13-Methylarachidonic Acid Is a Positive Allosteric Modulator of Endocannabinoid Oxygenation by Cyclooxygenase*

Shalley N. Kudalkar‡, Spyros P. Nikas†, Philip J. Kingsley‡, Shu Xu‡, James J. Galligan‡, Carol A. Rouzer¶
Surajit Banerjee***, Lipin Ji*, Marsha R. Eno*, Alexandros Makriyannis*, and Lawrence J. Marnett‡‡
From the †A. B. Hancock Jr. Memorial Laboratory for Cancer Research, Departments of Biochemistry, ‡Chemistry, and §‡‡Pharmacology, Vanderbilt Institute of Chemical Biology, ‡‡Vanderbilt Center in Molecular Toxicology, ‡§Vanderbilt Ingram Cancer Center, and ††Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, the †Department of Pharmaceutical Sciences, Northeastern University, Boston, Massachusetts 02115, and the ***Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, and the †††Northeastern Collaborative Access Team, Argonne National Laboratory, Argonne, Illinois 60439

Background: The structurally identical monomers of cyclooxygenase-2 act as distinct catalytic and allosteric subunits.

Results: 13-Methylarachidonic acid strongly potentiates cyclooxygenase-2-mediated endocannabinoid oxygenation without affecting arachidonic acid oxygenation.

Conclusion: 13-Methylarachidonic acid is a substrate-selective allosteric potentiator of cyclooxygenase-2-mediated endocannabinoid oxygenation.

Significance: 13-Methylarachidonic acid is a valuable tool for probing cyclooxygenase-dependent endocannabinoid oxygenation.

Cyclooxygenase-2 (COX-2) oxygenates arachidonic acid (AA) and the endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonylethanolamine to prostaglandins, prostaglandin glyceryl esters, and prostaglandin ethanolamides, respectively. A structural homodimer, COX-2 acts as a conformational heterodimer with a catalytic and an allosteric monomer. Prior studies have demonstrated substrate-selective negative allosteric regulation of 2-AG oxygenation. Here we describe AM-8138 (13(S)-methylarachidonic acid), a substrate-selective allosteric potentiator that augments 2-AG oxygenation by up to 3.5-fold with no effect on AA oxygenation. In the crystal structure of an AM-8138-COX-2 complex, AM-8138 adopts a conformation similar to the unproductive conformation of AA in the substrate binding site. Kinetic analysis suggests that binding of AM-8138 to the allosteric monomer of COX-2 increases 2-AG oxygenation by increasing \( k_{cat} \) and preventing inhibitory binding of 2-AG. AM-8138 restored the activity of COX-2 mutants that exhibited very poor 2-AG oxygenating activity and increased the activity of COX-1 toward 2-AG. Competition of AM-8138 for the allosteric site prevented the inhibition of COX-2-dependent 2-AG oxygenation by substrate-selective inhibitors and blocked the inhibition of AA or 2-AG oxygenation by nonselective time-dependent inhibitors. AM-8138 selectively enhanced 2-AG oxygenation in intact RAW264.7 macrophage-like cells. Thus, AM-8138 is an important new tool compound for the exploration of allosteric modulation of COX enzymes and their role in endocannabinoid metabolism.

The endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide are arachidonic acid (AA)-containing lipid signaling molecules that exert analgesic and anti-inflammatory effects via the activation of the cannabinoid receptors CB1 and CB2 (1, 2). Although the metabolic fate of these lipid mediators is dominated by their hydrolysis to free AA (3), growing evidence suggests that endocannabinoid oxygenation by cyclooxygenase-2 (COX-2) is an important alternative pathway (4, 5). The cyclooxygenase enzymes (COX-1 and COX-2) oxygenate free AA to the bicyclic endoperoxide prostaglandin (PG)H₂, which serves as the precursor for the production of multiple bioactive lipids that regulate a broad range of physiologic responses (6–8) (see Fig. 1A). Similarly, 2-AG and arachidonylethanolamide are oxygenated by COX-2 to PGH₂ glyceral ester and ethanolamide, respectively (5, 9, 10) (see Fig. 1A). The PG glyceral ester (PG-G) and PG ethanolamide derivatives that result from COX-2-dependent 2-AG oxygenation exhibit a wide array of physiologic effects, including activation of calcium mobilization in tumor cells and macrophages, modulation of inhibitory synaptic transmission, induction of neurotoxicity by enhancement of excitatory glutamatergic synaptic transmission, and induction of hyperalgesia and anti-inflammatory responses (11–16). In addition, mounting evidence suggests that COX-2-mediated oxygenation serves to regulate endocannabinoid tone in the central nervous system (4, 17).

The physiologic and pathophysiologic importance of the COX enzymes is illuminated by the fact that they are the primary targets of widely used nonsteroidal anti-inflammatory
Substrate-selective Activation of COX-2 by AM-8138

drugs such as aspirin, naproxen, and ibuprofen (18). The avail-
avility of so many COX inhibitors should provide useful tool
compounds for the exploration of COX-2-mediated endocan-
nabinoid oxygenation in vivo. However, under the conditions
that these inhibitors are normally used, they block both AA and
endocannabinoid oxygenation, making it impossible to differ-
entiate the roles of these distinct pathways. These consider-
ations highlight the importance of our recent discovery that
some weak, rapidly reversible inhibitors of AA oxygenation are
strong time-dependent inhibitors of 2-AG oxygenation (19,
20). This phenomenon, which we call substrate-selective inhibi-
tion, has been used to explore the role of COX-2 in modulat-
ing endocannabinoid tone in vivo, suggesting that suppression
of this pathway has anxiolytic effects (4, 21).

