Amino Acid Determinants in Cyclooxygenase-2 Oxygenation of the Endocannabinoid Anandamide†

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ABSTRACT: The endocannabinoid arachidonylethanolamide (AEA, anandamide) is an endogenous ligand for the cannabinoid receptors and has been shown to be oxygenated by cyclooxygenase-2 (COX-2). We examined the structural requirements for COX-mediated, AEA oxygenation using a number of substrate analogues and site-directed mutants of COX-2. Fourteen AEA analogues were synthesized and tested as COX substrates. These studies identified the hydroxyl moiety of AEA as a critical determinant in the ability of COX enzymes to effect robust endocannabinoid oxygenation. In addition, these studies suggest that subtle structural modifications of AEA analogues near the ethanolamide moiety can result in pronounced changes in their ability to serve as COX-2 substrates. Site-directed mutagenesis studies have permitted the development of a model of AEA binding within the COX-2 active site. As with arachidonic acid, the ω-terminus of AEA binds in a hydrophobic alcove near the top of the COX-2 active site. The polar ethanolamide moiety of AEA, like the carboxylate of arachidonate, interacts with Arg-120 at the bottom of the COX-2 active site. Mutation of Tyr-385 prevents AEA oxygenation, suggesting that, as in the case of other COX substrates, AEA metabolism is initiated by Tyr-385-mediated hydrogen abstraction. Thus, AEA binds within the COX-2 active site in a conformation roughly similar to that of arachidonic acid. However, important differences have been identified that account for the isoform selectivity of AEA oxygenation. Importantly, the COX-2 side pocket and Arg-513 in particular are critical determinants of the ability of COX-2 to efficiently generate prostaglandin H_2 ethanolamide. The reduced efficiency of COX-1-mediated, AEA oxygenation can thus be explained by the absence of an arginine residue at position 513 in this isoform. Mutational analysis of Leu-531, an amino acid located directly across from the COX-2 side pocket, suggests that AEA is shifted away from this hydrophobic residue and toward Arg-513 relative to arachidonic acid. Coupled with earlier observations with the endocannabinoid 2-arachidonylglycerol, these results indicate that one possible function of the highly conserved COX-2 active site side pocket is to promote endocannabinoid oxygenation.

The mammalian endogenous cannabinoid system is comprised of two G-protein-coupled receptors, CB1 and CB2, and their endogenous ligands. The first described endogenous ligand for cannabinoid receptors, anandamide, was reported in 1992 (*1*). Despite its relatively recent discovery, anandamide has been implicated in an array of pathological and physiological processes including reproduction, appetite regulation, cognition, motor control, analgesia/hyperalgesia, vascular homeostasis, gut motility, and immunomodulation (*2*−*9*). In addition to its cannabimimetic properties, anandamide has been implicated in vanilloid receptor signaling (*10*). In light of the myriad biological activities attributed to anandamide, it is not surprising that attention has been directed toward identifying and characterizing enzymes capable of endocannabinoid metabolism.

Cyclooxygenase-2 (COX-2)[1] selectively oxygenates the endocannabinoids 2-arachidonylglycerol (2-AG) and anandamide (AEA) (*11*−*14*). AEA oxygenation has been demonstrated with purified human COX-2 as well as cells expressing human COX-2 (*11*, *12*). In contrast, partially purified human COX-1 does not oxygenate AEA nor is any cyclooxygenase-mediated metabolism of AEA observed in human promonocytic THP cells expressing COX-1 (*11*). Despite these observations, only one product of COX-2 action on AEA has been identified, PGE_2 ethanolamide (PGE_2-EA) (*11*). No studies have been reported addressing whether COX-2 oxygenation of AEA represents a specific endocannabinoid oxygenase reaction or a general capacity of COX-2 to metabolize arachidonylamides. Finally, the molecular basis for the observed isoform selectivity has not been established.

To address these and other issues, we investigated arachidonylamide metabolism by wild-type and site-directed

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[1] Abbreviations: COX, cyclooxygenase; AEA, arachidonylethanolamide (anandamide); AG, arachidonylglycerol; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; EA, ethanolamide; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry.

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mutant COX enzymes. Substrate stucture−activity relationships were examined using synthetic derivatives of AEA. The products of cyclooxygenase action on AEA were characterized. Finally, we identified the molecular determinants that account for isoform-selective AEA oxygenation. Taken together, these results permit the development of a model of AEA binding within the COX-2 active site. In conjunction with previous results obtained with 2-AG, the current study indicates that a single, highly conserved amino acid, Arg-513, is the major determinant of the efficient endocannabinoid oxygenase activity of COX-2.

EXPERIMENTAL PROCEDURES

Materials. PGE_2-EA, PGD_2-EA, AEA, and purified soybean 15-lipoxygenase (P1) were purchased from Cayman Chemicals (Ann Arbor, MI). Arachidonic acid was obtained from NuChek Prep (Elysian, MN). Hematin was purchased from Sigma. Ram seminal vesicles were from Oxford Biomedical Research (Oxford, MI). All other chemicals were purchased from Aldrich.

Enzymology. COX-1 was purified from ram seminal vesicles as previously described (*15*). Site-directed mutagenesis of murine COX-2 was performed as described (*16*). COX-2 enzymes were expressed in *Sf*-9 insect cells using the pVL1393 transfer vector (PharMingen, San Diego, CA) and purified by ion-exchange chromatography and gel filtration as described previously (*16*). All enzymes tested were shown by densitometric scanning of SDS−polyacrylamide gels to be at least 80% pure. Apoenzymes were reconstituted with hematin prior to activity assays. COX activity was quantified as described (*17*). Initial reaction velocity data were obtained from the linear portion of oxygen uptake curves. Wild-type murine COX-2 and ovine COX-1 specific activities were 14.3 ± 1.0 and 19.9 ± 0.9 μmol of arachidonic acid min^{-1} (mg of protein)$^{-1}$, respectively. The PG-EA to hydroxyeicosatetraenoic acid ethanolamide (HETE-EA) ratios were determined as previously described (*14*).

