

Amino Acid Determinants in Cyclooxygenase-2 Oxygenation of the Endocannabinoid 2-Arachidonylglycerol*

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The endocannabinoid, 2-arachidonylglycerol (2-AG), is an endogenous ligand for the central (CB1) and peripheral (CB2) cannabinoid receptors and has been shown to be efficiently and selectively oxygenated by cyclooxygenase (COX)-2. We have investigated 2-AG/COX-2 interactions through site-directed mutagenesis. An evaluation of more than 20 site-directed mutants of murine COX-2 has allowed for the development of a model of 2-AG binding within the COX-2 active site. Most strikingly, these studies have identified Arg-513 as a critical determinant in the ability of COX-2 to efficiently generate prostaglandin H₂ glycerol ester, explaining, in part, the observed isoform selectivity for this substrate. Mutational analysis of Leu-531, an amino acid located directly across from Arg-513 in the COX-2 active site, suggests that 2-AG is shifted in the active site away from this hydrophobic residue and toward Arg-513 relative to arachidonic acid. Despite this difference, aspirin-treated COX-2 oxygenates 2-AG to afford 15-hydroxyicosatetraenoic acid glycerol ester in a reaction analogous to the C-15 oxygenation of arachidonic acid observed with acetylated COX-2. Finally, the differences in substrate binding do not alter the stereospecificity of the cyclooxygenase reaction; 2-AG-derived and arachidonic acid-derived products share identical stereochemistry.

Cyclooxygenase (COX; prostaglandin endoperoxide synthase, EC 1.14.99.1)¹ bis-dioxygenates arachidonic acid providing prostaglandin (PG) H₂, the precursor to the prostaglandins and thromboxanes (1). Two cyclooxygenase isoforms exist and differ in their regulation and tissue distribution (2). COX-1 is a constitutive enzyme expressed in most tissues, whereas COX-2 is inducible and highly regulated by a range of cytokines, growth factors, and tumor promoters (3–7). COX-1 appears to play a role in generating PGs, which serve cellular “housekeep-

ing” functions and account for PG and thromboxane synthesis in gastric mucosa, kidney, and platelets (8). In contrast, COX-2 activity is primarily responsible for PG biosynthesis in the central nervous system and inflammatory cells (9–11). It is now well established that the two COX isoforms play very different roles in an array of physiological and pathological processes *in vivo*.

The possibility that the different functions of COX enzymes may be mediated by isoform-specific products has recently been posited (12, 13). For example, the endocannabinoid anandamide has been shown to be selectively oxygenated by COX-2 generating PG ethanolamides (12). COX-2 also oxygenates the endocannabinoid 2-arachidonylglycerol (2-AG) providing glycerol esters of both PGs and, to a lesser extent, hydroxyicosatetraenoic acids (HETEs). 2-AG oxygenation by COX-2 has been demonstrated to occur in cultured macrophages. Both human and murine COX-2 metabolize 2-AG as efficiently as arachidonic acid (13).

The current study was initiated to define the binding of 2-AG in the COX-2 active site with a focus on protein residues that account for the observed isoform selectivity of this substrate. In addition, we determined the stereochemistry of oxygenation of 2-AG to PG glycerol esters and HETE glycerol esters (HETE-G) because product stereochemistry provides additional insight into the nature of substrate binding within the enzyme active site. These studies allow the development of a model for 2-AG binding in the COX-2 active site that helps explain the isoform selectivity of 2-AG oxygenation.

EXPERIMENTAL PROCEDURES

Materials—2-AG, PGF_{2α}, PGF_{2β}, 8-iso-PGF_{2α}, 11β-PGF_{2α}, and 15(R)-PGF_{2α} were purchased from Cayman Chemical (Ann Arbor, MI). Arachidonic acid was obtained from NuChek Prep (Elysian, MN). Hematin was purchased from Sigma. All molecular biology enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were purchased from Operon Technologies (Alameda, CA). Ram seminal vesicles were from Oxford Biomedical Research (Oxford, MI). All other chemicals were purchased from Aldrich.

Enzymology—Site-directed mutagenesis of murine COX-2 was performed as described (14). COX-2 enzymes were expressed in *Sf-9* insect cells by using the pVL1393 transfer vector (PharMingen, San Diego, CA) and purified by ion-exchange chromatography and gel filtration as described previously (14). Apoenzymes were reconstituted with hematin prior to activity assays. COX activity was quantified as described (15). Initial reaction velocity data were obtained from the linear portion of oxygen uptake curves. The PG-G to HETE-G product ratio for enzyme oxygenation of 2-AG was obtained by incubating 10 μg of protein with 10 μg of endocannabinoid in 150 μl of Tris-HCl buffer (100 mM, pH 8) containing 500 μM phenol for 10 min at 37 °C. Oxygenated products were extracted with EtOAc. The organic solvent was evaporated under a stream of argon, and the resultant residue was redissolved in 1:1 H₂O:MeCN and analyzed by LC/MS (see below). The PG-G to HETE-G ratio was calculated by dividing the area of peaks corresponding to

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¹ The abbreviations used are: COX, cyclooxygenase; mCOX, murine COX; AG, arachidonylglycerol; PG, prostaglandin; HETE, hydroxyicosatetraenoic acid; -G, glyceryl ester; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; PCR, polymerase chain reaction.