The COX enzymes are structural homodimers that behave as
functional heterodimers (22–26) (see Fig. 1B). Full catalytic
activity is obtained through binding a single molecule of the
required heme prosthetic group per dimer, suggesting that the
heme-containing subunit is catalytic, whereas the second sub-
unit serves an allosteric function (27). Indeed, growing evidence
indicates that various molecules, including non-substrate fatty
acids as well as some nonsteroidal anti-inflammatory drugs,
modulate COX activity by binding to the allosteric subunit (19,
20, 25, 26). We have proposed that substrate-selective inhibi-
tion results from high affinity binding of inhibitors to the allo-
steric site where they have little to no effect on AA oxygenation
but profoundly inhibit 2-AG oxygenation. These same inhibi-
tors only inhibit AA oxygenation when a second molecule binds
with lower affinity to the active site as well (20). Thus far, all
substrate-selective modulators of 2-AG oxygenation have been
inhibitory. Here, we report the discovery of AM-8138 (13(S,-
methylarachidonic acid) a substrate-selective positive allosteric
modulator of COX-2-mediated 2-AG oxygenation. This mole-
cule provides an important new tool for the elucidation of the
mechanism of allosteric control of COX enzymes and their role in
endocannabinoid metabolism.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma-Aldrich
unless otherwise stated. Phenol was obtained from Fisher Sci-
entific. Flurbiprofen was purchased from Cayman Chemicals
(Ann Harbor, MI), and hematin was procured from Frontier
Scientific (Newark, DE).

Enzyme Preparation—The expression and purification of
recombinant mCOX-2 from Sf9 cells were performed as
described previously (28) COX-1 was purified from sheep sem-
inal vesicles as described (29). To remove traces of heme from
mCOX-2, 5 mM glutathione and 0.05% deoxycholate (sodium
salt) were added to the size exclusion buffer. Site-directed
mutagenesis to generate COX-2 active site mutants was per-
formed as described previously (30).

Oxygen Consumption Assays—To determine COX activity,
pure mCOX-2 protein (50 nM) was reconstituted with 2 eq of
hematin and then incubated in a 1-ml thermostatted cuvette at
37 °C in a solution containing 100 mM Tris-HCl, pH 8.0 and 0.5
mM phenol. Substrates or AM compounds were added to a final
concentration of 0–100 μM, and oxygen consumption was moni-
tored using a Hansatech OXYG1 Plus connected to a
DW1 oxygen electrode chamber controlled with Oxyview soft-
ware (PP Systems Inc., Amesbury, MA). In the experiments
involving a combination of substrates and AM compounds, the
enzyme was either preincubated with AM compounds for 3–15
min, or the AM compounds were co-administered with sub-
strates. Oxygen consumption was measured for 5–10 min
depending on the assay.

Inhibition and Stimulation of Cyclooxygenase Activity by AM
Compounds—to determine the inhibitory effects of AM com-
ounds on COX activity, 50 nM mCOX-2 or oCOX-1 was incu-
bated with the desired compound for 15 min in a solution con-
taining 100 mM Tris, pH 8.0 and 0.5 mM phenol. Following the
preincubation, AA or 2-AG was added for 30 s at 37°C. The
reaction was quenched by addition of ice-cold ethyl acetate
containing 0.5% (v/v) acetic acid and 0.5 μM internal standards
(PGE2-d4 and PGE2-G-d3). The solution was then vigorously
mixed and cooled on ice, and the organic layer was removed
and dried under nitrogen for the analysis of PG or PG-G con-
tent by LC-MS/MS. Potentiation assays were carried out in a
similar manner as the inhibition assay; however, AM com-
pounds were added along with AA or 2-AG. For analysis of the
effects of AM-8138 on COX-2 kinetics, the concentration of
AA or 2-AG was varied while keeping the concentration of AM
compounds at 5 μM. AM compounds were co-added with
2-AG, and the reaction was quenched after 15 s. The kinetic
data were fit using Prism software to a substrate-dependent
inhibition model as described in Equation 1.

\[
V_0 = \frac{V_{max}[S]}{K_m + [S]} + \frac{[S]}{K_I}
\]

(Determination of AA and 2-AG Oxygenation Products by
LC-MS/MS—Samples were reconstituted in MeOH:H2O (1:1)
and chromatographed using a Shimadzu LC system equipped
with a Luna C18(2) column (50 × 2 mm, 3 μm) (Phenomenex)
with an isocratic elution method requiring 66% (v/v) 5 mM
ammonium acetate, pH 3.6 (solvent A) and 34% (v/v) acetonitrile
containing 6% (v/v) solvent A (solvent B) at a flow rate of
0.350 ml/min. MS/MS was conducted using an AB SCIEX MS
system with the following transitions: m/z 370 → 317 for PGE2/
D2, m/z 374 → 321 for PGE2-d4, m/z 444 → 391 for PGE2/D2-G,
and m/z 449 → 396 for PGE2-G-d3. Peak areas were normal-
ized to their deuterated internal standards for the quantification
of analytes.

Blocking or Reversibility of COX-2 Inhibition by Slow Tight
Binding Inhibitors and Weak Reversible Inhibitors—Reactions
were conducted under similar conditions as those used for the
inhibition assay. For the assessment of the effect of AM-8138 on
the potency of weak reversible inhibitors, mCOX-2 was prein-
cubated with 25 μM (R)-flurbiprofen for 3 min, and reactions
were initiated by the addition of AA or 2-AG and 5 μM
AM-8138. Reactions were quenched and analyzed via LC-MS/MS
as described above. For the assessment of the effect of
AM-8138 on the potency of slow, tight binding inhibitors,
enzyme at 50 nM was first preincubated with AM-8138 for 3
min at 37 °C prior to the addition of 10 μM indomethacin, 100
nM diclofenac, or vehicle (DMSO) for an additional 15 min at
37 °C. Reactions were initiated by the addition of 5 µM AA or 2-AG.