Chemistry. Acetylation of wild-type murine COX-2 was accomplished by treating the enzyme with 2 mM aspirin for 30 min at 37 °C. Arachidonylamides, with the exception of eicosa-5,8,11,14-tetraenoic acid (2-hydroxy-1-hydroxymethylethyl)amide (**14**), and eicosa-5,8,11,14-tetraenoic acid (2,3-dihydroxypropyl)amide (**15**), were synthesized by bis(2-oxo-3-oxazolidinyl)phosphinic chloride- or 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide-mediated coupling of arachidonic acid and the appropriate amine. Pure arachidonylamides were obtained by silica gel chromatography and characterized by mass spectrometry (MS) and ^{1}H and ^{13}C NMR sprectroscopy. Reaction and spectroscopic details can be found in Supporting Information. Eicosa-5,8,11,14-tetraenoic acid (2-hydroxy-1-hydroxymethylethyl)amide (**14**) was synthesized as previously described (*18*). Eicosa-5,8,-11,14-tetraenoic acid (2,3-dihydroxypropyl)amide (**15**) was prepared by coupling arachidonic acid chloride with 3-amino-1,2-propanediol. With the exception of (*R*)-2-methoxy-1-methylethylamine and 1-amino-2-methylpropan-2-ol, amines were obtained commercially. (*R*)-2-Methoxy-1-methylethylamine was prepared by methylating (*R*)-2-*tert*-butoxycarbonylamino-1-propanol with methyl iodide in the presence of NaH followed by BOC deprotection with gaseous HCl. 1-Amino-2-methylpropan-2-ol was synthesized as previously described (*19*). Incubation of purified soybean 15-lipoxygenase with AEA generated 15-hydroperoxyeicosatetraenoic acid ethanolamide, which, upon reduction with triphenylphosphine, provided 15-HETE-EA (*20*).

Mass Spectrometry. Liquid chromatography/mass spectrometry (LC/MS) was conducted with a Waters 2690 separations module with a Zorbax RX-C18 narrow-bore column (15 cm × 2.1 mm, 5 μm) interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer. Sodiated analytes were eluted with increasing concentrations of MeCN in 0.001% aqueous NaOAc and detected as positive ions. Electrospray ionization was carried out using nitrogen as sheath (78 psi) and auxiliary gas (14 psi) to assist with nebulization. A potential of 5.0 kV was applied to the ESI needle, and the capillary temperature was maintained at 220 °C. Mass spectral parameters were optimized to obtain maximum sensitivity without sacrificing unit resolution. Displayed mass chromatograms are representative examples of no fewer than three independent experiments.

Energy Minimization and Modeling. AEA was built into the protein coordinates of uninhibited mCOX-2 (PDB ID: 5COX). All amino acid positions were fixed except for the side chains of Arg-120, Arg-513, Glu-524, and Tyr-355. The hydroxyl group of AEA was restrained within 3.6 Å from the hydrogen bond donor/acceptor groups of Arg-513 and Glu-524 and the amide carbonyl within 3.6 Å of Arg-120. The Tyr-385 hydroxyl group was restrained within 3.6 Å from AEA C13 to ensure a productive conformation for oxygenation. The complexes were energy minimized for 1000 iterations using a conjugate gradient in the consistent valence force field. Molecular dynamic simulations were then run on the assemblies for 10000 iterations (10 ps) at 300 K. All simulations were performed using the Discover module of Insight II 2000 with a R12000 Silicon Graphics Octane workstation.

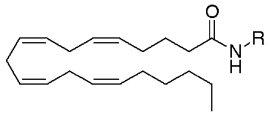
RESULTS

Arachidonylamide Metabolism by Wild-Type Cyclooxygenases. To examine the ability of wild-type murine COX-2 and ovine COX-1 to oxygenate arachidonylamides, AEA and 14 analogues were synthesized and tested as COX substrates (Table 1). The endocannabinoid AEA (**1**) was not the preferred arachidonylamide COX substrate. Several arachidonylamides were oxygenated more avidly than AEA by both COX isoforms. For example, the maximal rate of COX-2 oxygenation of arachidonic acid [2-hydroxy-1(*S*)-methylethyl]amide (**4**) was greater than twice that observed with AEA. In addition, the maximal rate of COX-1 oxygenation of **4** exceeded the AEA rate by nearly 4-fold.

With the exception of eicosatetraenoic acid [2-methoxy-1(*S*)-methylethyl]amide (**8**), arachidonylamides were preferentially oxygenated by COX-2 (Table 1). In the case of AEA, the rate of oxygen uptake observed when the endocannabinoid was incubated with COX-2 (27%) was more than double that observed with COX-1 (11%). Nonetheless, wild-type ovine COX-1 catalyzed modest oxygenation of most amides tested.

The structure−activity relationships shown in Table 1 identify the primary hydroxyl moiety of AEA as a determinant in the capacity of arachidonyl amides to serve as COX-2 substrates. Substitution of the hydroxyl group of AEA with

Table 1: Arachidonylamide Oxygenation by Wild-Type Murine COX-2 (200 nM) and Ovine COX-1 (150 nM)[a]



Compound	R =	mCOX-2	oCOX-1
AEA (1)		27.1 ± 1.5	11.2 ± 0.5
2		1.7 ± 0.3	0.5 ± 0.2
3		11.4 ± 0.5	0.5 ± 0.3
4		65.3 ± 2.7	40.0 ± 2.3
5		34.1 ± 1.1	2.9 ± 0.3
6		12.7 ± 0.6	7.2 ± 0.1
7		14.3 ± 0.9	9.8 ± 0.3
8		18.2 ± 1.3	20.8 ± 0.4
9		23.8 ± 0.7	17.5 ± 1.0
10		27.2 ± 1.6	7.9 ± 0.4
11		44.3 ± 1.6	20.8 ± 1.2
12		27.5 ± 0.3	4.0 ± 0.6
13		43.2 ± 0.8	11.6 ± 0.4
14		39.9 ± 1.8	7.8 ± 0.9
15		32.1 ± 1.9	13.6 ± 3.2

[a] Maximal rates of arachidonylamide (200 μM) oxygenation were determined as described in Experimental Procedures. Values are normalized to the rate of oxygen uptake catalyzed by wild-type enzyme with arachidonic acid (100 μM) and are expressed as a percentage of the arachidonic acid rate ($n \geq 3$, mean ± SE).

either a methyl (**2**) or methoxy (**3**) group resulted in markedly reduced oxygen uptake when these endocannabinoid analogues were incubated with COX-2. This reduction was more pronounced with the methyl substitution. In addition, substitution of the hydroxyl group of AEA with either methyl or methoxy groups decreased the maximal rate of COX-1-catalyzed oxygenation of these modified substrates. Similar trends are observed when the maximal rates of COX-2-mediated oxygenation of arachidonic acid (2-hydroxy-1-methylethyl)amides (**4**, **5**) are compared with the rates of oxygenation of the methyl- or methoxy-containing derivatives (**6**, **7** and **8**, **9**). It is noteworthy that substitution of the hydroxyl moiety in **1** and **4** with methyl or methoxy groups results in the generation of poorer COX-1 substrates whereas similar substitutions in **5** actually produce superior COX-1 substrates.

