the metabolism of PG-G under these conditions is warranted. The levels of PG-Gs produced in these cultures are at levels 50-fold higher than those shown for the action of PG-Gs on G-protein coupled receptors (GPCRs). PGE_2-G and $PGF_{2\alpha}$-G have been shown to mobilize Ca^{2+} at sub-picomolar concentrations and in a concentration dependent manner, in RAW264.7 macrophages and H1819 non-small cell carcinoma cells (178,215). This indicates that the levels of PG-Gs produced in response to inflammatory agonists are sufficient to activate these pathways, and warrant investigation. Dissection of the effects of the production of PG-Gs in DRGs could lead to

the identification of the unique and yet to be identified receptor that mediates the actions of PG-Gs already demonstrated.

Finally, this work demonstrates that primary DRG cultures are a robust *ex vivo* system to study the oxidative metabolism of 2-AG by PGHS-2. Here, we translate *in vitro* work demonstrating that a potent substrate selective inhibitor, *R*-flurbiprofen, is a potent selective inhibitor of 2-AG and AEA oxygenation. These studies could lead to the determination of the contribution of PGHS-2-mediated oxygenation of 2-AG and AEA in pain.

References

1. Blobaum, A. L., and Marnett, L. J. (2007) *J Med Chem* **50**, 1425-1441
2. Vane, J. R., Flower, R. J., and Botting, R. M. (1990) *Stroke* **21**, IV12-23
3. Jack, D. B. (1997) *Lancet* **350**, 437-439
4. Stone, E. (1763) *Philos Trans R Soc Lond* **53**, 195-200
5. Leroux, M. (1830) *J Chim Med* **6**, 341
6. Maclagan, T. J. (1876) *Lancet* **1**, 342-343
7. Brune, K., and Hinz, B. (2004) *Arthritis Rheum* **50**, 2391-2399
8. Kurzrok, R. a. L., C. (1930) *Proc. Soc. Exp. Biol. N.Y.* **28**, 268-272
9. Von Euler, U. S. (1935) *Kiln. Wschr.* **14**, 1182-1183
10. Von Euler, U. S. (1936) *J. Physiol.* **88**, 213-234
11. Von Euler, U. S. (1939) *Skand. Arch. Physiol.* **81**, 65-80
12. Goldblatt, M. W. (1935) *Biochem J* **29**, 1346-1357
13. Goldblatt, M. W. (1935) *J Physiol* **84**, 208-218
14. Hamberg, M., and Samuelsson, B. (1967) *J Biol Chem* **242**, 5336-5343
15. Smith, J. B., and Willis, A. L. (1971) *Nat New Biol* **231**, 235-237
16. Vane, J. R. (1971) *Nat New Biol* **231**, 232-235
17. Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., and Herschman, H. R. (1991) *J Biol Chem* **266**, 12866-12872
18. Xie, W. L., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) *Proc Natl Acad Sci U S A* **88**, 2692-2696
19. Marnett, L. J. (2009) *Annu Rev Pharmacol Toxicol* **49**, 265-290
20. van der Ouderaa, F. J., Buytenhek, M., Slikkerveer, F. J., and van Dorp, D. A. (1979) *Biochim Biophys Acta* **572**, 29-42
21. Roth, G. J., Siok, C. J., and Ozols, J. (1980) *J Biol Chem* **255**, 1301-1304
22. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and Van Dorp, D. A. (1977) *Biochim Biophys Acta* **487**, 315-331
23. Yu, Y., Fan, J., Chen, X. S., Wang, D., Klein-Szanto, A. J., Campbell, R. L., FitzGerald, G. A., and Funk, C. D. (2006) *Nat Med* **12**, 699-704
24. Picot, D., Loll, P. J., and Garavito, R. M. (1994) *Nature* **367**, 243-249
25. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) *Nat Struct Biol* **3**, 927-933
26. Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988) *J Biol Chem* **263**, 3550-3553
27. Rouzer, C. A., and Marnett, L. J. (2003) *Chem Rev* **103**, 2239-2304
28. Dietz, R., Nastainczyk, W., and Ruf, H. H. (1988) *Eur J Biochem* **171**, 321-328
29. Marnett, L. J., Wlodawer, P., and Samuelsson, B. (1975) *J Biol Chem* **250**, 8510-8517
30. Markey, C. M., Alward, A., Weller, P. E., and Marnett, L. J. (1987) *J Biol Chem* **262**, 6266-6279
31. DeFelippis, M. R., Murthy, C. P., Faraggi, M., and Klapper, M. H. (1989) *Biochemistry* **28**, 4847-4853
32. Koppenol, W. H. (1990) *FEBS Lett* **264**, 165-167
33. Tsai, A., and Kulmacz, R. J. (2000) *Prostaglandins Other Lipid Mediat* **62**, 231-254