Effects of AM-8138 in RAW264.7 Macrophages—RAW264.7 macrophages were plated at 10% confluence in DMEM + GlutaMAX supplemented with 10% fetal bovine serum. After 24 h, the medium was replaced with serum-free DMEM + GlutaMAX containing 15 µM AM-8138 (31). After 24 h, the cells were washed with 1 volume of serum-free DMEM + GlutaMAX to remove any unincorporated AM-8138 and treated with DMEM + GlutaMAX ± 100 ng/ml Kdo2-lipid A (KLA) for 6 h followed by the addition of 5 µM ionomycin for 45 min. Cells were then scraped into the medium, and the resulting samples were extracted with an equivalent volume of ethyl acetate containing 0.5% acetic acid and the appropriate internal standards. The organic layer was dried under an inert gas stream and dissolved in methanol for LC-MS/MS analysis as described above.

Crystallization, Data Collection, Structure Determination, and Refinement—Crystallization of mCOX-2 was carried out as described previously with modest modification (30). mCOX-2 was reconstituted with a 2-fold molar excess of Co3+ protoporphyrin IX. The enzyme solution was dialyzed at 4 °C against a solution containing 20 mM sodium phosphate buffer, pH 6.7 containing 100 mM NaCl, 0.6% (w/v) β-octyl glucoside, and 0.1% NaN3. The concentration of β-octyl glucoside was adjusted to 1.2%, and a 10-fold molar excess of AM-8138 was added to the protein solution for 30 min prior to crystallization. Crystallization was performed using the hanging drop method by mixing 3.5 ml of the protein-inhibitor complex with 3.5 ml of a crystallization solution consisting of 50 mM EPPS buffer, pH 8.0 containing 120 mM MgCl2 and 28% PEG monomethyl ether 550. Crystals were transferred into a solution consisting of 50 mM EPPS, pH 8.0 containing 120 mM MgCl2 and 28% PEG monomethyl ether 550 for about 30 s, and the samples were then frozen in liquid nitrogen for data collection.

Diffraction data were collected using the synchrotron radiation x-ray source with 100 K liquid nitrogen streaming at beamline 24-ID-C in the Advance Photon Source at Argonne National Laboratory. Diffraction data were collected and processed with XDS (32) and determined as C2 space group. Initial phases were determined by molecular replacement using monomer coordinates (Protein Data Bank code 3NT1, chain A) with Phaser (33). The model was improved with several rounds of model building in Coot (34) and PHENIX (35). Only one monomer was observed in the asymmetric unit. Chemical constraints for AM-8138 were generated through SMILES in Coot (34). Water molecules were added during the last cycles of refinement, and translation-libration-screw refinement was applied (36). The potential of phase bias was excluded by simulated annealing using PHENIX (37). The values of the Ramachandran plot for the final refinement of the structure were obtained by use of the PHENIX suite. X-ray diffraction data collection and structural refinement statistics are reported in Table 1. The illustrations contain representations of monomer A and were prepared with PyMOL version 1.5.0.4 (Schrödinger, LLC).

RESULTS

Characterization of Fatty Acid Analogs—We initially investigated AM-8138 along with its glyceryl ester (AM-8125) and ethanolamide (AMG-313) analogs (Fig. 1C) as substrates and allosteric modulators of COX-2 activity. A polarographic assay that measures oxygen consumption demonstrated that AM-8125 and AMG-313 were poor substrates for mCOX-2, exhibiting total oxygen consumption of ~5–7% of that recorded when AA was the substrate (Fig. 2A). In contrast, no oxygenation of AM-8138 by mCOX-2 was observed (Fig. 2A).

Each methylarachidonate analog was further tested for its ability to regulate the COX-2-dependent oxygenation of AA or 2-AG in vitro by measuring PG and PG-G formation, respectively, by LC-MS/MS or total oxygen consumption polarographically. Following a 15-min preincubation with the enzyme, AMG-313 weakly inhibited AA and 2-AG oxygenation with 50% inhibition observed at ~50 µM compound (Fig. 2B). No inhibitory effects on AA or 2-AG oxygenation were seen when the enzyme was incubated with either AM-8125 or AM-8138. In contrast, these fatty acid analogs increased 2-AG oxygenation by ~3.5-fold (Fig. 3, A and C) but exerted no effect on the ability of the enzyme to oxygenate AA (Fig. 3, B and D). Because, AM-8125 and AMG-313 were metabolized by mCOX-2, we focused our attention on AM-8138, which was neither a substrate nor an inhibitor.

Potentiation of 2-AG Oxygenation by AM-8138—AM-8138 caused a concentration-dependent increase in 2-AG oxygenation with a maximal effect observed at 10 µM (Fig. 3C). The ~3-fold increase in total oxygen consumption observed upon co-incubation of 5 µM 2-AG with 20 µM AM-8138 in the presence of 50 nM mCOX-2 correlated well with the increase in total product formation as determined by LC-MS/MS (Fig. 4, A and B). Total products in this assay include PG-Gs, the primary products of COX-2-mediated oxygenation of 2-AG, and hydroxyeicosatetraenoic acid glyceryl esters (HETE-Gs), which are minor products. Further evaluation of the concentration dependence of AM-8138-mediated activation of 2-AG oxygenation provided us with an EC50 of ~0.7 µM (Fig. 4C).