Enzyme−substrate interactions near the polar terminus of arachidonylamides were further investigated using chiral, 1- and 2-methyl-substituted AEA derivatives. The incorporation of a 1-methyl group in an (*S*)-orientation on AEA produced the most avidly oxygenated substrate examined (**4**). The rate of COX-2-mediated oxygenation of **4** was halved by the simple inversion of the chiral center at the 1-position (**5**). This stereochemical modification also dramatically reduced the ability of COX-1 to act on **5** (13-fold). To determine if these relationships were dictated by a dominant effect of the 1(*S*)-methyl substitution, the 1,1-dimethyl analogue, **10**, was synthesized and tested as a COX substrate. Interestingly, COX-2 oxygenated **10** with a maximal rate comparable to both AEA and **5**, suggesting that 1(*R*)-methyl substitution prevents the "substrate-enhancing" effects of AEA 1(*S*)-methyl substitution.

Similarly, the stereochemistry of methyl substitution at the AEA 2-position affected the ability of COX enzymes to act on arachidonylamide substrates. Incorporation of a 2(*S*)-methyl group (**11**) produced a better COX substrate than AEA. This enhancement was not observed when the 2-methyl group was present in the (*R*)-orientation (**12**). In contrast to the results with the 1,1-dimethyl-AEA analogue, **10**, the maximal rate of COX-2-mediated oxygenation of the 2,2-dimethyl analogue, **13**, was comparable to **11** and significantly higher than that observed with either AEA or **12**. This observation suggests that 2(*R*)-methyl substitution does not abrogate the substrate-enhancing effects of AEA 2(*S*)-methyl substitution.

The maximal rate of oxygenation of the amide derivative of 2-AG, arachidonic acid (2-hydroxy-1-hydroxymethylethyl)amide (**14**), approached the maximal rate of oxygenation for the cannabimimetic ester and exceeded that observed with AEA by 50% (*13*). The amide analogue of 1-AG, **15**, was also tested. The maximal rates of COX-mediated oxygenation of the regioisomers **14** and **15** were comparable; however, **14** proved to be a slightly superior COX-2 substrate and a slightly inferior COX-1 substrate.

AEA was incubated with ovine COX-1 or murine COX-2, and oxygenated products were analyzed by LC/MS (Figure 1). Both COX-1 and COX-2 generated four primary oxygenated AEA products. Two closely eluting polar products were detected with mass-to-charge ratios of 418, consistent with sodiated PG-EAs. Identification of these products as PGE_2- and PGD_2-EA was accomplished by coelution with synthetic standards of PGE_2- and PGD_2-EA under multiple chromatographic conditions. Two closely eluting nonpolar AEA metabolites with *m*/*z* 386, consistent with HETE-EA products, were also detected. The more polar *m*/*z* 386 product coeluted with an enzymatically generated standard of 15-

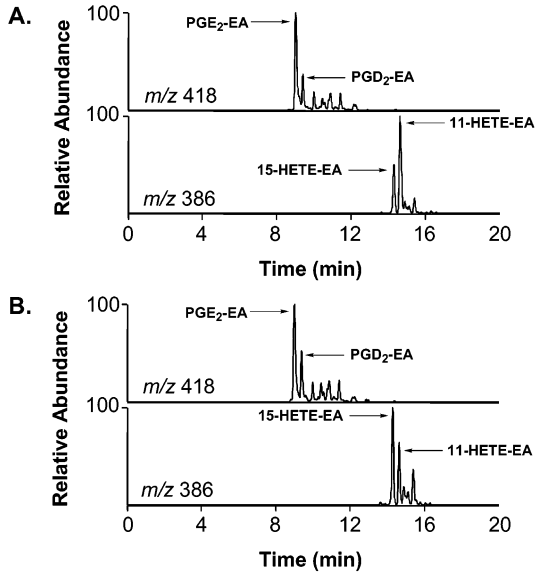


FIGURE 1: Oxygenated products formed by cyclooxygenase action on AEA. Selected ion mass chromatograms of oxygenated AEA products generated by (A) wild-type murine COX-2 (10 μg) and (B) wild-type ovine COX-1 (10 μg) incubated with AEA (10 μg) for 10 min at 37 °C in 100 mM Tris-HCl containing 500 μM phenol (pH 8.0) are shown. 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* 418 (top panels) and *m*/*z* 386 (bottom panels). Individual chromatograms are normalized to the largest peak detected.

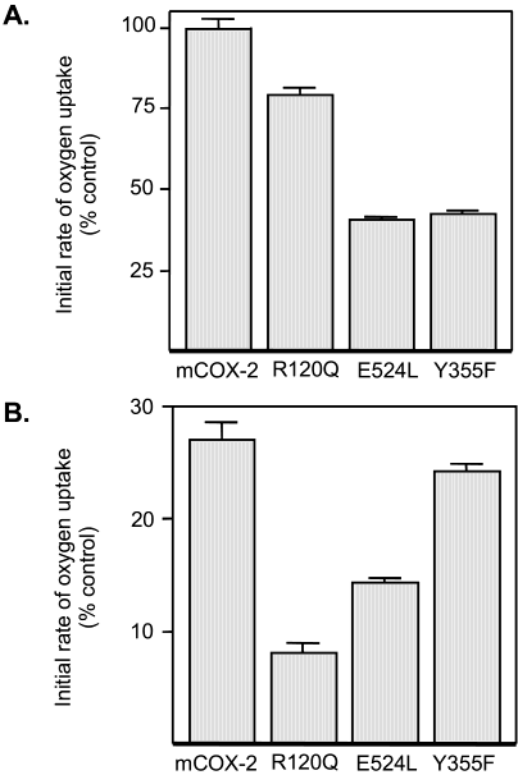


FIGURE 2: Oxygenation of (A) arachidonic acid and (B) AEA by constriction site mutants of murine COX-2. Initial O_2 uptake rates by wild-type and mutant COX-2 enzymes (200 nM) with arachidonic acid (100 μM) and AEA (200 μM) are shown and are normalized to the initial rate of O_2 uptake for arachidonic acid with wild-type murine COX-2 (mean ± SE, $n \geq 3$).