34. Goodwin, D. C., Rowlinson, S. W., and Marnett, L. J. (2000) *Biochemistry* **39**, 5422-5432
35. Kulmacz, R. J., and Wang, L. H. (1995) *J Biol Chem* **270**, 24019-24023
36. Hemler, M. E., Graff, G., and Lands, W. E. (1978) *Biochem Biophys Res Commun* **85**, 1325-1331
37. Hemler, M. E., Cook, H. W., and Lands, W. E. (1979) *Arch Biochem Biophys* **193**, 340-345
38. Marshall, P. J., Kulmacz, R. J., and Lands, W. E. (1987) *J Biol Chem* **262**, 3510-3517
39. Kulmacz, R. J., and Lands, W. E. (1983) *Prostaglandins* **25**, 531-540
40. Wu, G., Wei, C., Kulmacz, R. J., Osawa, Y., and Tsai, A. L. (1999) *J Biol Chem* **274**, 9231-9237
41. Hemler, M. E., and Lands, W. E. (1980) *J Biol Chem* **255**, 6253-6261
42. Wu, G., Rogge, C. E., Wang, J. S., Kulmacz, R. J., Palmer, G., and Tsai, A. L. (2007) *Biochemistry* **46**, 534-542
43. Mbonye, U. R., Wada, M., Rieke, C. J., Tang, H. Y., Dewitt, D. L., and Smith, W. L. (2006) *J Biol Chem* **281**, 35770-35778
44. Mbonye, U. R., Yuan, C., Harris, C. E., Sidhu, R. S., Song, I., Arakawa, T., and Smith, W. L. (2008) *J Biol Chem* **283**, 8611-8623
45. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) *Annu Rev Biochem* **69**, 145-182
46. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) *Nature* **384**, 644-648
47. Wong, E., Bayly, C., Waterman, H. L., Riendeau, D., and Mancini, J. A. (1997) *J Biol Chem* **272**, 9280-9286
48. Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M., and Seibert, K. (1996) *J Biol Chem* **271**, 15810-15814
49. Guo, Q., Wang, L. H., Ruan, K. H., and Kulmacz, R. J. (1996) *J Biol Chem* **271**, 19134-19139
50. Roth, G. J., Stanford, N., and Majerus, P. W. (1975) *Proc Natl Acad Sci U S A* **72**, 3073-3076
51. Shimokawa, T., and Smith, W. L. (1992) *J Biol Chem* **267**, 12387-12392
52. Holtzman, M. J., Turk, J., and Shornick, L. P. (1992) *J Biol Chem* **267**, 21438-21445
53. Meade, E. A., Smith, W. L., and DeWitt, D. L. (1993) *J Biol Chem* **268**, 6610-6614
54. Rowlinson, S. W., Crews, B. C., Goodwin, D. C., Schneider, C., Gierse, J. K., and Marnett, L. J. (2000) *J Biol Chem* **275**, 6586-6591
55. Milton, A. S., and Wendlandt, S. (1970) *J Physiol* **207**, 76P-77P
56. Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., and Narumiya, S. (1998) *Nature* **395**, 281-284
57. Saper, C. B., and Breder, C. D. (1994) *N Engl J Med* **330**, 1880-1886
58. Ek, M., Engblom, D., Saha, S., Blomqvist, A., Jakobsson, P. J., and Ericsson-Dahlstrand, A. (2001) *Nature* **410**, 430-431

59. Breder, C. D., Dewitt, D., and Kraig, R. P. (1995) *J Comp Neurol* **355**, 296-315
60. Breder, C. D., and Saper, C. B. (1996) *Brain Res* **713**, 64-69
61. Li, S., Wang, Y., Matsumura, K., Ballou, L. R., Morham, S. G., and Blatteis, C. M. (1999) *Brain Res* **825**, 86-94
62. Willis, A. L. (1969) *Prostaglandins, peptides and amines* **31**
63. Woolf, C. J., and Salter, M. W. (2000) *Science* **288**, 1765-1769
64. Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V., and Woolf, C. J. (2001) *Nature* **410**, 471-475
65. Vikman, K. S., Hill, R. H., Backstrom, E., Robertson, B., and Kristensson, K. (2003) *Pain* **106**, 241-251
66. Fehrenbacher, J. C., Burkey, T. H., Nicol, G. D., and Vasko, M. R. (2005) *Pain* **113**, 113-122
67. Zhang, J., and Chen, C. (2008) *J Biol Chem* **283**, 22601-22611
68. Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K., Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y., Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., and Narumiya, S. (2000) *Science* **287**, 2013-2017
69. Bennett, A. (2000) *Thorax* **55 Suppl 2**, S54-56
70. Ojeda, S. R., and Campbell, W. B. (1982) *Endocrinology* **111**, 1031-1037
71. Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. (1999) *Proc Natl Acad Sci U S A* **96**, 10501-10506
72. Kennedy, C. R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C. D., Magnuson, M. A., Oates, J. A., Breyer, M. D., and Breyer, R. M. (1999) *Nat Med* **5**, 217-220
73. Karim, S. M. (1968) *Br Med J* **4**, 618-621
74. Karim, S. M., Trussell, R. R., Patel, R. C., and Hillier, K. (1968) *Br Med J* **4**, 621-623
75. Sugimoto, Y., Yamasaki, A., Segi, E., Tsuboi, K., Aze, Y., Nishimura, T., Oida, H., Yoshida, N., Tanaka, T., Katsuyama, M., Hasumoto, K., Murata, T., Hirata, M., Ushikubi, F., Negishi, M., Ichikawa, A., and Narumiya, S. (1997) *Science* **277**, 681-683
76. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M., and et al. (1995) *Nature* **378**, 406-409
77. Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) *Cell* **91**, 197-208
78. Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chulada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., Kluckman, K. D., Kim, H. S., and Smithies, O. (1995) *Cell* **83**, 483-492
79. Shio, H., and Ramwell, P. (1972) *Nat New Biol* **236**, 45-46
80. Shio, H., and Ramwell, P. W. (1972) *Science* **175**, 536-538
81. Shio, H., Ramwell, P. W., and Jessup, S. J. (1972) *Prostaglandins* **1**, 29-36
82. Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H., and Coffman, T. M. (1998) *J Clin Invest* **102**, 1994-2001

83. Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., and Narumiya, S. (1997) *Nature* **388**, 678-682
84. Patrono, C., and Rocca, B. (2009) *J Thromb Haemost* **7 Suppl 1**, 258-261
85. Collins, R., Peto, R., Baigent, C., and Sleight, P. (1997) *N Engl J Med* **336**, 847-860
86. Smith, G. C. (1998) *Pharmacol Rev* **50**, 35-58
87. Nguyen, M., Camenisch, T., Snouwaert, J. N., Hicks, E., Coffman, T. M., Anderson, P. A., Malouf, N. N., and Koller, B. H. (1997) *Nature* **390**, 78-81
88. Patrono, C., and Dunn, M. J. (1987) *Kidney Int* **32**, 1-12
89. Oates, J. A., FitzGerald, G. A., Branch, R. A., Jackson, E. K., Knapp, H. R., and Roberts, L. J., 2nd. (1988) *N Engl J Med* **319**, 761-767
90. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A., and Smithies, O. (1995) *Cell* **83**, 473-482
91. Pollard, M., and Luckert, P. H. (1981) *Science* **214**, 558-559
92. Kudo, T., Narisawa, T., and Abo, S. (1980) *Gann* **71**, 260-264
93. Waddell, W. R., and Loughry, R. W. (1983) *J Surg Oncol* **24**, 83-87
94. Waddell, W. R., Ganser, G. F., Cerise, E. J., and Loughry, R. W. (1989) *Am J Surg* **157**, 175-179
95. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. (1991) *N Engl J Med* **325**, 1593-1596
96. Peleg, II, Maibach, H. T., Brown, S. H., and Wilcox, C. M. (1994) *Arch Intern Med* **154**, 394-399
97. Smalley, W., Ray, W. A., Daugherty, J., and Griffin, M. R. (1999) *Arch Intern Med* **159**, 161-166
98. Kune, G. A., Kune, S., and Watson, L. F. (1988) *Cancer Res* **48**, 4399-4404
99. Smith, B. J., Wills, M. R., and Savory, J. (1983) *Ann Clin Lab Sci* **13**, 359-365
100. Oka, M., Inaba, A., Uchiyama, T., Hazama, S., Shimoda, K., Suzuki, M., and Suzuki, T. (1994) *Am J Surg* **167**, 264-267
101. Seyberth, H. W., Segre, G. V., Morgan, J. L., Sweetman, B. J., Potts, J. T., Jr., and Oates, J. A. (1975) *N Engl J Med* **293**, 1278-1283
102. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* **107**, 1183-1188
103. Hanahan, D., Christofori, G., Naik, P., and Arbeit, J. (1996) *Eur J Cancer* **32A**, 2386-2393
104. Hanahan, D., and Folkman, J. (1996) *Cell* **86**, 353-364
105. Baron, J. A., Sandler, R. S., Bresalier, R. S., Quan, H., Riddell, R., Lanas, A., Bolognese, J. A., Oxenius, B., Horgan, K., Loftus, S., and Morton, D. G. (2006) *Gastroenterology* **131**, 1674-1682
106. Bertagnolli, M. M., Eagle, C. J., Zauber, A. G., Redston, M., Solomon, S. D., Kim, K., Tang, J., Rosenstein, R. B., Wittes, J., Corle, D., Hess, T. M., Woloj, G. M., Boisserie, F., Anderson, W. F., Viner, J. L., Bagheri, D., Burn, J., Chung, D. C., Dewar, T., Foley, T. R., Hoffman, N., Macrae, F., Pruitt, R. E., Saltzman, J. R., Salzberg, B., Sylwestrowicz, T., Gordon, G. B., and Hawk, E. T. (2006) *N Engl J Med* **355**, 873-884

107. Arber, N., Eagle, C. J., Spicak, J., Racz, I., Dite, P., Hajer, J., Zavoral, M., Lechuga, M. J., Gerletti, P., Tang, J., Rosenstein, R. B., Macdonald, K., Bhadra, P., Fowler, R., Wittes, J., Zauber, A. G., Solomon, S. D., and Levin, B. (2006) *N Engl J Med* **355**, 885-895
108. Behne, D., Kyriakopoeulos, A., Weiss-Nowak, C., Kalckloesch, M., Westphal, C., and Gessner, H. (1996) *Biol Trace Elem Res* **55**, 99-110
109. Brigelius-Flohe, R. (1999) *Free Radic Biol Med* **27**, 951-965
110. Seiler, A., Schneider, M., Forster, H., Roth, S., Wirth, E. K., Culmsee, C., Plesnila, N., Kremmer, E., Radmark, O., Wurst, W., Bornkamm, G. W., Schweizer, U., and Conrad, M. (2008) *Cell Metab* **8**, 237-248
111. Mills, G. C. (1957) *J Biol Chem* **229**, 189-197
112. Clerch, L. B., and Massaro, D. (1993) *J Clin Invest* **91**, 499-508
113. Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M., and Funk, C. D. (1997) *J Biol Chem* **272**, 16644-16651
114. de Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998) *J Biol Chem* **273**, 22528-22536
115. Durak, I., Beduk, Y., Kavutcu, M., Ozturk, S., Canbolat, O., and Ulutepe, S. (1997) *Int Urol Nephrol* **29**, 5-11
116. Zachara, B. A., Szewczyk-Golec, K., Tyloch, J., Wolski, Z., Szylberg, T., Stepien, S., Kwiatkowski, S., Bloch-Boguslawska, E., and Wasowicz, W. (2005) *Neoplasma* **52**, 248-254
117. Gladyshev, V. N., Factor, V. M., Housseau, F., and Hatfield, D. L. (1998) *Biochem Biophys Res Commun* **251**, 488-493
118. Esworthy, R. S., Baker, M. A., and Chu, F. F. (1995) *Cancer Res* **55**, 957-962
119. Chu, F. F., and Esworthy, R. S. (1995) *Arch Biochem Biophys* **323**, 288-294
120. Florian, S., Wingler, K., Schmehl, K., Jacobasch, G., Kreuzer, O. J., Meyerhof, W., and Brigelius-Flohe, R. (2001) *Free Radic Res* **35**, 655-663
121. Walshe, J., Serewko-Auret, M. M., Teakle, N., Cameron, S., Minto, K., Smith, L., Burcham, P. C., Russell, T., Strutton, G., Griffin, A., Chu, F. F., Esworthy, S., Reeve, V., and Saunders, N. A. (2007) *Cancer Res* **67**, 4751-4758
122. Esworthy, R. S., Aranda, R., Martin, M. G., Doroshow, J. H., Binder, S. W., and Chu, F. F. (2001) *Am J Physiol Gastrointest Liver Physiol* **281**, G848-855
123. Banning, A., Deubel, S., Kluth, D., Zhou, Z., and Brigelius-Flohe, R. (2005) *Mol Cell Biol* **25**, 4914-4923
124. Kobayashi, M., and Yamamoto, M. (2005) *Antioxid Redox Signal* **7**, 385-394
125. Singh, A., Rangasamy, T., Thimmulappa, R. K., Lee, H., Osburn, W. O., Brigelius-Flohe, R., Kensler, T. W., Yamamoto, M., and Biswal, S. (2006) *Am J Respir Cell Mol Biol* **35**, 639-650
126. Banning, A., Kipp, A., Schmitmeier, S., Lowinger, M., Florian, S., Krehl, S., Thalmann, S., Thierbach, R., Steinberg, P., and Brigelius-Flohe, R. (2008) *Cancer Res* **68**, 9746-9753
127. Flohe, L. (1978) *Ciba Found Symp*, 95-122
128. Yoshimura, S., Watanabe, K., Suemizu, H., Onozawa, T., Mizoguchi, J., Tsuda, K., Hatta, H., and Moriuchi, T. (1991) *J Biochem* **109**, 918-923