Binding Mode of AM-8138 in mCOX-2—We solved the structure of AM-8138 bound in the active site of mCOX-2 complexed with Co3+ protoporphyrin IX at a resolution of 2.16 Å (C2 space group; Table 1). All four monomers in the asymmetric unit were very similar, as indicated by root mean square deviation values in the range of 0.11–0.27 Å for the main chain atoms. Clear electron density was observed in the active sites of all four monomers, indicating that the ligand is bound in the active site. Further crystallographic modeling suggested that AM-8138 is bound in a conformation in which its carboxylic acid group forms two hydrogen bonds to Tyr-385 and Ser-530 at the top of the active site with a distance of 2.3 and 2.6 Å, respectively (Fig. 5). This “inverted” binding of AM-8138 contrasts to the productive binding of AA in which the carboxylate is at the base of the active site; however, a similar, catalytically non-productive conformation of AA complexed with mCOX-2 has been reported (38–40). Some flexibility in the electron density was noted in the ω-end of AM-8138, and occupancy refinement from the PHENIX suite (35) suggested that this low quality of electron density in the active site was not due to lower...
Substrate-selective Activation of COX-2 by AM-8138

FIGURE 1. Metabolic pathways initiated by COX-2 and the foundation for allosteric modulation of the enzyme. A, COX-2 catalyzes the conversion of AA, 2-AG, and arachidonylethanolamide (AEA) to PGH₂, PGH₂-G, and PGH₂ ethanolamide (EA), respectively. These intermediates are subject to further metabolism by specific synthases, which produce bioactive prostaglandins or their glyceryl ester or ethanolamide analogs. B, COX-2 is a structural homodimer that functions as a heterodimer. Complete activity is achieved with binding of only a single molecule of heme to one subunit. This subunit serves as the catalytic monomer, whereas the other subunit exerts allosteric regulation. In the figure, heme (red) is shown in the catalytic site (cat) (orange), and the allosteric site (allo) is show in blue. C, structures of AM-8138, its glyceryl ester (AM-8125), and ethanolamide (AMG-313) analogs.

FIGURE 2. Characterization of fatty acid analogs as COX-2 substrates or inhibitors. A, oxygenation of 50 μM AA, 2-AG, AM-8125, and AMG-313 by 100 nM mCOX-2. No oxygenation of AM-8138 was observed. The oxygen consumption assay was carried out as described under “Experimental Procedures.” The data are representative of different experiments involving triplicate determinations. The error bars indicate ± S.D. B, inhibition of AA and 2-AG oxygenation by AMG-313. Product formation was monitored following the oxygenation of AA and 2-AG by purified COX-2 to form PGs (solid lines) and PG-Gs (dashed lines). IC₅₀ values were calculated using nonlinear regression. The results are from three experiments involving triplicate determinations. The error bars indicate ± S.D. ND, not detected.
occupancies of the ligand but rather flexibility/multiple conformations of AM-8138. The final model contains the major conformation in which the (S)-methyl group at the C-13 position of AM-8138 is inserted into the pocket above the constriction site near Val-349, Leu-359, and Tyr-355. It is notable that the side chain of Leu-531 in all four monomers is flipped away from the active site to accommodate the \( /H9275 \)-tail of AM-8138 as has been observed in the crystal structure of 1-AG bound to COX-2 (41).

**AM-8138 Restores 2-AG Oxygenation of Inactive Mutants**—Arg-120, Tyr-355, and Ser-530, which are critical residues for 2-AG binding and oxygenation by mCOX-2 (39, 41, 42), are also involved in the interaction of AM-8138 with the enzyme (Fig. 5). Hence, we evaluated the effect of mutation of these residues, i.e. Y355F, Y355A, R120A, R120Q, and S530A, on the ability of AM-8138 to potentiate 2-AG oxygenation. Of these, the R120A and Y355F mutations were moderately inhibitory to AA oxygenation, whereas the other mutants retained wild-type activity with this substrate. Addition of AM-8138 had no significant effect on AA oxygenation by any of the mutants with the exception of Y355A in which it was inhibitory (Fig. 6A). The S530A and Y355F mutations had no effect on baseline activity or on the ability of AM-8138 to effect a 3-fold increase in 2-AG oxygenation as observed in the wild-type enzyme (Fig. 6B). In contrast, Y355A, R120A, and R120Q exhibited marked reductions in 2-AG oxygenating capacity that were restored by AM-8138 to levels observed in the AM-8138-stimulated wild-type COX-2. Interestingly, in the presence of AM-8138, 2-AG oxygenation by R120Q was higher than that of the wild-type

**FIGURE 3. Effects of AM-8138 and AM-8125 on mCOX-2-dependent oxygenation of 2-AG and AA.** Following co-incubation of 5 \( \mu \text{M} \) 2-AG (A and C) or AA (B and D) with AM-8138 (A and B) or AM-8125 (C and D) in a solution containing 50 \( \text{nm} \) mCOX-2 at 37 °C, PG-Gs (A and C) and PGs (B and D) were analyzed by LC-MS/MS as described under “Experimental Procedures.” The results are from three different experiments involving triplicate determinations. The error bars indicate ±S.D., and statistical significance of \( p = 0.01–0.05 \) (*), \( p = 0.001–0.01 \) (**), and \( p < 0.0001 \) (****) relative to zero concentration of AM-8138 is indicated. ns, not significant.

**FIGURE 4. Comparison of maximum oxygen consumption with products formed upon oxygenation of 2-AG by mCOX-2 in the presence of AM-8138.** \( \text{O}_2 \) uptake was measured by polarographic assay (A) or LC-MS/MS (B) during a 30-s incubation of 5 \( \mu \text{M} \) 2-AG with 50 \( \text{nm} \) mCOX-2 in the presence or absence of AM-8138 at concentrations of 1 and 20 \( \mu \text{M} \). The data are representative of three experiments involving triplicate determinations. The error bars indicate ±S.D.