HETE-EA. Comparison of the product profiles of AEA oxygenation with similar profiles for arachidonic acid and 2-AG oxygenation strongly suggests that the less polar *m*/*z* 386 product represents 11-HETE-EA (13). Finally, in both COX-1 and COX-2 incubations with AEA, a minor product of intermediate polarity with *m*/*z* 346 was observed, consistent with the PGH_2-EA degradation product, hydroxyheptadecatrienoic acid ethanolamide (HHT-EA).

The demonstration that AEA incubation with purified ovine COX-1 results in the production of PG-EAs indicates that the oxygen uptake detected is not the result of nonspecific oxygenation. Furthermore, although differences were observed in the regiospecificity of AEA monooxygenation by COX-1 and COX-2, no statistically significant difference in the ratios of total PG-EAs to HETE-EAs produced by these enzymes was observed (Figure 1 and data not shown). Taken together, these results indicate that COX-1 and COX-2 oxygenate the endocannabinoid AEA to provide PGE_2-EA, PGD_2-EA, and both 11- and 15-HETE-EAs.

Site-Directed Mutagenesis: Constriction Site Mutations. The cyclooxygenase active site residues, Arg-120, Tyr-355, and Glu-524, participate in a hydrogen-bonding network which forms a constriction at the bottom of the substrate (and inhibitor) binding site (*21*−*25*). These residues also play roles in binding the polar carboxylate of arachidonic acid and glycerol ester of 2-AG (*14*, *25*). To identify interactions between these residues and AEA, site-directed mutants of mCOX-2, including R120Q, Y355F, and E524L, were tested for their ability to oxygenate this endocannabinoid. Incorporation of a glutamine for Arg-120 had little impact on the ability of the enzyme to oxygenate arachidonic acid at high concentrations (<25% reduction). In contrast, the ability to oxygenate AEA was reduced more than 3-fold, suggesting that Arg-120 is critical in permitting COX-2 oxygenation of AEA. Incorporation of a leucine residue for Glu-524 reduced the ability of the mutant enzyme to oxygenate both arachidonic acid and AEA to a similar extent. Finally, although the Y355F mutant enzyme oxygenated arachidonic acid at approximately half the rate of wild-type enzyme, AEA oxygenation was essentially unaffected (Figure 2).

Site-Directed Mutagenesis: Catalytic Tyrosine and Hydrophobic Alcove Mutations. The COX-2 active site residue, Tyr-385, is required for both arachidonic acid and 2-AG oxygenation. A tyrosyl radical formed from Tyr-385 initiates the cyclooxygenase reaction by 13-*pro-S* hydrogen abstraction, and consequently, the Y385F mutant enzyme is inactive (*13*, *26*). Not surprisingly, no oxygen uptake was detected when AEA was incubated with Y385F mCOX-2. The ω-termini of arachidonic acid and 2-AG bind in the hydrophobic alcove near the top of the cyclooxygenase active site (*14*, *16*, *25*). Gly-533 is located near the end of this alcove, and mutations that introduce steric bulk at position 533 disrupt arachidonic acid and 2-AG binding and, consequently, prevent oxygenation (*13*, *16*). Consistent with previous results with other COX substrates, no oxygen uptake was detected when AEA was incubated with the G533V enzyme.

Site-Directed Mutagenesis: Side Pocket Mutations. The demonstration that the cyclooxygenase isoform selectivity observed with 2-AG can be attributed, in large part, to the COX-2 side pocket, and Arg-513 in particular, prompted an investigation of the role of this structural feature in the preferential metabolism of AEA by COX-2 (*13*, *14*). Mutation of side pocket residues does not detract from the ability

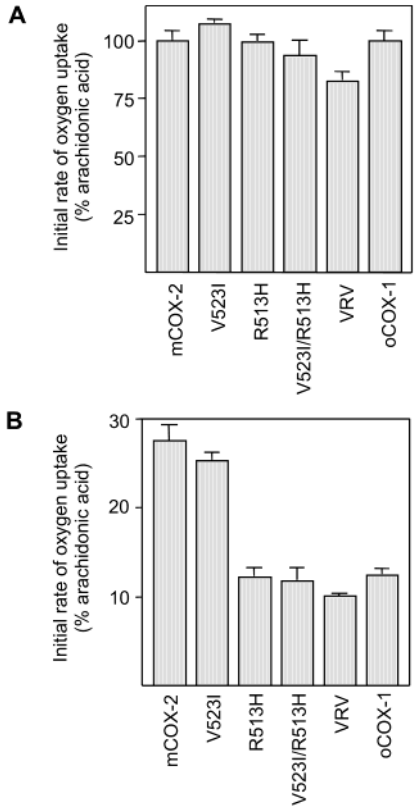


FIGURE 3: Oxygenation of arachidonic acid and AEA by side pocket mutants of COX-2. Initial O_2 uptake rates by wild-type and mutant mCOX-2 enzymes (200 nM) and wild-type oCOX-1 (150 nM) with (A) arachidonic acid (100 μM) and (B) AEA (200 μM) are shown and are normalized to the initial rate of O_2 uptake for arachidonic acid with wild-type enzyme (mean ± SE, $n \geq 3$).

of mutant COX-2 enzymes to oxygenate arachidonic acid (Figure 3A). In contrast, the triple mutant of COX-2, V523I/R513H/V434I (VRV) which comprises the major side pocket differences between the two enzymes, behaved almost identically to wild-type ovine COX-1 when treated with AEA (Figure 3B). Examination of single mutations revealed that Arg-513 completely accounts for the preferential metabolism of AEA by COX-2 over COX-1. Reduced AEA oxygenation rates were observed with all enzymes containing a His-513 residue, including wild-type COX-1 (Figure 3B).