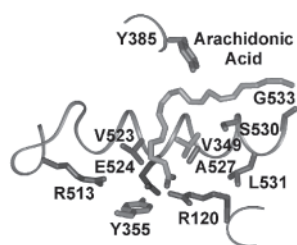


FIG. 1. Model of arachidonic acid bound in the COX-2 active site. A model of the predicted interactions between arachidonic acid and the active site residues of mCOX-2 is shown.

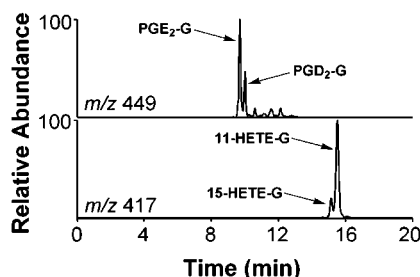


FIG. 2. Oxygenated products formed by COX-2 action on 2-AG. Selected ion mass chromatograms of oxygenated 2-AG products generated by wild-type mCOX-2 (10 μ g) incubated with an equal mass of 2-AG. The products were eluted with a 15-min gradient of 20–100% acetonitrile in H_2O (0.001% sodium acetate) and detected by monitoring m/z 449 (top panel) and 417 (bottom panel).

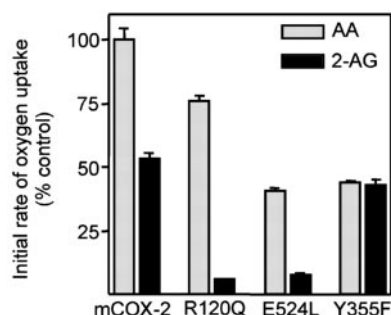


FIG. 3. Oxygenation of arachidonic acid and 2-AG by constriction site mutants of COX-2. Initial O_2 uptake rates by wild-type and mutant murine COX-2 enzymes (200 nM) with arachidonic acid (100 μ M) and 2-AG (200 μ M) are shown and are normalized to the initial rate of O_2 uptake for arachidonic acid with wild-type enzyme (mean $\pm$ S.E., $n = 3$).

PGE_2 -G and PGD_2 -G (m/z 449, $M + Na^+$) by the area of peaks corresponding to 11- and 15-HETE-G (m/z 417, $M + Na^+$) followed by normalization to the ratio of products generated by wild-type enzyme.

Chemistry—Acetylation of wild-type murine COX-2 was accomplished by treating the enzyme with 2 mM aspirin for 30 min at 37 °C. PGF_2 -G was prepared by incubating 300 μ g of 2-AG with 450 μ g of heme-reconstituted mCOX-2 in 3.0 ml of Tris-HCl buffer (100 mM, pH 8) containing 500 μ M phenol for 2 min at 37 °C followed immediately by treatment with 3.0 ml of ice-cold EtOAc containing triphenylphosphine (5 mg/ml). The biphasic mixture was centrifuged, and the organic solvent was removed and dried under a stream of argon. The resultant residue was dissolved in 500 μ l of 1:1 H_2O :MeCN, filtered (0.22 μ m), and applied to a C18 solid phase extraction column (Varian, Walnut Creek, CA). PGF_2 -G was eluted with 1:1 H_2O :MeCN. The eluant was lyophilized, and glycerol esters were saponified by treatment with 1 N NaOH for 1 h at 37 °C. The reaction mixture was acidified with 1 N HCl and extracted with EtOAc. Thin layer chromatographic analysis was conducted with a solvent of $CHCl_3$:MeOH:HOAc: H_2O (90:10:1:1), and the products were visualized by iodine staining. HETE-Gs were prepared by incubating mCOX-2 (500 nM) with 2-AG (50 μ M) for 10 min at 37 °C in Tris-HCl buffer (100 mM, pH 8) containing 500 μ M phenol followed by the addition of ice-cold EtOAc. The biphasic mixture was centrifuged, and the organic solvent was removed and dried under a stream of argon. The resultant residue was saponified by treatment

TABLE I
Normalized ratio of PG-G to HETE-G products of 2-AG oxygenation by mCOX-2 enzymes

The PG-G to HETE-G product ratio was obtained by analyzing products by LC/MS and was calculated by dividing the area of peaks corresponding to PGE_2 -G and PGD_2 -G (m/z 449, $M + Na^+$) by the area of peaks corresponding to 11- and 15-HETE-G (m/z 417, $M + Na^+$) followed by normalization to the ratio of products generated by wild-type enzyme. The values represent the means $\pm$ S.E., and the indicated statistical differences are for comparisons with wild-type enzyme.