129. Bjornstedt, M., Xue, J., Huang, W., Akesson, B., and Holmgren, A. (1994) *J Biol Chem* **269**, 29382-29384
130. Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., Motta, L., Richardson, A., and Prolla, T. A. (2003) *Free Radic Biol Med* **34**, 496-502
131. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) *Biochim Biophys Acta* **710**, 197-211
132. Roveri, A., Maiorino, M., Nisii, C., and Ursini, F. (1994) *Biochim Biophys Acta* **1208**, 211-221
133. Thomas, J. P., Geiger, P. G., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *Biochim Biophys Acta* **1045**, 252-260
134. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990) *J Biol Chem* **265**, 454-461
135. Arai, M., Imai, H., Koumura, T., Yoshida, M., Emoto, K., Umeda, M., Chiba, N., and Nakagawa, Y. (1999) *J Biol Chem* **274**, 4924-4933
136. Nomura, K., Imai, H., Koumura, T., Kobayashi, T., and Nakagawa, Y. (2000) *Biochem J* **351**, 183-193
137. Shi, Z. Z., Osei-Frimpong, J., Kala, G., Kala, S. V., Barrios, R. J., Habib, G. M., Lukin, D. J., Danney, C. M., Matzuk, M. M., and Lieberman, M. W. (2000) *Proc Natl Acad Sci U S A* **97**, 5101-5106
138. Jung, G., Yang, D. C., and Nakao, A. (1985) *Biochem Biophys Res Commun* **130**, 559-566
139. Schewe, T., Halangk, W., Hiebsch, C., and Rapoport, S. M. (1975) *FEBS Lett* **60**, 149-152
140. Chen, C. J., Huang, H. S., and Chang, W. C. (2003) *FASEB J* **17**, 1694-1696
141. Sakamoto, H., Imai, H., and Nakagawa, Y. (2000) *J Biol Chem* **275**, 40028-40035
142. Stafford, P. (1993)
143. Zias, J., Stark, H., Sellgman, J., Levy, R., Werker, E., Breuer, A., and Mechoulam, R. (1993) *Nature* **363**, 215
144. Piomelli, D. (2003) *Nat Rev Neurosci* **4**, 873-884
145. Gaoni, Y., and Mechoulam, R. (1964) *J Am Chem Soc* **86**, 1646-1647
146. Devane, W. A., Dysarz, F. A., 3rd, Johnson, M. R., Melvin, L. S., and Howlett, A. C. (1988) *Mol Pharmacol* **34**, 605-613
147. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) *Nature* **346**, 561-564
148. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) *Nature* **365**, 61-65
149. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258**, 1946-1949
150. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., and Waku, K. (1995) *Biochem Biophys Res Commun* **215**, 89-97
151. Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C., and Piomelli, D. (1994) *Nature* **372**, 686-691
152. Cadas, H., Gaillet, S., Beltramo, M., Venance, L., and Piomelli, D. (1996) *J Neurosci* **16**, 3934-3942
153. Hillard, C. J., and Jarrahian, A. (2000) *Chem Phys Lipids* **108**, 123-134

154. Beltramo, M., Stella, N., Calignano, A., Lin, S. Y., Makriyannis, A., and Piomelli, D. (1997) *Science* **277**, 1094-1097
155. Schmid, P. C., Zuzarte-Augustin, M. L., and Schmid, H. H. (1985) *J Biol Chem* **260**, 14145-14149
156. Kozak, K. R., and Marnett, L. J. (2002) *Prostaglandins Leukot Essent Fatty Acids* **66**, 211-220
157. Yu, M., Ives, D., and Ramesha, C. S. (1997) *J Biol Chem* **272**, 21181-21186
158. Wang, L. H., Matijevic-Aleksic, N., Hsu, P. Y., Ruan, K. H., Wu, K. K., and Kulmacz, R. J. (1996) *J Biol Chem* **271**, 19970-19975
159. Stella, N., Schweitzer, P., and Piomelli, D. (1997) *Nature* **388**, 773-778
160. Bisogno, T., Berrendero, F., Ambrosino, G., Cebeira, M., Ramos, J. A., Fernandez-Ruiz, J. J., and Di Marzo, V. (1999) *Biochem Biophys Res Commun* **256**, 377-380
161. Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., and Lichtman, A. H. (2001) *Proc Natl Acad Sci U S A* **98**, 9371-9376
162. Farooqui, A. A., Rammohan, K. W., and Horrocks, L. A. (1989) *Ann N Y Acad Sci* **559**, 25-36
163. Higgs, H. N., and Glomset, J. A. (1994) *Proc Natl Acad Sci U S A* **91**, 9574-9578
164. Pete, M. J., Ross, A. H., and Exton, J. H. (1994) *J Biol Chem* **269**, 19494-19500
165. Goparaju, S. K., Ueda, N., Taniguchi, K., and Yamamoto, S. (1999) *Biochem Pharmacol* **57**, 417-423
166. Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., Kathuria, S., and Piomelli, D. (2002) *Proc Natl Acad Sci U S A* **99**, 10819-10824
167. Hoover, H. S., Blankman, J. L., Niessen, S., and Cravatt, B. F. (2008) *Bioorg Med Chem Lett* **18**, 5838-5841
168. Marrs, W. R., Blankman, J. L., Horne, E. A., Thomazeau, A., Lin, Y. H., Coy, J., Bodor, A. L., Muccioli, G. G., Hu, S. S., Woodruff, G., Fung, S., Lafourcade, M., Alexander, J. P., Long, J. Z., Li, W., Xu, C., Moller, T., Mackie, K., Manzoni, O. J., Cravatt, B. F., and Stella, N. (2010) *Nat Neurosci* **13**, 951-957
169. Marnett, L. J. (2009) *Nat Chem Biol* **5**, 8-9
170. Long, J. Z., Li, W., Booker, L., Burston, J. J., Kinsey, S. G., Schlosburg, J. E., Pavon, F. J., Serrano, A. M., Selley, D. E., Parsons, L. H., Lichtman, A. H., and Cravatt, B. F. (2009) *Nat Chem Biol* **5**, 37-44
171. Nomura, D. K., Dix, M. M., and Cravatt, B. F. (2010) *Nat Rev Cancer*
172. Nomura, D. K., Long, J. Z., Niessen, S., Hoover, H. S., Ng, S. W., and Cravatt, B. F. (2010) *Cell* **140**, 49-61
173. Kozak, K. R., Gupta, R. A., Moody, J. S., Ji, C., Boeglin, W. E., DuBois, R. N., Brash, A. R., and Marnett, L. J. (2002) *J Biol Chem* **277**, 23278-23286
174. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J Biol Chem* **275**, 33744-33749
175. Kozak, K. R., Crews, B. C., Morrow, J. D., Wang, L. H., Ma, Y. H., Weinander, R., Jakobsson, P. J., and Marnett, L. J. (2002) *J Biol Chem* **277**, 44877-44885
176. Rouzer, C. A., and Marnett, L. J. (2005) *J Biol Chem* **280**, 26690-26700
177. Kozak, K. R., Crews, B. C., Ray, J. L., Tai, H. H., Morrow, J. D., and Marnett, L. J. (2001) *J Biol Chem* **276**, 36993-36998