Site-Directed Mutagenesis: Main Channel Mutations. Our demonstration of a role for the side pocket in AEA binding in the COX-2 active site resembles findings obtained for 2-AG. In the case of 2-AG, the endocannabinoid is shifted toward the COX-2 side pocket with respect to arachidonic acid, thus facilitating Arg-513 interactions with the primary hydroxyl groups of the glycerol moiety (*14*). This proposed shift was experimentally supported by the demonstration that mutations made in Leu-531, an amino acid positioned directly across from the side pocket in the cyclooxygenase active site, reduced arachidonic acid oxygenation rates more markedly than those obtained with the endocannabinoid (*14*, *25*). To conduct a similar test with AEA, Leu-531 mutant enzymes were evaluated for their ability to oxygenate this endocannabinoid. The ability of these mutant enzymes to oxygenate both arachidonic acid and AEA was markedly decreased compared to wild-type COX-2; L531A, the least active mutant, oxygenated arachidonic acid at a rate approximately 1% that observed with wild-type enzyme

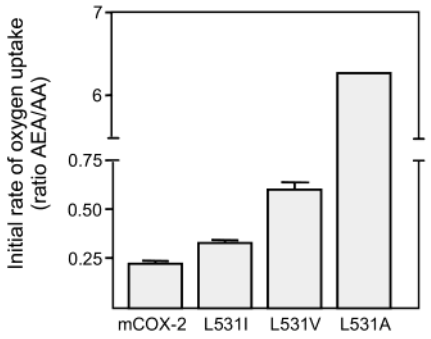


FIGURE 4: Effects of Leu-531 mCOX-2 mutations on the oxygenation rates of arachidonic acid and AEA. The ratio of initial O_2 uptake rates of AEA (200 μM) to arachidonic acid (100 μM) by wild-type and mutant murine COX-2 enzymes (400 nM) are shown (mean ± SE, $n \geq 3$).

(specific activity = 0.26 μmol of O_2 min^{-1} mg^{-1}). However, as observed with 2-AG, 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 AEA to arachidonic acid oxygenation rates further increased (Figure 4). The L531A mutant enzyme shifted the ratio of AEA to arachidonic acid oxygenation rates so significantly that AEA became a far superior substrate for this enzyme being oxygenated at a rate exceeding that observed for arachidonic acid by more than 6-fold.

COX substrates bind within the enzyme active site in a L-shaped conformation (*16*, *25*). The side chain of Val-349 projects into the hydrophobic L-shaped channel and is within the van der Waals distance of arachidonic acid (*25*). Val-349 has been implicated in stabilizing a conformation of arachidonic acid that is optimal for cyclization and has been shown to participate in 2-AG oxygenation (*14*, *27*). To examine a possible role for this hydrophobic residue in AEA metabolism, V349I, V349L, and V349A site-directed mutant COX-2 enzymes were incubated with AEA, and oxygenated products were analyzed by LC/MS. As observed with 2-AG, the V349A and V349L mutant enzymes generated a greater proportion of monooxygenated, HETE-EA, products than bis-dioxygenated, PG-EA, products when compared to wild-type enzyme (data not shown). In addition, V349L and, to a lesser extent, V349A displayed shifts in HETE-EA regiochemistry, from C11 to C15, with the leucine-containing mutant providing almost exclusively 15-HETE-EA (Figure 5).

Acetylated COX-2 and S530M Generation of 15-HETE-EA. Both aspirin-acetylated COX-2 and the S530M enzyme have been shown to oxygenate arachidonic acid and 2-AG to provide 15-HETE and 15-HETE-G, respectively (*14*, *28*−*33*). To investigate the ability of aspirin-treated COX-2 and the S530M mutant to bind and metabolize AEA, these enzymes were incubated with endocannabinoid, and oxygenated products were analyzed by LC/MS. Neither enzyme generated a significant amount of PGE_2- or PGD_2-EA (data not shown). However, HETE-EA products were observed, and regiochemical analysis demonstrated that oxygenation occurred preferentially at C15 (Figure 6).

DISCUSSION

Despite demonstrations that the endogenous cannabinoid system plays a critical role in an array of pathological and

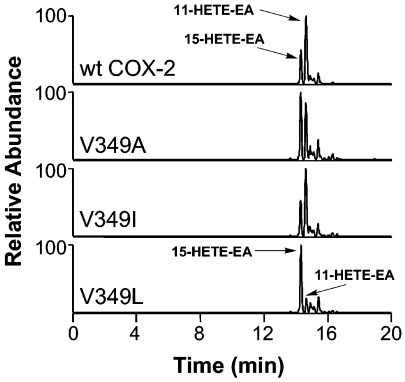


FIGURE 5: Generation of 15-HETE-EA by V349L mCOX-2. Selected ion mass chromatograms of HETE-EA products generated by wild-type or Val-349 mutant mCOX-2 enzymes (10 μg) incubated with AEA (10 μg) are shown. HETE-EA products were eluted with a 15 min gradient of 20−100% acetonitrile in H_2O (0.001% sodium acetate) and detected by monitoring *m*/*z* 386.