Enzyme	Ratio
Wild type	1.0 $\pm$ 0.1
R120A	1.1 $\pm$ 0.1
R120Q	1.1 $\pm$ 0.2
Y348F	0.90 $\pm$ 0.07
V349A	<0.1 ^a
V349L	0.15 $\pm$ 0.02 ^a
V349I	1.2 $\pm$ 0.1
Y355A	1.2 $\pm$ 0.1
Y355F	1.5 $\pm$ 0.1 ^b
W387F	<0.1 ^a
L503F ^c	0.84 $\pm$ 0.08
Y504F	1.4 $\pm$ 0.1 ^b
R513H	1.1 $\pm$ 0.2
R513H/V523I	0.88 $\pm$ 0.04
V523I	0.76 $\pm$ 0.02
V523I/R513H/V434I	0.57 $\pm$ 0.08 ^b
E524L	0.39 $\pm$ 0.07 ^b
A527V	0.6 $\pm$ 0.1
S530A	0.52 $\pm$ 0.02 ^b
S530M	<0.1 ^a
L531A	0.21 $\pm$ 0.02 ^a
L531V	0.33 $\pm$ 0.03 ^a
L531I	0.69 $\pm$ 0.08

^a $p < 0.01$ (Student's t test).

^b $p < 0.05$ (Student's t test).

^c COX-2 $\rightarrow$ COX-1 mutations are in bold.

with 1 N NaOH for 1 h at 37 °C. The reaction mixture was acidified with 1 N HCl, and HETEs were extracted with EtOAc. HETE stereochemistry was established as previously described (16).

Mass Spectrometry—LC/MS was conducted as previously described (13). All displayed chromatograms are representative of at least three separate experiments.

Molecular Biology—Polymerase chain reaction (PCR) primers (5'-GTCGCAAGCTTGGCTGCAGAGTTAGAAAGCGCTCTACGGAGACATAGATGC-3' and 5'-GCTGAGAGCTCGAGGTGTGCATCTTGAACATGATGAGGTAAAGGGAC-3') were designed according to *Ovis aries* COX-2 cDNA (GenBankTM accession number U68486) and used to amplify a 300-nucleotide fragment from *O. aries* genomic DNA that included the codon for residue 513. Genomic DNA was isolated from ram seminal vesicle tissue with a DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. The primers encoded for the addition of *Hind*III and *Sac*I restriction sites (underlined above). These enzymes were used to subclone the PCR product into the pBS+ vector (Stratagene, La Jolla, CA) for amplification and subsequent sequencing.

Energy Minimization and Modeling—2-AG was built into the protein coordinates of uninhibited mCOX-2 (Protein Data Bank code 5COX). All amino acid positions were fixed except for the side chains of Arg-120, Arg-513, Glu-524, and Tyr-355. The glycerol ester moiety of 2-AG was restrained within 3.6 Å from the hydrogen bond donor/acceptor groups of Arg-513, Arg-120, and Glu-524. The Tyr-385 hydroxyl group was restrained within 3.6 Å from C-13 to ensure a productive conformation for oxygenation. The complexes were energy minimized for 1000 iterations using a conjugate gradient in the consistent valence forcefield. Molecular dynamic simulations were then run on the energy minimized assemblies for 1000 iterations at 300 K. All simulations were performed using the Discover module of Insight II 2000 with a R12000 Silicon Graphics Octane work station.

RESULTS AND DISCUSSION

Site-directed Mutagenesis and Cyclooxygenase Activity Assays—Wild-type and mutant COX-2 enzymes were expressed in insect cells and then purified by ion exchange and gel filtration. The mutations were made in enzyme residues throughout the cyclooxygenase active site with particular attention paid to

A.