178. Nirodi, C. S., Crews, B. C., Kozak, K. R., Morrow, J. D., and Marnett, L. J. (2004) *Proc Natl Acad Sci U S A* **101**, 1840-1845
179. Hu, S. S., Bradshaw, H. B., Chen, J. S., Tan, B., and Walker, J. M. (2008) *Br J Pharmacol*
180. Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R., and Rice, K. C. (1990) *Proc Natl Acad Sci U S A* **87**, 1932-1936
181. Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J. F., Beslot, F., Bohme, G. A., Imperato, A., Pedrazzini, T., Roques, B. P., Vassart, G., Fratta, W., and Parmentier, M. (1999) *Science* **283**, 401-404
182. Di Marzo, V., Melck, D., De Petrocellis, L., and Bisogno, T. (2000) *Prostaglandins Other Lipid Mediat* **61**, 43-61
183. Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N., and Ishima, Y. (1999) *J Biol Chem* **274**, 2794-2801
184. Sugiura, T., Kondo, S., Kishimoto, S., Miyashita, T., Nakane, S., Kodaka, T., Suhara, Y., Takayama, H., and Waku, K. (2000) *J Biol Chem* **275**, 605-612
185. Burkey, T. H., Quock, R. M., Consroe, P., Ehlert, F. J., Hosohata, Y., Roeske, W. R., and Yamamura, H. I. (1997) *Eur J Pharmacol* **336**, 295-298
186. Kearn, C. S., Greenberg, M. J., DiCamelli, R., Kurzawa, K., and Hillard, C. J. (1999) *J Neurochem* **72**, 2379-2387
187. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., and Hogestatt, E. D. (1999) *Nature* **400**, 452-457
188. Sagar, D., Gaw, A. G., Okine, B., Woodhams, S., Wong, A., Kendall, D., and Chapman, V. (2009) *Molecular Pain* **5**, 59
189. Iversen, L., and Chapman, V. (2002) *Curr Opin Pharmacol* **2**, 50-55
190. Jennings, E. A., Vaughan, C. W., and Christie, M. J. (2001) *J Physiol* **534**, 805-812
191. Meng, I. D., Manning, B. H., Martin, W. J., and Fields, H. L. (1998) *Nature* **395**, 381-383
192. Lichtman, A. H., Cook, S. A., and Martin, B. R. (1996) *J Pharmacol Exp Ther* **276**, 585-593
193. Telleria-Diaz, A., Schmidt, M., Kreusch, S., Neubert, A. K., Schache, F., Vazquez, E., Vanegas, H., Schaible, H. G., and Ebersberger, A. (2010) *Pain* **148**, 26-35
194. Caille, S., Alvarez-Jaimes, L., Polis, I., Stouffer, D. G., and Parsons, L. H. (2007) *J Neurosci* **27**, 3695-3702
195. Alvarez-Jaimes, L., Stouffer, D. G., and Parsons, L. H. (2009) *J Neurochem* **111**, 37-48
196. Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T., and Kunos, G. (2001) *Nature* **410**, 822-825
197. Pi-Sunyer, F. X., Aronne, L. J., Heshmati, H. M., Devin, J., and Rosenstock, J. (2006) *Jama* **295**, 761-775
198. Scheen, A. J., Finer, N., Hollander, P., Jensen, M. D., and Van Gaal, L. F. (2006) *Lancet* **368**, 1660-1672