C, analysis of 2-AG oxygenation in the presence of varying concentrations of AM-8138 was carried out as described under “Experimental Procedures.” The data are from a single experiment involving triplicate determinations. The error bars indicate ±S.E., and ** indicates \( p = 0.001–0.01 \).
TABLE 1
X-ray data collection and refinement statistics

| Statistic | Value |
|-----------|-------|
| Wavelength (Å) | 0.9792 |
| Resolution range (Å) | 2.16 (2.24–2.16) |
| Space group | C2 |
| Unit cell | 215.8Å, 121.5Å, 134.8Å, 90°, 123.6°, 90° |
| Total reflections | 867,679 (86,519) |
| Unique reflections | 152,390 (15,303) |
| Multiplicity | 5.7 (5.7) |
| Completeness (%) | 98.12 (95.94) |
| Mean I/σ(I) | 10.12 (1.48) |
| Wilson B-factor | 39.66 |
| Rmerge (%) | 0.136 (1.35) |

In contrast, the less disruptive L531I mutant displayed 35 and 30% of wild-type mCOX-2 activity for AA and 2-AG oxygenation, respectively. The oxygenation of AA was unaffected upon addition of AM-8138 (Fig. 6A); however, a 3-fold increase in 2-AG oxygenation was observed in the presence of AM-8138 (Fig. 6B).

Finally, we targeted Val-349, which lines the hydrophobic channel adjacent to Ser-530 in the COX-2 active site. Mutation of Val-349 to Ala results in an increase in monooxgenated products (i.e. HETE-Gs) relative to PG-Gs upon oxygenation of 2-AG (42). The V349A mutant generated HETE-Gs instead of PG-Gs, and the amount of HETE-Gs generated in the presence of AM-8138 was 7-fold higher than that produced in the absence of AM-8138 (Fig. 7).

Steady-state Kinetics of Potentiation—To gain insight into the mechanism of potentiation of AM-8138 and its effect on catalytic efficiency of the enzyme, we carried out a steady-state kinetic analysis of 2-AG oxygenation in the presence and absence of the compound (Fig. 8). In the absence of AM-8138, initial rates of 2-AG oxygenation by mCOX-2 increased with substrate concentration up to 5 μM and then decreased (Fig. 8A, red circles). These results are consistent with a model of substrate-dependent inhibition in which binding of substrate to a second site on the enzyme produces an inactive complex. In the case of COX-2, the most likely binding site is in the allosteric subunit. Fitting the data to this substrate inhibition model yielded an inhibition constant (K_i) of 45 ± 11 μM. AM-8138 markedly reduced substrate inhibition as indicated by a 5.6-fold increase in K_i (Table 2). Furthermore, addition of AM-8138 produced an ~2-fold increase in both K_m and k_{cat} for 2-AG oxygenation (Table 2). These results indicate that AM-8138 stimulates 2-AG oxygenation by increasing the maximal velocity of the reaction and relieving substrate inhibition at the expense of a modest decrease in apparent substrate affinity. A likely explanation of this effect is that AM-8138 binds to the allosteric site of COX-2, precluding inhibitory binding of 2-AG in that site and inducing a conformational change in the catalytic site that results in increased k_{cat} and K_m for 2-AG oxygenation.

We extended our kinetic analysis to evaluate the effects of active site mutants on AM-8138-dependent augmentation of COX-2 activity. The AM-8138-mediated increase in 2-AG oxygenation by Y355F was similar to that of the wild-type enzyme in that both K_m and k_{cat} were higher in the presence of AM-8138 than in its absence, and the apparent substrate-dependent inhibition was markedly reduced (Fig. 8B and Table 2). The S530A mutant exhibited behavior consistent with very strong substrate-dependent inhibition in the absence of AM-8138, whereas in the presence of AM-8138, the S530A mutant produced kinetic behavior similar to that of the Y355F mutant (Fig. 8C and Table 2). The inability of the R120Q mutant to oxygenate 2-AG in the absence of AM-8138 precluded the assessment of its kinetic parameters. However, the presence of AM-8138 increased the catalytic efficiency (k_{cat}/K_m) of this mutant enzyme to levels 2-fold higher than those of the AM-8138-treated wild-type protein primarily as a result of a 2-fold lower value of K_m for 2-AG in the mutant (Fig. 8D and Table 2).

FIGURE 5. Binding of AM-8138 in the mCOX-2 active site. A stereodrawing of the crystal structure of AM-8138 (cyan) bound in the active site of mCOX-2 (gray) is shown. The interacting residues are colored as yellow sticks, the two H-bonds are illustrated as dashes, and the simulated annealing omit map (Feo–F, blue) is contoured in 100% and 50% at the level of 3σ.

enzyme (Fig. 6B). The most remarkable effect was seen with a double alanine mutant of Arg-120 and Tyr-355 (R120A/Y355A) as illustrated in Fig. 6, C and D. This double mutant was unable to utilize AA or 2-AG in the absence of AM-8138. However, addition of AM-8138 restored the oxygenation of 2-AG by the R120A/Y355A mutant to levels observed with the wild-type enzyme incubated with 2-AG alone but had no effect on AA oxygenation.

Our crystal structure suggests that binding of AM-8138 to COX-2 causes the side chain of Leu-531 to adopt a conformation similar to its conformation when 1-AG is bound to the active site (41). This observation suggests that Leu-531 may play an important role in the binding of these ligands. Hence, we tested the effect of a range of Leu-531 mutants on the potentiating ability of AM-8138 (Fig. 6). Very low to undetectable amounts of PGs and PG-Gs were produced by the L531V and L531A mutants with or without AM-8138, consistent with the hypothesis that Leu-531 is important to AM-8138 potentiation.
Allosteric Modulation of Substrate Oxygenation by AM-8138 — As described earlier, substrate-selective inhibitors of COX-2 are believed to inhibit 2-AG oxygenation by binding to the allosteric site (19, 43). Our hypothesis is that AM-8138 also exerts its effects by binding to this site. Thus, we further hypothesized that AM-8138 will compete with substrate-selective inhibitors for binding to the enzyme, thereby preventing inhibition of 2-AG oxygenation. Indeed, our data support this hypothesis as we...
observed that co-incubation of AM-8138 with 2-AG in the presence of 25 μM substrate-selective inhibitor (R)-flurbiprofen completely prevented inhibition of 2-AG oxygenation and potentiated the oxygenation levels severalfold compared with those of uninhibited enzyme (Fig. 9A). Similarly, the inhibitory effect of 100 nM mefenamic acid was prevented upon co-addition of AM-8138 with 2-AG, and the resultant enzyme activity was comparable with that of uninhibited enzyme (Fig. 9B).