COX-1	510	535
Homo sapiens	e k c h p n s i f g e s m i e i g a p f s l k g l l	
Ovis aries	e k c h p n s i f g e s m i e m g a p f s l k g l l	
Oryctolagus cuniculus	e k c q p n s i f g e s m i e i g a p f s l k g l l	
Rattus norvegicus	e k c q p n s l f g e s m i e m g a p f s l k g l l	
Mus musculus	e k c q p n s i f g e s m i e m g a p f s l k g l l	
Oncorhynchus mykiss	e k t r p n a i f g e s m v e m g a p f s l k g l l	
Salvelinus fontinalis	e k t r p n a i f g e s m v e m g a p f s l k g l l	

COX-2	510	535
Homo sapiens	e k p r p d a i f g e t m v e v g a p f s l k g l m	
Ovis aries	e k p a p d a i f g e t m v e a g a p f s l k g l m	
Sus scrofa	e k p r p d a i f g e t m v e a g a p f s l k g l m	
Bos taurus	e k p r p d a i f g e t m v e a g a p f s l k g l m	
Equus caballus	e k p r p d a i f g e t m v e l g a p f s l k g l m	
Cavia porcellus	e k p r p d a i f g e t m v e m g a p f s l k g l m	
Mustela vison	e k p r p d a i f g e t m v e i g a p f s l k g l m	
Oryctolagus cuniculus	e r p r p d a i f g e t m v e m g a p f s l k g l m	
Rattus norvegicus	e k p r p d a i f g e t m v e l g a p f s l k g l m	
Mus musculus	e k p r p d a i f g e t m v e l g a p f s l k g l m	
Gallus gallus	e k p r p g a i f g e t m v e i g a p f s l k g l m	
Oncorhynchus mykiss	e r p r p n a v f g e t m v e m g a p y s l k g l l	
Salvelinus fontinalis	e r p r p n a v f g e t m v e m g a p y s l k g l l	

FIG. 4. **Sequence alignments for COX-1 and COX-2.** Amino acids 510–535 are shown for all previously reported COX sequences (A) and the revised ovine COX-2 sequence (B). Residues 513, 523, and 530 are indicated in boxes.

B.

COX-2	510	535
Ovis aries - revised	e k p r p d a i f g e t m v e a g a p f s l k g l m	

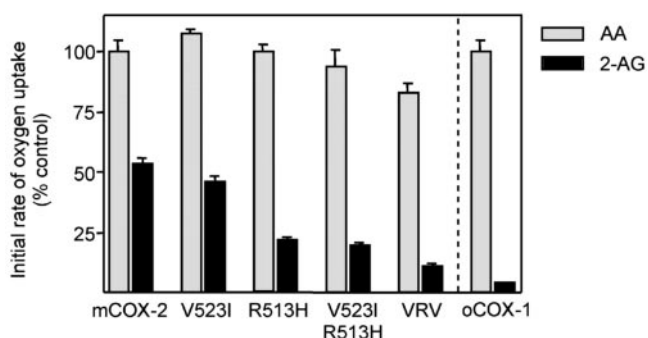


FIG. 5. **Oxygenation of arachidonic acid and 2-AG by side pocket mutants of COX-2.** Initial O_2 uptake rates by wild-type and mutant murine COX-2 enzymes (200 nM) and wild-type ovine COX-1 (150 nM) with arachidonic acid (100 μ M) and 2-AG (200 μ M) are shown and are normalized to the initial rate of O_2 uptake for arachidonic acid with wild-type enzyme (mean $\pm$ S.E., $n = 3$).

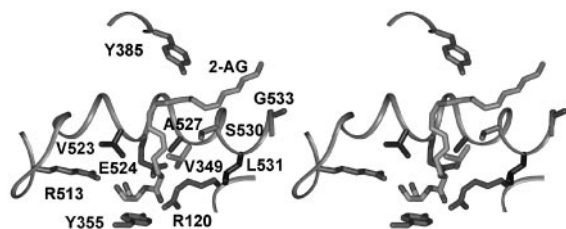


FIG. 6. **Model of 2-AG bound in the COX-2 active site.** A stereo view of the predicted interactions between 2-AG and the active site residues of mCOX-2 is shown.

amino acids implicated in arachidonic acid and 2-AG binding (Fig. 1). All of the proteins were shown by densitometric scanning of SDS-polyacrylamide gels to be at least 80% pure with the following exceptions: W387F (50%) and Y504F (60%). COX activity was determined by oxygen uptake. This necessitated the use of purified proteins to minimize possible hydrolysis of 2-AG to arachidonic acid by contaminating esterases/lipases.

Analyses of COX reaction product profiles typically employ radiolabeled arachidonic acid substrate and take advantage of

the availability of all necessary synthetic standards. The expense and technical difficulties associated with the synthesis of the requisite quantity of radiolabeled 2-AG prevented a similar strategy from being employed in these studies. Thus, products were identified and quantified by LC/MS analysis. Fig. 2 shows a typical LC/MS chromatogram for wild-type mCOX-2 incubated with 2-AG.