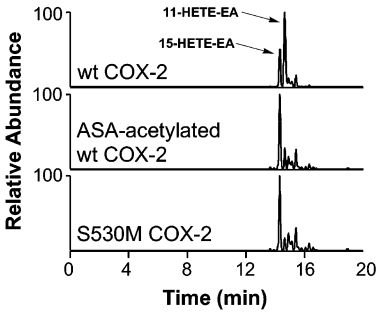


FIGURE 6: Production of 15-HETE-EA by acetylated mCOX-2 and S530M mCOX-2. Selected ion mass chromatograms of HETE-EA products generated by wild-type mCOX-2 enzymes (10 μg), acetylated wild-type mCOX-2 (10 μg), or S530M mCOX-2 (10 μg) incubated with AEA (10 μg) are shown. HETE-EA products were eluted with a 15 min gradient of 20−100% acetonitrile in H_2O (0.001% sodium acetate) and detected by monitoring *m*/*z* 386.

physiological processes and growing evidence that fatty acid oxygenase-mediated endocannabinoid metabolism is biologically important, little is known about the molecular details of COX action on AEA (*11*, *14*, *34*−*37*). The current study was designed to (1) identify strutural features of AEA that promote oxygenation by COX-2, (2) characterize the products of COX action on AEA, (3) define the molecular basis for COX isoform selectivity, and (4) develop a comprehensive model for AEA binding in the COX-2 active site. These aims were accomplished through the use of substrate structure−activity relationship studies, site-directed mutagenesis, and molecular modeling.

Murine COX-2 metabolized AEA at 27% of the maximal rate observed for arachidonic acid oxygenation. This contrasts markedly from the value of 60−85% initially reported for human COX-2 (*11*). However, these results are in good agreement with subsequent studies of human COX-2 that demonstrated the ratio of k_{cat} values for AEA and arachidonic acid is approximately 30% (*12*). Our finding that ovine COX-1 oxygenates AEA differs from initial reports that wild-type human COX-1 is unable to catalyze AEA metabolism (*11*). To confirm our finding, the products of COX-1 action on AEA were analyzed by LC/MS. COX-1 generated both PG and HETE ethanolamides from AEA, indicating that the observed oxygen uptake was neither nonspecific oxygenation nor experimental artifact (Figure 1). This discrepancy may reflect differences in enzyme preparation, cyclooxygenase activity assay conditions, or species differences between ovine and human COX-1.

Evaluation of the oxygenation of 14 AEA structural analogues highlighted the importance of the endocannabinoid primary alcohol in promoting COX action. The finding that substitution of the AEA hydroxyl group with either a methyl or methoxy group reduced the ability of COX-2 to catalyze oxygenation indicates that a hydrogen bond donor/acceptor is an important determinant in enzyme−substrate interactions.

The capacity of COX enzymes to catalyze AEA oxygenation was shown to be sensitive to modest substrate structural modifications near the polar terminus. Incorporation of a methyl group in the (*S*)-orientation at the 1-position of the ethanolamine moiety of AEA (**4**) more than doubled the rate at which COX-2 catalyzed substrate oxygenation. Stereoinversion of the methyl group (**5**) eliminated the rate enhancement. Interestingly, this methyl-substituted AEA analogue, also known as (*R*)-methanandamide, has been employed in endocannabinoid studies due to its cannabimimetic activity and an increased metabolic stability (*38*−*40*). Our results demonstrate that, in addition to the reported hydrolytic stability, **5** appears to be relatively resistant to COX-1 oxygenation.

The endocannabinoid 2-AG has been shown to be an excellent substrate for both murine and human COX-2 (*13*). This ester is oxygenated by COX-2 as efficiently as arachidonic acid and is a far superior substrate when compared to AEA. Thus, it is not surprising that the amide analogue of 2-AG, **14**, was oxygenated by COX-2 more avidly than AEA. The more stable arachidonylglycerol regioisomer, 1-AG, is a poorer COX-2 substrate than the endocannabinoid, 2-AG (13). Similarly, the 1-AG amide analogue, **15**, proved to be an inferior COX-2 substrate when compared to **14**, although this difference was less pronounced than that observed with the related esters.

A general understanding of AEA binding within COX-2 is provided by examination of the effect of mutations in the enzyme constriction site, the hydrophobic alcove, and Tyr-385. The constriction site residue, Arg-120, appears critical in facilitating AEA oxygenation whereas Tyr-355 appears to be essentially uninvolved in AEA metabolism (Figure 2). Glu-524 has previously been identified as an important constriction site residue in 2-AG oxygenation (*14*). The results presented in Figure 2 suggest that although this amino acid may play a modest role in both arachidonic acid and AEA oxygenation, it is not as critical in AEA oxygenation as it is in 2-AG oxygenation. The observations that endocannabinoid oxygenase activity was eliminated by mutation of Tyr-385 or by incorporation of increased steric bulk in the COX-2 hydrophobic alcove at residue 533 further aided in the construction of a model of AEA binding within COX-2. In total, these results suggest that AEA, like 2-AG, binds within the COX-2 active site in a conformation similar to that of arachidonic acid with the polar ethanolamide moiety near Arg-120 at the constriction site, the 13-*pro*-*S* hydrogen positioned near Tyr-385, and the ω-end situated in the hydrophobic alcove. The demonstration that mutations in Val-349 shift AEA product distribution toward monooxygenated products and, with V349A and V349L enzymes, toward 15-HETE-EA, specifically, supports the general

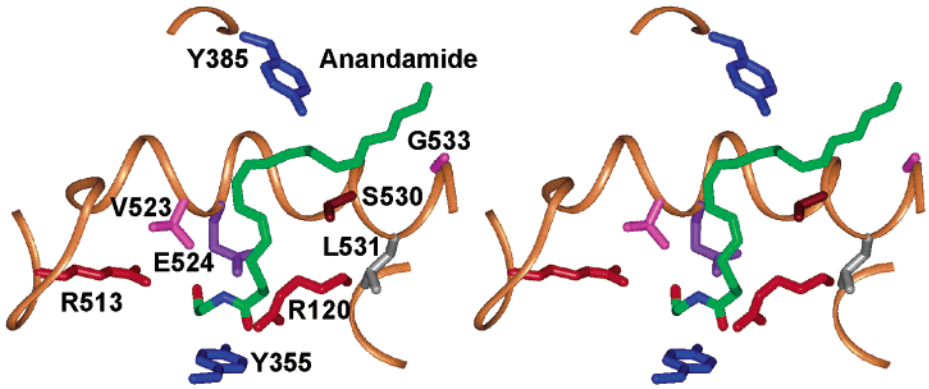


FIGURE 7: Model of AEA bound in the COX-2 active site. A stereoview demonstrates the predicted interactions between AEA and the active site residues of mCOX-2.