We extended our hypothesis concerning the effect of AM-8138 on inhibition of 2-AG oxygenation to include the slow, tight binding COX inhibitors indomethacin and diclofenac, which are not substrate-selective. Slow, tight binding inhibitors display a two-step mechanism of inhibition with the rapid formation of an initial reversible complex followed by a slow transformation to a much more tightly bound form that inhibits COX-2 activity (44–46). Theoretically, binding of such an inhibitor could occur at the catalytic site, directly blocking access to substrate, or at the allosteric site, leading to a conformational change that inactivates the catalytic site. Our extended hypothesis was based on the previously reported finding that binding of a single molecule of these inhibitors to COX-2 is sufficient to inhibit oxygenation of both AA and 2-AG (19, 43, 44). Indeed, preincubation of mCOX-2 with AM-8138 blocked the inhibitory effects of 100 nM mefenamic acid was prevented upon co-addition of AM-8138 with AA or 2-AG for 15 s before quenching the reaction, and LC-MS/MS was used to quantify the products of the reaction as described under "Experimental Procedures." The data are from a single experiment involving triplicate determinations. The error bars indicate ±S.E. The kinetic data were fit using Prism software to a substrate-dependent inhibition model ("Experimental Procedures").

TABLE 2

| Enzyme     | K<sub>m</sub> (μM) | k<sub>cat</sub> (μM s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | K<sub>i</sub> (μM) | k<sub>i</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | k<sub>i</sub>/K<sub>m</sub> (μM<sup>-1</sup> s<sup>-1</sup>) |
|------------|-------------------|-----------------------------|-----------------------------|-------------------|-----------------------------|-----------------------------|
| WT mCOX-2  | 1.3 ± 0.6         | 0.1 ± 0.04                  | 45 ± 11                     | 4.7 ± 0.8         | 1.3 ± 0.1                  | 130 ± 40                    |
| S530A      | ND                | ND                          | ND                          | 1.2 ± 0.5         | 2 ± 0.1                    | 65 ± 12                     |
| Y335F      | 1.3 ± 0.4         | 0.33 ± 0.04                 | 12 ± 3                      | 1.6 ± 0.3         | 1 ± 0.1                    | 2 ± 0.4                     |
| R120Q      | ND                | ND                          | ND                          | 1.3 ± 0.3         | 0.77 ± 0.06                | 33 ± 8                      |
| WT oCOX-1  | 1.2 ± 0.3         | 0.18 ± 0.02                 | 14 ± 4                      | 3.9 ± 0.5         | 0.25 ± 0.05                | 4.7 ± 0.05                  |

a Data were analyzed using a kinetic model for substrate inhibition.

b Curve fitting of the data was successful, but large errors were observed.

c Enzyme activity was too low for evaluation of kinetic constants.

FIGURE 8. Steady-state kinetic analysis of potentiation by AM-8138. Analyses are provided for wild-type mCOX-2 (A), Y335F (B), S530A (C), and R120Q (D). The data points represent the amount of PG-Gs produced with (black circles) or without (red circles) 5 μM AM-8138. AM-8138 was added along with AA or 2-AG for 15 s before quenching the reaction, and LC-MS/MS was used to quantify the products of the reaction as described under "Experimental Procedures." The data are from a single experiment involving triplicate determinations. The error bars indicate ±S.E. The kinetic data were fit using Prism software to a substrate-dependent inhibition model ("Experimental Procedures").

TABLE 2

Steady-state kinetic parameters of 2-AG oxygenation by wild-type mCOX-2, S530A, Y335F, R120Q, and wild-type oCOX-1 with or without AM-8138

AM-8138 (5 μM) was added to 50 nM wild-type (WT) mCOX-2, oCOX-1, or the indicated mutant along with 2-AG ranging in concentration from 0 to 50 μM. Reactions were quenched after 15 s for mCOX-2 and its mutants or 30 s for oCOX-1 with organic solvent containing deuterated internal standards. Product formation was analyzed by LC-MS/MS using selected reaction monitoring and normalized to DMSO control values. Data are the mean ± S.E. of triplicate determinations. ND, not determined.

| Enzyme     | K<sub>m</sub> (μM) | k<sub>cat</sub> (μM s<sup>-1</sup>) | K<sub>i</sub> (μM) | k<sub>i</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | k<sub>i</sub>/K<sub>m</sub> (μM<sup>-1</sup> s<sup>-1</sup>) |
|------------|-------------------|-----------------------------|-------------------|-----------------------------|-----------------------------|
| WT mCOX-2  | 1.3 ± 0.6         | 0.61 ± 0.04                 | 45 ± 11           | 4.7 ± 0.8                   | 1.3 ± 0.1                   | 130 ± 40                    |
| S530A      | ND                | ND                          | ND                | 1.2 ± 0.5                   | 2 ± 0.1                    | 65 ± 12                     |
| Y335F      | 1.3 ± 0.4         | 0.33 ± 0.04                 | 12 ± 3            | 1.6 ± 0.3                   | 1 ± 0.1                    | 2 ± 0.4                     |
| R120Q      | ND                | ND                          | ND                | 1.3 ± 0.3                   | 0.77 ± 0.06                | 33 ± 8                      |
| WT oCOX-1  | 1.2 ± 0.3         | 0.18 ± 0.02                 | 14 ± 4            | 3.9 ± 0.5                   | 0.25 ± 0.05                | 4.7 ± 0.05                  |

a Data were analyzed using a kinetic model for substrate inhibition.

b Curve fitting of the data was successful, but large errors were observed.

c Enzyme activity was too low for evaluation of kinetic constants.
at the catalytic site. This latter model would be in agreement with prior work suggesting that indomethacin binds in the catalytic site of COX-2 (22).