Constriction Site Mutations—In both COX isoforms, Arg-120, Tyr-355, and Glu-524 participate in a hydrogen bonding network that forms a constriction at the bottom of the substrate (and inhibitor) binding site (17, 18). In addition to forming the constriction, these residues play a role in binding the polar carboxylate of both arachidonic acid and acidic nonsteroidal anti-inflammatory drugs (19–23). To identify interactions between these residues and 2-AG, site-directed mutants of mCOX-2 were generated, including R120Q and R120A, Y355F and Y355A, and E524L. The site-directed mutants R120Q, Y355F, and E524L displayed modestly reduced capacities to oxygenate arachidonic acid at high substrate concentrations (<2.5-fold reductions) (Fig. 3). However, the R120Q and E524L mutants demonstrated ~9- and 7-fold reductions in 2-AG oxygenation rates, respectively, when compared with wild-type enzyme (Fig. 3). In contrast, although Y355F oxygenated arachidonic acid at approximately half the rate of wild-type enzyme, 2-AG oxygenation remained essentially unaffected (Fig. 3). A role for Glu-524 in 2-AG oxygenation is further supported by the demonstration that the E524L mutant generates a smaller proportion of PG-G products in comparison with wild-type enzyme (Table I). In contrast, Tyr-355 mutants actually generate a greater proportion of PG-G products in comparison with wild-type enzyme (Table I). Taken together, these results suggest that both Arg-120 and Glu-524 are critical residues in facilitating 2-AG oxygenation and that Glu-524 plays a role in establishing or maintaining a substrate conformation that is amenable to cyclization to PG-G products. Tyr-355 appears to be relatively uninvolved in 2-AG oxygenation because mutations in this residue do not detract from 2-AG oxygenation rates or PG-G formation.

Side Pocket Mutations—Previous investigations have identified the COX-2 side pocket as a structural determinant of the

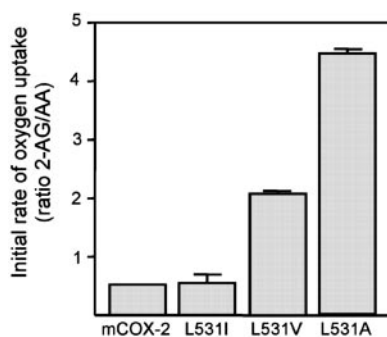


FIG. 7. Effects of Leu-531 mCOX-2 mutations on the oxygenation rates of arachidonic acid and 2-AG. The ratio of initial O_2 uptake rates of 2-AG (200 μM) to arachidonic acid (100 μM) by wild-type and mutant murine COX-2 enzymes (400 nM) are shown (mean $\pm$ S.E., $n = 3$).

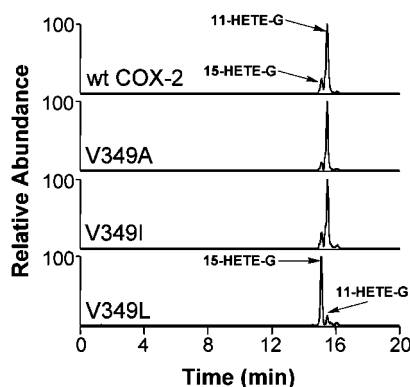


FIG. 8. Generation of 15-HETE-G by V349L mCOX-2. Selected ion mass chromatograms of HETE-G products generated by wild-type mCOX-2 (10 μg), V349A mCOX-2 (10 μg), V349I mCOX-2 (10 μg), or V349L mCOX-2 (10 μg) incubated with an equal mass of 2-AG are shown. HETE-G products were eluted with a 15-min gradient of 20–100% acetonitrile in H_2O (0.001% sodium acetate) and detected by monitoring m/z 417.

isoform selectivity observed for 2-AG oxygenation (13). The COX-2 side pocket is an additional solvent accessible region off the main cyclooxygenase active site formed by the conserved change of Ile-523 in COX-1 for the sterically less demanding Val-523 in COX-2 (17, 24). In addition, COX-1 and COX-2 differ in this region at positions 434 and 513. In human and ovine COX-1, these residues are Ile-434 and His-513, and in all COX-2 genes reported to date these residues are Val-434 and Arg-513 with a single exception; the reported ovine COX-2 sequence encodes for an alanine at position 513 (Fig. 4A) (25). To identify the side pocket residue(s) responsible for the COX-2 selectivity of 2-AG oxygenation, site-directed mCOX-2 mutants were generated incorporating conserved COX-2 $\rightarrow$ COX-1 changes. As expected, all side pocket mutants efficiently oxygenated arachidonic acid (Fig. 5). Investigations into 2-AG oxygenation began with Val-523 because this residue dictates the COX-2 selectivity of diarylheterocycle inhibitors such as rofecoxib and celecoxib; the V523I mutant enzyme is resistant to inhibition by these inhibitors (26, 27). Substitution of Ile for Val-523 only modestly reduced the 2-AG oxygenation rate (Fig. 5). However, substitution of His for Arg-513 markedly reduced the rate of 2-AG metabolism (Fig. 5). In support of a critical role for Arg-513 and minimal involvement of Val-523 in 2-AG turnover, the double mutant incorporating both changes was essentially indistinguishable from the R513H mutant enzyme (Fig. 5). In fact, all enzymes examined that did not contain Arg at position 513, including wild-type COX-1 and the triple mutant V523I/R513H/V434I, showed dramatically reduced activity to-