conclusion that AEA binds in COX-2 in a conformation similar to those of arachidonic acid and 2-AG (Figure 5). Comparable effects of Val-349 mutations are observed with these COX substrates (*14*, *27*). The observation that both acetylated COX-2 and S530M COX-2 generate 15-HETE-EA when incubated with AEA further supports the conclusion that AEA binds in a conformation similar to those of arachidonic acid and 2-AG as these substrates are also converted to 15-monoxygenated products when treated with these enzymes (Figure 6) (*14*, *28*−*33*). The results with V349 and S530 mutants demonstrate that AEA oxygenation regiochemistry responds to COX-2 active site modifications in a manner similar to that observed with arachidonic acid and 2-AG oxygenation. More generally, these results demonstrate that arachidonic acid and both endocannabinoids are capable of adopting a conformation in the sterically congested active site of acetylated COX-2 that leads to selective C-15 oxygenation in addition to the more typical conformation in unmodified enzyme that leads preferentially to prostanoid formation. However, as each of the residues discussed above is conserved in COX-1, these results provide no insight into the isoform selectivity observed for AEA metabolism.

To address the question of isoform-selective AEA oxygenation, we focused on the residues known to be involved in the selective oxygenation of 2-AG. Previous studies demonstrated that the COX-2 side pocket, in large part, accounts for the ability of COX-2 to efficiently oxygenate 2-AG (*14*). Thus, side pocket mutants were evaluated for AEA oxygenase activity. As in the case of 2-AG, the side pocket residue, Val-523, plays no role in the capacity of COX-2 to oxygenate AEA (Figure 3B). Consistent with studies on 2-AG metabolism, Arg-513 appears to be critical in the observed isoform-selective oxygenation of AEA. Furthermore, all COX enzymes containing a histidine at position 513, including ovine COX-1, displayed reduced activity toward the endocannabinoid. Interestingly, the single mutant, R513H, oxygenated AEA at an initial rate indistinguishable from that of wild-type ovine COX-1 (Figure 3B). This result suggests that the enhanced capacity of COX-2 to metabolize AEA can be entirely attributed to the presence of an arginine residue at position 513. This contrasts with previous studies with 2-AG that indicated that Arg-513 contributes to, but does not entirely account for, the isoform-selective oxygenation of the endocannabinoid (*14*). These results, coupled with the fact that Arg-513 is conserved in all COX-2 proteins reported to date, support the assertion that endocannabinoid oxygenation may represent a fundamental COX-2 function.

A model of 2-AG binding within the COX-2 active site has recently been proposed (*14*). In this model, 2-AG is shifted within the COX-2 active site with respect to the crystallographically determined position of arachidonic acid. This shift was interpreted as a means to promote interactions between the endocannabinoid glycerol alcohols and Arg-513. As a consequence of this shift, 2-AG is positioned further from Leu-531, an active site residue opposite the side pocket, than arachidonic acid. A test of this model was provided by examining the effects of Leu-531 mutations on 2-AG and arachidonic acid turnover. Mutations in this residue had more pronounced deleterious effects on arachidonic acid oxygenation than on 2-AG metabolism, supporting the conclusion that the endocannabinoid is positioned further from Leu-531 than the free acid. Our data support an interaction between AEA and Arg-513 similar to that observed with 2-AG. Consequently, a similar confirmatory test of this interaction could be applied by examining the effects of Leu-531 mutations on both AEA and arachidonic acid turnover. As shown in Figure 4, mutations in Leu-531 reduce the ability of mutant enzymes to oxygenate arachidonic acid much more significantly than they reduce the ability to oxygenate AEA. Remarkably, the L531A enzyme catalyzes oxygen uptake more than 6-fold more rapidly when incubated with the endocannabinoid than with arachidonic acid. These results suggest that AEA, like 2-AG, is displaced toward the side pocket of COX-2, and away from Leu-531, relative to arachidonic acid.

Guided by the results of the site-directed mutagenesis studies, a model for AEA binding to COX-2 was developed (Figure 7). Distance restraints were imposed between the implicated polar residues in COX-2 (Arg-120, Arg-513, Glu-524) and the ethanolamide moiety of AEA, and molecular dynamic simulations were performed to optimize substrate−enzyme interactions. These simulations resulted in the positioning of the amide carbonyl between Arg-120 and Tyr-355. The calculated distances between the AEA carbonyl oxygen and the closest Arg-120 guanidinium nitrogen and Tyr-355 hydroxyl groups were 3.1 and 2.8 Å, respectively. The primary alcohol of AEA adopted a conformation that suggests hydrogen bonding to the Arg-513 guanidinium group. The distance between the alcohol oxygen of AEA and the closest Arg-513 guanidinium nitrogen was measured to be 3.1 Å. This arrangement resembles that reported for

2-AG binding within COX-2 in which the glycerol alcohol oxygens are positioned 2.9 and 3.3 Å from the closest Arg-513 guanidinium nitrogen (*14*). To facilitate the interaction between the AEA hydroxyl group and Arg-513, the substrate is shifted toward the side pocket within the active site. Modeling results positioned the AEA carbonyl carbon greater than 7 Å from the closest Leu-531 methyl group, which contrasts sharply from the experimentally determined distance of 4.6 Å between C-1 of arachidonic acid and Leu-531 (*25*). This conformation closely resembles that reported for 2-AG in which the endocannabinoid carbonyl carbon is positioned 7.1 Å from Leu-531 (*14*). Thus, COX-2 accommodates an AEA binding conformation that provides for close interaction between polar enzyme residues implicated in AEA oxygenation and hydrogen bond donors/acceptors of the endocannabinoid.

The present results suggest that endocannabinoids bind in a productive conformation in COX-2 similar to arachidonic acid. However, subtle differences have been identified and have proven consistent with both 2-AG and AEA. The endocannabinoid polar headgroups bind near the cyclooxygenase active site constriction interacting with polar residues in this enyzme region including Arg-120 and Arg-513. The universal presence of arginine 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 endocannabinoid metabolism. When compared to the binding of arachidonic acid in the COX-1 active site, the polar termini of endocannabinoids are displaced toward Arg-513 and away from Leu-531, facilitating a critical interaction between endocannabinoid primary alcohol moieties and the guanidinium of Arg-513. Despite this different binding mode, endocannabinoids, like arachidonic acid, are oxygenated by wild-type COX-2 to provide prostaglandin-like lipids and by aspirin-acetylated COX-2 to provide 15-HETE derivatives. Finally, although AEA was oxygenated by ovine COX-1, the endocannabinoid proved to be a superior COX-2 substrate. This is consistent with previous findings that human COX-2 oxygenated AEA more efficiently than human COX-1 (*11*). Taken together, these findings support the hypothesis that endocannabinoid oxygenation is a COX-2 selective function and may represent a significant evolutionary impetus for the existence of two cyclooxygenase isoforms.