**Potentiation of COX-1-dependent 2-AG Oxygenation by AM-8138**—Previous reports have shown that 2-AG is a poor substrate for COX-1 (47), leading us to question whether AM-8138 would also potentiate COX-1-dependent oxygenation of 2-AG. Indeed, a 10–12-fold increase in PG-G formation was observed when COX-1 was incubated with 2-AG in the presence of 5 μM AM-8138 (Fig. 10A). The levels of PG-Gs produced by COX-1 were ~78% of those produced by AM-8138-treated mCOX-2 (Fig. 10B). AM-8138 caused a concentration-dependent increase in 2-AG oxygenation with a maximal effect observed at 10 μM (Fig. 10A). As was observed with mCOX-2, the presence of AM-8138 did not affect AA oxygenation by oCOX-1 (Fig. 10C). Steady-state kinetic analysis of oCOX-1 revealed that the presence of 5 μM AM-8138 increased $k_{cat}$ by 4-fold and reduced $K_i$ by 2.4-fold with no change in $K_m$ for 2-AG, bringing the catalytic efficiency of oCOX-1 close to that of mCOX-2 in the absence of AM-8138 (Fig. 10D and Table 2).

**Substrate-selective Potentiation of 2-AG Oxygenation in RAW264.7 Cells**—To determine whether allosteric potentiation of 2-AG oxygenation can occur within a cellular context, we preincubated RAW264.7 cells with AM-8138 for 24 h followed by a 6-h activation with the chemically defined lipopolysaccharide KLA to induce COX-2 expression (Fig. 11A). A subsequent 45-min stimulation with ionomycin triggered PG and PG-G biosynthesis. Quantification of the levels of PGs and PG-Gs produced by the cells revealed that AM-8138 exposure had no effect on PG release (Fig. 11B) but resulted in a 4-fold increase in PG-G formation compared with that of untreated cells (Fig. 11C). The levels of KLA-induced COX-2 expression were unaffected by AM-8138, and COX-1 expression was unaffected by treatment with AM-8138 and/or KLA (Fig. 11A). Levels of AA (Fig. 11D) and 2-AG (Fig. 11E) were unaffected by treatment with AM-8138. Analysis of AM-8138 levels at the end of the KLA preincubation confirmed that the compound had been retained by the cells. No significant changes in the levels of AM-8138 were observed as a result of the KLA treatment (Fig. 11F), and we were unable to detect any oxygenated products of AM-8138 upon further inspec-
tion of culture medium or cell lysates, consistent with our in vitro finding that AM-8138 is not a substrate for COX-2. These results demonstrate that AM-8138 is capable of selectively activating 2-AG oxygenation within intact, stimulated RAW264.7 cells.

**DISCUSSION**

Smith and co-workers (24, 25) first proposed that non-substrate fatty acids of COX-2, particularly palmitic acid, positively regulate 2-AG and AA oxygenation by allosteric modulation of oxygenase activity. Kinetic studies indicated that palmitic acid potentiates AA oxygenation by ~2-fold by decreasing its $K_m$ from 8.5 to 3.5 $\mu M$; however, the potentiating effect on 2-AG oxygenation is more modest (22, 23). Their work as well as our own observations of substrate-selective inhibition of 2-AG oxygenation provides the foundation for a model of allosteric regulation of COX-2 in which the heme-bound subunit catalyzes the COX and peroxidase reactions, whereas the heme-free subunit exerts allosteric control, influencing substrate binding and turnover in the catalytic subunit (23).

The current results expand on the concept of positive allosteric modulation of COX activity by non-substrate fatty acids. The data suggest that changes in the catalytic monomer of the COX enzymes brought about by the binding of a non-substrate fatty acid analog of AA to the allosteric monomer potentiate endocannabinoid oxygenation selectively. Our proposed mechanism of allosteric modulation by AM-8138 is illustrated in Fig. 12, which shows that binding of AM-8138 in the allosteric subunit induces a conformational change that favors 2-AG oxygenation by increasing $k_{cat}$ but also prevents inhibitory binding of 2-AG in the allosteric subunit, thereby relieving the substrate-dependent inhibition observed with 2-AG alone. Further support for this model comes from the ability of AM-8138 to protect the enzyme from the inhibition of 2-AG oxygenation by substrate-selective and slow, tight binding inhibitors (Fig. 9).

The model outlined in Fig. 12 suggests that AM-8138 and 2-AG competitively bind to the allosteric subunit, exerting stimulatory and inhibitory effects on 2-AG oxygenation, respectively. If this model is correct, then the AM-8138-induced increase in $K_j$ (Table 2) is a reflection of competition between AM-8138 and 2-AG as described by Equation 2.

$$K_{(app)} = K_j \left(1 + \frac{[P]}{K_p}\right)$$  (Eq. 2)

where $K_{(app)}$ is the observed value of $K_j$ in the presence of the potentiator (AM-8138), $K_j$ is the value in its absence, $[P]$ is the concentration of AM-8138, and $K_p$ is its equilibrium dissociation constant. Using the data in Table 2 and Equation 2 provides an estimated value for $K_p$ of 1 $\mu M$, which is in good agreement with the observed EC$_{50}$ value of 0.7 $\mu M$ (Fig. 4C). These results...
indicate that AM-8138 has a much higher affinity for the allosteric site than 2-AG with a $K_I$ of 45 nM. Thus, AM-8138 is an effective competitor for 2-AG at that site.