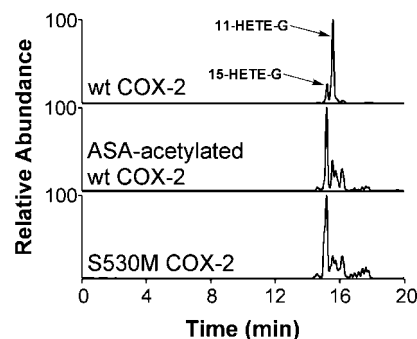


FIG. 9. Production of 15-HETE-G by acetylated mCOX-2 and S530M mCOX-2. Selected ion mass chromatograms of HETE-G products generated by wild-type mCOX-2 (10 μg), acetylated wild-type mCOX-2 (100 μg), or S530M mCOX-2 (100 μg) incubated with an equal mass of 2-AG are shown. HETE-G products were eluted with a 15-min gradient of 20–100% acetonitrile in H_2O (0.001% sodium acetate) and detected by monitoring m/z 417.

ward the endocannabinoid (Fig. 5). Thus, Arg-513 appears, in part, to dictate the isoform selectivity observed for COX metabolism of 2-AG.

These and prior results led to the hypothesis that a capacity to metabolize 2-AG represented an important evolutionary impetus for COX-2 enzymes. To conclude that endocannabinoid metabolism represents a fundamental COX-2 function, residues critical for endocannabinoid oxygenase activity should be conserved in all COX-2 enzymes. Consequently, Arg-513 would be expected to be universally conserved. The residue at position 513 is Arg in all COX-2 sequences from teleosts to human except for sheep, which contains an Ala (25). We sought to confirm the identity of the amino acid at position 513 of sheep COX-2 by PCR analysis of sheep seminal vesicle genomic DNA. Using the reported sheep COX-2 cDNA sequence as a guide, PCR primers were designed which, upon amplification, would provide a 300-base pair fragment spanning the codon for residue 513. PCR amplification of sheep genomic DNA and sub-cloning of the subsequent products provided multiple clones containing the expected size fragment. Sequencing of three unique clones revealed that residue 513 is an Arg encoded by CGT (Fig. 4B). Thus, Arg-513 appears to be conserved in all known COX-2 genes, supporting the assertion that this residue may be critical for COX-2 function.

Taken together these results suggest that three polar residues within the COX-2 active site, Arg-120, Arg-513, and Glu-524, play critical roles in promoting 2-AG oxygenation. This endocannabinoid contains three distinct hydrogen bond donor/acceptors including the two primary alcohols and the ester carbonyl. Directed by the results of the site-directed mutagenesis studies, a model for 2-AG binding to COX-2 was developed. Distance restraints were imposed between the three implicated polar residues in COX-2 and the glycerol moiety of 2-AG, and molecular dynamic simulations were performed to optimize substrate-enzyme interactions. These simulations resulted in the positioning of the ester carbonyl between Arg-120 and Tyr-355. The calculated distances of the 2-AG carbonyl oxygen from the closest Arg-120 guanidinium nitrogen and Tyr-355 hydroxyl groups were 3.3 and 2.9 angstroms, respectively. The primary alcohols of 2-AG adopted a conformation that suggests chelation of the Arg-513 guanidinium group (Fig. 6). The distances between the two alcohols of 2-AG and the closest Arg-513 guanidinium nitrogen were measured to be 2.9 angstroms and 3.2 angstroms. One of these primary alcohols was located only 3.2 angstroms from the carboxylate moiety of Glu-524. As shown in Fig. 6, COX-2 accommodates a 2-AG binding conformation that provides for close interaction between each