199. Topol, E. J., Bousser, M. G., Fox, K. A., Creager, M. A., Despres, J. P., Easton, J. D., Hamm, C. W., Montalescot, G., Steg, P. G., Pearson, T. A., Cohen, E., Gaudin, C., Job, B., Murphy, J. H., and Bhatt, D. L. (2010) *Lancet* **376**, 517-523
200. Boekholdt, S. M., and Peters, R. J. (2010) *Lancet* **376**, 489-490
201. Romero, J., Lastres-Becker, I., de Miguel, R., Berrendero, F., Ramos, J. A., and Fernandez-Ruiz, J. (2002) *Pharmacol Ther* **95**, 137-152
202. Rouzer, C. A., and Marnett, L. J. (2008) *J Biol Chem* **283**, 8065-8069
203. Rouzer, C. A., Tranguch, S., Wang, H., Zhang, H., Dey, S. K., and Marnett, L. J. (2006) *Biochem J* **399**, 91-99
204. Kulmacz, R. J., Miller, J. F., Jr., Pendleton, R. B., and Lands, W. E. (1990) *Methods Enzymol* **186**, 431-438
205. Jiang, Z. Y., Woollard, A. C., and Wolff, S. P. (1991) *Lipids* **26**, 853-856
206. Rowlinson, S. W., Crews, B. C., Lanzo, C. A., and Marnett, L. J. (1999) *J Biol Chem* **274**, 23305-23310
207. Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. R., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., and Eling, T. E. (1984) *Mol Pharmacol* **26**, 328-335
208. Funk, M. O., Isacc, R., and Porter, N. A. (1976) *Lipids* **11**, 113-117
209. Landino, L. M., Crews, B. C., Timmons, M. D., Morrow, J. D., and Marnett, L. J. (1996) *Proc Natl Acad Sci U S A* **93**, 15069-15074
210. Tsai, A., Wei, C., Baek, H. K., Kulmacz, R. J., and Van Wart, H. E. (1997) *J Biol Chem* **272**, 8885-8894
211. Strieder, S., Schaible, K., Scherer, H. J., Dietz, R., and Ruf, H. H. (1992) *J Biol Chem* **267**, 13870-13878
212. Wada, M., DeLong, C. J., Hong, Y. H., Rieke, C. J., Song, I., Sidhu, R. S., Yuan, C., Warnock, M., Schmaier, A. H., Yokoyama, C., Smyth, E. M., Wilson, S. J., FitzGerald, G. A., Garavito, R. M., Sui de, X., Regan, J. W., and Smith, W. L. (2007) *J Biol Chem* **282**, 22254-22266
213. Malkowski, M. G., Thuresson, E. D., Lakkides, K. M., Rieke, C. J., Micielli, R., Smith, W. L., and Garavito, R. M. (2001) *J Biol Chem* **276**, 37547-37555
214. Kozak, K. R., Prusakiewicz, J. J., Rowlinson, S. W., Schneider, C., and Marnett, L. J. (2001) *J Biol Chem* **276**, 30072-30077
215. Richie-Jannetta, R., Nirodi, C. S., Crews, B. C., Woodward, D. F., Wang, J. W., Duff, P. T., and Marnett, L. J. (2010) *Prostaglandins Other Lipid Mediat* **92**, 19-24
216. Brigelius-Flohe, R., and Kipp, A. (2009) *Biochim Biophys Acta* **1790**, 1555-1568
217. Kingsley, P. J., Rouzer, C. A., Saleh, S., and Marnett, L. J. (2005) *Anal Biochem* **343**, 203-211
218. Milne, G. L., Sanchez, S. C., Musiek, E. S., and Morrow, J. D. (2007) *Nat Protoc* **2**, 221-226
219. Kulmacz, R. J., and Lands, W. E. (1985) *J Biol Chem* **260**, 12572-12578
220. Prusakiewicz, J. J., Duggan, K. C., Rouzer, C. A., and Marnett, L. J. (2009) *Biochemistry* **48**, 7353-7355
221. Vasquez, E., Bar, K. J., Ebersberger, A., Klein, B., Vanegas, H., and Schaible, H. G. (2001) *J Neurosci* **21**, 9001-9008

222. Chubb, A. J., Fitzgerald, D. J., Nolan, K. B., and Moman, E. (2006) *Biochemistry* **45**, 811-820
223. Srinivasan, N. S., and Lee Donald, G. (1979) *Journal of Organic Chemistry* **44**, 1574
224. Miyashita, A., Suzuki, Y., Iwamoto, K.-i., and Higashino, T. (1994) *Chem. Pharm. Bull.* **42**, 2633-2635
225. Khurana, J. M., and Kandpal, B. M. (2003) *Tetrahedron Letters* **44**, 4909-4912
226. Bredereck, H. G., R.; Schuh, H. G. V.; Theilig, G. (1959) *Angewandte Chemie* **71**, 753-774
227. Wolkenberg Scott, E., Wisnoski David, D., Leister William, H., Wang, Y., Zhao, Z., and Lindsley Craig, W. (2004) *Org Lett* **6**, 1453-1456
228. Duggan, K. C., Musee, J., and Marnett, L. J. (2010) *Methods Mol Biol* **644**, 55-65
229. Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K., and Marnett, L. J. (1998) *Science* **280**, 1268-1270
230. Duggan, K. C., Musee, J and L. J. Marnett. (2010) *Methods Molecular Biology* **644**, 55-67
231. Landino, L. M., Crews, B. C., Gierse, J. K., Hauser, S. D., and Marnett, L. J. (1997) *J Biol Chem* **272**, 21565-21574
232. Staniaszek, L. E., Norris, L. M., Kendall, D. A., Barrett, D. A., and Chapman, V. (2010) *Br J Pharmacol* **160**, 669-676
233. Calignano, A., La Rana, G., Giuffrida, A., and Piomelli, D. (1998) *Nature* **394**, 277-281
234. Strangman, N. M., Patrick, S. L., Hohmann, A. G., Tsou, K., and Walker, J. M. (1998) *Brain Res* **813**, 323-328
235. Amaya, F., Samad, T. A., Barrett, L., Broom, D. C., and Woolf, C. J. (2009) *Pain* **142**, 59-67
236. Tegeder, I., Niederberger, E., Vetter, G., Brautigam, L., and Geisslinger, G. (2001) *J Neurochem* **79**, 777-786
237. Yaksh, T. L., Dirig, D. M., Conway, C. M., Svensson, C., Luo, Z. D., and Isakson, P. C. (2001) *J Neurosci* **21**, 5847-5853
238. Consiglio, A. R., and Lucion, A. B. (2000) *Brain Res Brain Res Protoc* **5**, 109-114
239. Rouzer, C. A., Kingsley, P. J., Wang, H., Zhang, H., Morrow, J. D., Dey, S. K., and Marnett, L. J. (2004) *J Biol Chem* **279**, 34256-34268
240. Buczynski, M. W., Svensson, C. I., Dumlao, D. S., Fitzsimmons, B. L., Shim, J. H., Scherbart, T. J., Jacobsen, F. E., Hua, X. Y., Yaksh, T. L., and Dennis, E. A. (2010) *J Neurochem* **114**, 981-993