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SUPPORTING INFORMATION AVAILABLE

Reaction details and spectroscopic data for arachidonylamides. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. 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*, 1882−1884.
2. Maccarrone, M., Falciglia, K., Di Rienzo, M., and Finazzi-Agro, A. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 309−317.
3. Wenger, T., and Moldrich, G. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 301−307.
4. Lichtman, A. H., Varvel, S. A., and Martin, B. R. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 269−285.
5. Fernandez-Ruiz, J., Lastres-Becker, I., Cabranes, A., Gonzalez, S., and Ramos, J. A. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 257−267.
6. Walker, J. M., and Huang, S. M. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 235−242.
7. Hogestatt, E. D., and Zygmunt, P. M. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 343−351.
8. Pinto, L., Capasso, R., Di Carlo, G., and Izzo, A. A. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 333−341.
9. Parolaro, D., Massi, P., Rubino, T., and Monti, E. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 319−332.
10. Di Marzo, V., De Petrocellis, L., Fezza, F., Ligresti, A., and Bisogno, T. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 377−391.
11. Yu, M., Ives, D., and Ramesha, C. S. (1997) *J. Biol. Chem. 272*, 21181−21186.
12. So, O. Y., Scarafia, L. E., Mak, A. Y., Callan, O. H., and Swinney, D. C. (1998) *J. Biol. Chem. 273*, 5801−5807.
13. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J. Biol. Chem. 275*, 33744−33749.
14. Kozak, K. R., Prusakiewicz, J. J., Rowlinson, S. W., Schneider, C., and Marnett, L. J. (2001) *J. Biol. Chem. 276*, 30072−30077.
15. Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. D., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., and Eling, T. E. (1984) *Mol. Pharmacol. 26*, 328−335.
16. Rowlinson, S. W., Crews, B. C., Lanzo, C. A., and Marnett, L. J. (1999) *J. Biol. Chem. 274*, 23305−23310.
17. Kalgutkar, A. S., and Marnett, L. J. (1994) *Biochemistry 33*, 8625−8628.
18. Khanolkar, A. D., Abadji, V., Lin, S., Hill, W. A. G., Taha, G., Abouzid, K., Meng, Z., Fan, P., and Makriyannis, A. (1996) *J. Med. Chem. 39*, 4515−4519.
19. Cairns, T. L., and Fletcher, J. H. (1941) *J. Am. Chem. Soc. 63*, 1034−1035.
20. Ueda, N., Yamamoto, K., Yamamoto, S., Tokunaga, T., Shirakawa, E., Shinkai, H., Ogawa, M., Sato, T., Kudo, I., Inoue, K., Takizawa, H., Nagano, T., Hirobe, M., Matsuki, N., and Saito, H. (1995) *Biochim. Biophys. Acta 1254*, 127−134.
21. Picot, D., Loll, P. J., and Garavito, R. M. (1994) *Nature 367*, 243−249.
22. Kiefer, J. R., Pawlitz, J. L., Moreland, K. T., Stegeman, R. A., Hood, W. F., Gierse, J. K., Stevens, A. M., Goodwin, D. C., Rowlinson, S. W., Marnett, L. J., Stallings, W. C., and Kurumbail, R. G. (2000) *Nature 405*, 97−101.
23. 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.
24. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) *Nat. Struct. Biol. 3*, 927−933.
25. Malkowski, M. G., Ginell, S. L., Smith, W. L., and Garavito, R. M. (2000) *Science 289*, 1933−1937.
26. Shimokawa, T., Kulmacz, R. J., DeWitt, D. L., and Smith, W. L. (1990) *J. Biol. Chem. 265*, 20073−20076.
27. Thuresson, E. D., Lakkides, K. M., Rieke, C. J., Sun, Y., Wingerd, B. A., Micielli, R., Mulichak, A. M., Malkowski, M. G., Garavito, R. M., and Smith, W. L. (2001) *J. Biol. Chem. 276*, 10347−10359.
28. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L. (1994) *J. Biol. Chem. 269*, 13207−13215.
29. Xiao, G., Tsai, A.-L., Palmer, G., Boyar, W. C., Marshall, P. J., and Kulmacz, R. J. (1997) *Biochemistry 36*, 1836−1845.
30. Mancini, J. A., Vickers, P. J., O'Neill, G. P., Boily, C., Falgueyret, J. P., and Riendeau, D. (1997) *Mol. Pharmacol. 51*, 52−60.
31. Schneider, C., and Brash, A. R. (2000) *J. Biol. Chem. 275*, 4743−4746.
32. Meade, E. A., Smith, W. L., and DeWitt, D. L. (1993) *J. Biol. Chem. 268*, 6610−6614.
33. 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.
34. Moody, J. S., Kozak, K. R., Ji, C., and Marnett, L. J. (2000) *Biochemistry 40*, 861−866.
35. 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.

36. Kozak, K. R., and Marnett, L. J. (2002) *Prostaglandins, Leukotrienes Essent. Fatty Acids 66*, 211−220.
37. Smith, W. L., and Langenbach, R. (2001) *J. Clin. Invest. 107*, 1491−1495.
38. Abadji, V., Lin, S., Taha, G., Griffin, G., Stevenson, L. A., Pertwee, R. G., and Makriyannis, A. (1994) *J. Med. Chem. 37*, 1889−1893.
39. Baker, D., Pryce, G., Croxford, J. L., Brown, P., Pertwee, R. G., Huffman, J. W., and Layward, L. (2000) *Nature 404*, 84−87.
40. Calignano, A., La Rana, G., Giuffrida, A., and Piomelli, D. (1998) *Nature 394*, 277−281.

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