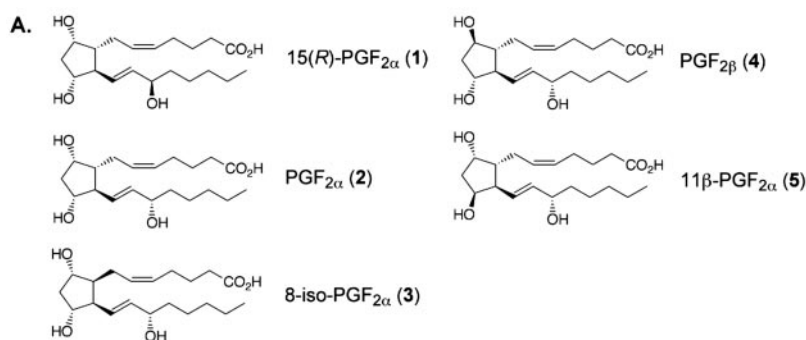
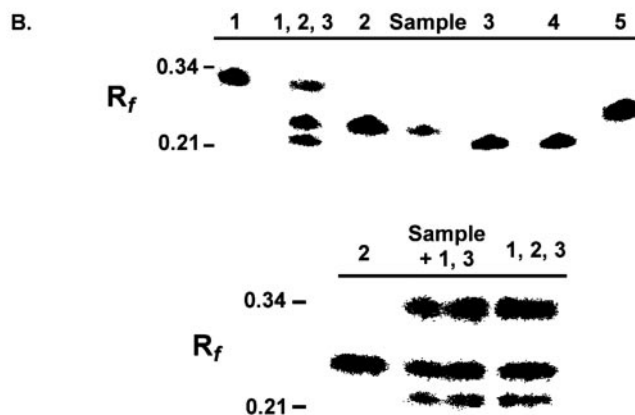


FIG. 10. **Stereochemistry of 2-AG oxygenation.** A, structures of PGF₂ diastereomers. B, thin layer chromatographic analysis of PGF₂ generated by COX-2 oxygenation of 2-AG followed by triphenylphosphine reduction and saponification (*Sample*). The products were visualized by iodine staining.



of the three polar enzyme residues implicated in 2-AG metabolism and hydrogen bond donors/acceptors of the endocannabinoid.

Hydrophobic Channel Mutations—Both COX isoforms contain Leu at position 531 (Fig. 4A). In COX-2, Leu-531 is positioned directly across from the side pocket in the cyclooxygenase active site (Fig. 6) (17, 24, 28). Crystallographic analysis of complexes of COX-1 with arachidonic acid indicate that Leu-531 is beyond van der Waals' contact distances from substrate; however, site-directed mutagenesis studies demonstrate that this residue promotes high affinity, productive binding of arachidonic acid within the active site (23, 29). Reduction of the hydrophobic side chain length at position 531 in COX-1 affects the positioning of arachidonic acid near Arg-120 and substantially decreases oxygenation rates (29). The demonstrated role of the COX-2 side pocket, and Arg-513 in particular, in 2-AG oxygenation suggests that this endocannabinoid may bind nearer the side pocket and consequently, further from Leu-531 than arachidonic acid. To test the hypothesis that Leu-531 may have a less significant role in positioning 2-AG than that seen with arachidonic acid, three mutations (L531I, L531V, and L531A) were made in mCOX-2 to reduce the size of the hydrophobic side chain. The ability of these mutant enzymes to oxygenate both arachidonic acid and 2-AG decreased markedly. However, the deleterious effects of Leu-531 mutations were less pronounced with the endocannabinoid substrate. In addition, as the side chain of residue 531 decreased in size, the ratio of 2-AG/arachidonic acid oxygenation rates increased (Fig. 7). Thus, changes in Leu-531 of COX-2 affect 2-AG oxygenation rates less significantly than arachidonic acid oxygenation rates. These results are consistent with our model in which 2-AG is displaced toward the side pocket of COX-2 and away from Leu-531 relative to arachidonic acid (Fig. 6). In fact, modeling results positioned C-1 of 2-AG 7.3 angstroms from the closest Leu-531 methyl group. In com-

parison, the crystal structure of arachidonic acid bound in COX-1 reveals a distance of 4.6 angstroms from C-1 to the closest Leu-531 methyl group (23).

Arachidonic acid binds within COX enzymes in an L-shaped conformation placing the 13-*pro-S*-hydrogen close to Tyr-385 and the ω -end near Gly-533 (14, 23). Previous investigations demonstrated that 2-AG binds within COX-2 in a similar conformation; neither the G533V nor the Y385F mutant COX-2 enzymes oxygenated 2-AG (13). Residues Ala-527 and Val-349 line the hydrophobic L-shaped channel adjacent to Ser-530 (Fig. 6). Ala-527 and Val-349 are within van der Waals' distances of arachidonic acid, and Val-349 has been implicated in stabilizing a conformation of arachidonic acid that is optimal for cyclization (23, 29). Mutations were made in COX-2 at positions 527 and 349 to examine whether these residues also affect 2-AG oxygenation. The mutant enzymes were incubated with 2-AG, and the products were analyzed by LC/MS. The A527V and V349I mutations did not show significant increases in monooxygenated products (Table I). However, V349A and V349L did show relative increases in monooxygenated products (Table I). V349L also displayed a dramatic shift in HETE-G regiochemistry, from C-11 to C-15 (Fig. 8). Therefore, one of the methyl groups of Val-349 seems to interact with 2-AG to promote PG-G generation. These findings are consistent with arachidonic acid product profiles from mutant COX-1 enzymes (29).