CHAPTER VI

SUMMARY

PGHSs catalyze the oxygenation of fatty acyl substrates (FAH) such as arachidonic acid (AA). This is the committed step in the generation of prostaglandin H_2 (PGH_2). PGH_2 is the substrate for five downstream isomerases that lead to the generation of prostaglandins. Prostaglandins mediate a variety of physiological effects, such as pain, inflammation, fever, vascular homeostasis, and parturition, by their actions at several prostaglandin-specific G-protein coupled receptors (GPCRs). The endogenous ligands for the cannabinoid receptors, arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG) are substrates for the second isoform of PGHS, PGHS-2. Their oxygenation leads to the generation of AEA and 2-AG derived prostaglandins (PG-EAs and PG-Gs). PG-Gs have been shown to have unique actions at yet to be identified GPCRs including, mobilizing Ca^{2+} at the picomolar level in cell culture, and causing a concentration dependent hyperalgesia (exaggerated pain) and allodynia (pain in response to non-pain evoking stimuli). Despite these findings, PG-Gs have been detected at low levels *in vivo*, and *ex vivo* are only detectable at levels 1000-fold lower than their free acid counterparts, PGs. The *in vitro* k_{cat}/K_m values for 2-AG and AA with PGHS-2 are comparable, and this disparity in product formation *ex vivo* has led us to hypothesize that there are factors not apparent in the k_{cat}/K_m of 2-AG with PGHS-2, that affect its oxygenation relative to AA. The work presented herein, represents efforts to determine the *in vitro* biochemical determinants of 2-AG oxygenation by PGHS-2, and as an extension of this work, I demonstrate that these determinants are similar *ex vivo*, and

furthermore, that in the neuronal milleu, 2-AG is robustly oxygenated by PGHS-2, leading to the formation of PG-Gs. In sum, this work provides further credence to the physiological relevance of PGHS-2-mediated oxygenation of 2-AG.

The activation of oxygenase activity of PGHS requires turnover at the peroxidase (POX) active site of PGHS, leading to the activation of the catalytic residue, Tyr385, in the oxygenase site. This converts it to a tyrosyl radical, which initiates oxgenation of FAH. Since the turnover of 2-AG in murine macrophages was so low, I hypothesized that 2-AG derived peroxides such as 15-HpETE-G and PGG_2-G are poorer substrates at the POX active site, leading to poorer activation of 2-AG oxygenation relative to AA. To test this hypothesis, I generated a surrogate for the chemically unstable 2-AG-derived PGG_2-G, 15-HpETE-G, and its free acid AA-derived counterpart 15-HpETE. 15-HpETE and 15-HpETE-G did not differ significantly in their disposition as POX substrates and are equivalent substrates and activators of PGHS's oxygenase activity. Furthermore, qualitative examination of the levels of reduction of *in situ* generated PGG_2 and PGG_2-G demonstrates that both AA derived PGG_2 and 2-AG derived PGG_2-G are reduced to a similar extent by the POX activity of PGHS. This eliminated the possibility that the glyceryl ester on PGG_2-G affects the rate of its reduction by the POX active site of PGHS. While the K_ms for 15-HpETE and 15-HpETE-G for the POX active site of PGHS were comparable, the concentration of peroxide required to activate the COX active site of PGHS-2 for oxygenation of 2-AG had not been determined. This value, K_p, is a concentration that can be determined from co-incubations of PGHS and the substrate in question, in the face of increasing levels of glutathione peroxidase (GPx). Testing of 2-AG and AA led to a dimunition of oxygenation of 2-AG that was significantly greater

than that of AA, at any of the concentrations of GPx tested. Furthermore, calculation of the K_p from linear regression analysis of the traces of the decay of oxygenation with AA and 2-AG demonstrated that the K_p of 2-AG was ~ 3-fold higher than AA's. This indicated that the activation of oxygenation of 2-AG by PGHS-2 requires at least 3-fold higher concentrations of peroxide to activate the oxygenation of 2-AG by PGHS-2. Further confirmation of the exquisite sensitivity of 2-AG oxygenation to peroxide-mediated activation was demonstrated by the severely impaired turnover of 2-AG by a POX active site mutant of PGHS-2, H388Y PGHS-2, which retains less than 1/300 of wt PGHS-2's POX activity. H388Y PGHS-2 can still oxygenate AA to the same extent as wt PGHS-2, however its reaction trajectory is characterized by a lag phase, during which PGG_2 levels reach levels sufficient to achieve activation of COX activitity in latent H388Y PGHS-2. Examination of the levels of PGG_2 and PGG_2-G over the course of oxygenation by H388Y PGHS-2 reveals that PGG_2-G levels are greatly diminished over the entire time course, and never approach the levels of PGG_2 determined under these conditions. Wt PGHS-2 levels of 2-AG oxygenation were restored by exogenous 15-HpETE or 15-HpETE-G and in a concentration dependent manner. 2-AG oxygenation by PGHS-2 bears striking resemblance to the oxygenation of an ω-3 fatty acid, eicosapentaenoic acid (EPA). EPA is also substrate for PGHS-2 and its oxygenation by H388Y PGHS-2 is also severely impaired, and can be restored to wt PGHS-2 levels via co-incubation with increasing concentrations of peroxide. The crystal structure of EPA with PGHS reveals that the *13-pro-(S)*- hydrogen on the FAH backbone of EPA is shifted away from COX catalytic residue, Tyr385. Determination of the K_p of EPA with PGHS-2 reveals that its K_p is 20-fold greater than AA's with PGHS-2. The binding mode of 2-

AG in the COX active site of PGHS has been proposed by our lab, based on extensive mutagenesis studies, however, these findings have not been confirmed by crystallography. It is therefore possible that the *13-pro-(S)-* hydrogen on the FAH backbone of 2-AG, like EPA, is shifted away from Tyr385, leading to an increased K_p.