Acetylated COX-2 and S530M Generation of 15-HETE-G—Both aspirin-acetylated COX-2 and the S530M enzyme have been shown to oxygenate arachidonic acid to provide 15-HETE (30–35). These findings have led to the suggestion that some of the therapeutic actions of aspirin may be mediated by the generation of 15-HETE and subsequent metabolites (36). Both 2-AG oxygenation and 15-HETE generation by acetylated-COX-2 are dependent, in part, on the COX-2 side pocket, and the possibility existed that these two overlapping spatial re-

quirements would prevent 2-AG oxygenation by acetylated-COX-2 (35). To assess the capacity of aspirin-treated COX-2 and the S530M mutant to bind and metabolize 2-AG, these enzymes were incubated with the endocannabinoid, and the products were evaluated by LC/MS. Neither enzyme generated a significant amount of PGE₂-G or PGD₂-G (data not shown and Table I). However, HETE-G products were observed, and regiochemical analysis demonstrated that oxygenation occurred preferentially at C-15 (Fig. 9). Thus, 2-AG and arachidonic acid are both capable of adopting a conformation in the active site of aspirin-acetylated COX-2, which leads to selective C-15 oxygenation in addition to the more typical conformation in unmodified enzyme that leads preferentially to prostaglandin formation.

Stereochemistry of Oxygenated 2-AG Products—COX-2 oxygenation of anandamide and 2-AG to generate PG ethanolamides and glyceryl PGs has been reported (12, 13). However, the stereochemistry of the products has not been conclusively established. Structural changes in the active site of cyclooxygenase enzymes can lead to altered product profiles and product stereochemistry. As a result, product stereochemical analysis provides an additional test of substrate-enzyme interactions. In addition, PG and HETE stereoisomers generally possess dramatically different biological activities and/or potencies. For example, 15(*R*)-PGF_{2α} displays negligible binding to rat vascular smooth muscle cells and binds ovine luteal cell FP receptors with 15-fold less affinity when compared with the natural stereoisomer (37, 38). Similarly, 8(*S*)-HETE activates peroxisome proliferator-activated receptor α ~10-fold more potently than 8(*R*)-HETE (39). Consequently, we investigated the relative stereochemistry of PG and HETE glyceryl esters generated by COX-2 metabolism of 2-AG. PGF₂-G was obtained by rapidly reducing the endoperoxide PGH₂-G generated in COX-2 incubations with 2-AG. Following saponification to generate the PGF₂-free acid, the sample was analyzed by thin layer chromatography and compared with PGF_{2α} and diastereomers (Fig. 10). The sample generated from 2-AG eluted as a single spot with an identical R_f as PGF_{2α}. Under the chromatographic conditions employed, PGF_{2β}, 8-iso-PGF_{2α}, 11β-PGF_{2α}, and 15(*R*)-PGF_{2α} were readily distinguished from PGF_{2α}. Thus, 2-AG oxygenation by COX-2 provides PG-Gs with the same relative stereochemistry found for arachidonic acid-derived PGs. HETE-G stereochemistry also was investigated and found to be similar to that observed with HETEs generated by arachidonic acid oxygenation (15-HETE-G, 40 ± 2% *R*; 11-HETE-G, > 99% *R*; mean ± S.E.).

The present results suggest that the COX-2 active site accommodates the endocannabinoid, 2-AG, in a productive conformation similar to arachidonic acid; however, subtle differences have been identified. The endocannabinoid glyceryl moiety interacts with three polar residues in the COX-2 active site, Arg-120, Arg-513, and Glu-524. The universal presence of Arg at position 513 in the side pocket of COX-2 enzymes and its absence in mammalian COX-1 enzymes explains, in part, the isoform selectivity observed for 2-AG metabolism. When compared with the binding of arachidonic acid in the COX-1 active site, the polar terminus of 2-AG shifts toward the COX-2 side pocket and Arg-513 and away from Leu-531. Despite this difference, 2-AG, like arachidonic acid, is oxygenated by aspirin-acetylated COX-2 to provide 15-HETE-G and is *bis*-dioxigen-

ated by the unmodified enzyme to provide PG-Gs with the natural prostaglandin stereochemistry. These findings support the hypothesis that 2-AG is a natural COX-2 substrate and that the active site of this enzyme has evolved to promote efficient 2-AG turnover.

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