These results indicated that the initiation of PGHS-2 mediated oxygenation of 2-AG is sensitive to peroxide tone. I hypothesized that this sensitivity partly explains the poor recovery of PG-Gs from the *ex vivo* setting. To test my hypothesis, I used shRNA to stably deplete the levels of two of the major components of the endogenous peroxide detoxification mechanisms, glutathione peroxidase 1 (GPx1) and glutathione peroxidase 4 (GPx4) in murine fibroblasts. Murine fibroblasts can oxygenate both AA and 2-AG leading to the recovery of PGs and PG-Gs. Depletion of GPx4 in these fibroblasts led to a 3-fold increase in lipid peroxidation and a significant increase in the levels of isoprostanes in these cells relative to control. A concomitant increase in PGE_2-G (2-fold) and $PGF_{2\alpha}$-G (4-fold) production following inflammatory stimulus was also observed in cells with depleted GPx4, relative to control. GPx1 depleted cells did not differ from control. These results indicated peroxide tone is important for PGHS-2 mediated 2-AG oxygenation, and indicates that under conditions of increased oxidant stress, 2-AG oxygenation in augmented.

These results indicated that the POX active site of PGHS could be a veritable pharmacological target for the modulation of 2-AG oxygenation. To test this hypothesis, I designed a ligand based one the binding parameters for PGG_2 in the POX active site of PGHS. Testing of our lead compound 2-methyl-4,5-dihexylimidazole demonstrated that it was a modest POX inhibitor, and the first of its kind. It was exciting to demonstrate

that even with modest inhibition of the POX active site, I could selectively and potently inhibit the PGHS-2-mediated oxygenation of 2-AG over that of AA. Furthermore, testing in a murine macrophage cell line treated with inflammatory agonist revealed that 2-methyl-4,5-dihexylimidazole was an extremely potent and selective inhibitor of 2-AG oxygenation. These efforts have generated an extremely useful tool for the dissection of the PGHS-2/2-AG axis, and could lead to a delineation of the role of PGHS-2 in endocannbinoid metabolism.

Finally, and very importantly, I examined the formation of PG-Gs and PG-EAs in the neuronal milieu in the context of inflammation. For the first time, we demonstrate that primary murine neuronal cultures oxygenate endogenously produced AEA and 2-AG to form PG-EAs and PG-Gs. This finding finally thrusts PGHS-2 into the fore in terms of its role in endocannabinoid metabolism in the neuronal context. Even more powerful, is the demonstration that the primary neuronal culture is an extremely useful platform for the testing of agents that can selectively inhibit the oxygenation of 2-AG over that of AA. This could potentially lead to compounds that could be useful in dissection of the PGHS-2/2-AG axis, especially in determining the role of PGHS-2 mediated oxygention of 2-AG and AEA in mediating pain.

This work opens up new avenues of inquiry by providing validation that the PGHS-2 mediated oxygenation of endocannabinoids AEA and 2-AG is relevant to the cell, and has created tools that can be used to further examine the relevance of this metabolism in both physiology and pathology.

DETERMINANTS OF CYCLOOXYGENASE-2-MEDIATED OXIDATIVE METABOLISM OF THE ENDOCANNABINOID, 2-ARACHIDONOYL GLYCEROL, *IN VITRO* AND *EX VIVO*

Joel Musee

Dissertation under the direction of Professor Lawrence J. Marnett

PGHSs catalyze the oxygenation of fatty acyl substrates (FAH) such as arachidonic acid (AA). This is the committed step in the generation of prostaglandin H_2 (PGH_2). PGH_2 is the substrate for five downstream isomerases that lead to the generation of prostaglandins. Prostaglandins mediate a variety of physiological effects, such as pain, inflammation, fever, vascular homeostasis, and parturition, by their actions at several prostaglandin-specific G-protein coupled receptors (GPCRs). The endogenous ligands for the cannabinoid receptors, arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG) are substrates for the second isoform of PGHS, PGHS-2. Their oxygenation leads to the generation of AEA and 2-AG derived prostaglandins (PG-EAs and PG-Gs). PG-Gs have been shown to have unique actions at yet to be identified GPCRs including, mobilizing Ca^{2+} at the picomolar level in cell culture, and causing a concentration dependent hyperalgesia (exaggerated pain) and allodynia (pain in response to non-pain evoking stimuli). To determine the biochemical determinants of the oxygenation of 2-AG by PGHS-2, I compared the abilities of AA derived hydroperoxides to 2-AG derived peroxides as substrates and activators of the oxygenase function of PGHS. I demonstrated that PGG_2 and PGG_2-G were both equivalent substrates for PGHS-2. Interestingly, the oxygenation of 2-AG demonstrated and increased need for the concentrations of peroxide required to activate its oxygenation. In the presence of

increased peroxide scavenging by glutathione peroxide (GPx), the oxygenation of 2-AG was almost abrogated compared to relatively unaffected AA oxygenation. Specifically, I demonstrated that the depletion of the membrane associated GPx (GPx4), leads to increased oxidant stress and peroxide tone, and significantly increased oxygenation of 2-AG in murine derived cells.

These results led us to conclude that the oxygenation of 2-AG is exquisitely sensitive to the concentration of oxygenase activating peroxide. Chemical ligands specifically designed to target the peroxidase active site of PGHS have to date not been reported. I designed a chemical mimic of PGHS-2 substrate PGG2 and developed a chemical ligand that inhibited the turnover of peroxide at the peroxidase active site of PGHS. This lead compound forms a new class of molecule that can specifically and potently inhibit the oxygenation of 2-AG by PGHS-2, while having no impact on AA oxygenation. It could form a useful tool for the dissection of the physiological role of PGHS-2 mediated 2-AG oxygenation.