Crystallographic studies indicate that AM-8138 binds in a conformation similar to the previously reported non-productive conformation of AA (39). In this conformation, the C-13
methyl group of AM-8138 rests near the floor of the L-shaped channel where it interacts with Arg-120, Tyr-355, and Val-349. Ser-530 and the catalytically important Tyr-385 are hydrogen-bonded to the carboxylic acid of AM-8138. This conformation of AM-8138 does not allow abstraction of any of its allylic hydrogen atoms by the tyrosyl radical of Tyr-385, a required first step in oxygenation. Thus, the crystal structure helps to explain why AM-8138 is not a substrate for COX-2. The crystal structure also shows that binding of AM-8138 to COX-2 causes the side chain of Leu-531 to flip into the position that is observed upon the binding of 1-AG in a productive conformation or AA in a non-productive conformation (38, 39, 41). These results suggest that the flexibility of Leu-531 is key to accommodation of bulkier ligands. Consistently, we found that mutations of Leu-531 to alanine or valine result in marked reduction of the 2-AG oxygenation activity of COX-2 that cannot be rescued by AM-8138, although it is noted that others have reported minimal effects of various Leu-531 mutations on oxygenation of 2-AG (41). In contrast, the less disruptive L531I mutation retained some 2-AG oxygenation capacity and responsiveness to AM-8138 potentiation. Perhaps more intriguing were the effects of mutations at Tyr-355 and Arg-120, which demonstrated striking reductions in 2-AG oxygenation activity that were completely rescued by AM-8138. The results suggest that interaction of AM-8138 with any one of these residues is not required for its potentiating effects. Clearly, further exploration of the interaction of AM-8138 with the COX-2 active site is required to fully decipher the molecular basis of its ability to selectively potentiate 2-AG oxygenation.

The potentiation of 2-AG oxygenation by COX-1 is also consistent with the model in Fig. 12. Application of Equation 2 to the data in Table 2 yields a K_I value of 3.7 μM for binding of AM-8138 to the COX-1 allosteric site, suggesting a somewhat lower affinity than is seen with COX-2. Note that the K_I of 14 μM indicates a higher affinity of 2-AG for the allosteric site of COX-1, implying greater substrate inhibition than is observed with COX-2 (Table 2). Although AM-8138 appears to not compete as well against 2-AG for binding to the allosteric site of COX-1, it exerts a larger effect on k_cat in COX-1 (4-fold) than in COX-2 (2-fold). The increase in catalytic efficiency combined with relief of substrate inhibition results in a substantial stimulation of activity. This result suggests the interesting hypothesis that an endogenous potentiator might act similarly to AM-8138, rendering COX-1 capable of significant PG-G synthesis in vivo. Further work is obviously needed to explore the possible existence of such a naturally occurring potentiating molecule, which could modulate PG-G biosynthesis by both COX isozymes.

The selective effects of AM-8138 on cyclooxygenase activity translated well to a cellular system. Our studies with KLA/isonicmycin-treated RAW264.7 cells demonstrated that AM-8138 maintains its ability to allosterically potentiate cyclooxygenase-dependent oxygenation of 2-AG without affecting the levels of either substrate. Our data suggest that COX-2, which is expressed in large quantities in response to KLA, is likely the major contributor of PG-G synthesis in AM-8138-treated cells. However, the ability of AM-8138 to stimulate PG-G production by COX-1 suggests that at least some of the increase in PG-Gs in the presence of the potentiator may be attributable to COX-1. Further investigation using selective inhibitors is required to evaluate the contribution of each isoform of COX in PG-G synthesis, although the lack of PG-G production by AM-8138-treated RAW264.7 cells not pretreated with KLA (Fig. 11C) argues against a significant role for COX-1. Irrespective of the isoform involved, AM-8138 clearly potentiated PG-G synthesis in the cells and can serve as a useful tool compound for probing the biological activity of PG-Gs and other COX-derived oxygenation products of 2-AG that are typically found at very low levels in cellular systems.

In summary, these findings uncover a mechanism of substrate-selective modulation of COX activity by a non-substrate fatty acid analog of AA. The ability of this analog to selectively augment the oxygenation of 2-AG, partially due to allosteric effects on the active site of the enzyme and partially due to its ability to eliminate substrate inhibition, supports a model of allosteric modulation of the enzyme. The discovery of AM-8138 provides a valuable tool to explore how the binding of an allosteric regulator regulates COX activity toward endocannabinoid oxygenation and may be a useful tool for studying COX-dependent production of PG-Gs and modulation of endocannabinoid tone in a cellular setting.

Acknowledgments—We thank James A. Wepy for valuable input in developing the AM-8138 potentiation scheme. We also thank William N. Beavers for helpful discussions and suggestions. This work is based upon research conducted at the Advanced Photon Source on the Northeastern Collaborative Access Team beamlines, which are supported by National Institutes of Health Grant P41 GM103403 from the NIGMS. Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the United States Department of Energy under Contract DE-AC02-06CH11357.

Note Added in Proof—Surajit Banerjee’s contributions to this article fulfill the JBC authorship criteria but his authorship was inadvertently omitted from the version of the article that was published on February 2, 2015 as a Paper in Press.

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) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949
2. Stella, N., Schweitzer, P., and Piomelli, D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. Nature 388, 773–778
3. Blankman, J. L., and Cravatt, B. F. (2013) Chemical probes of endocannabinoid metabolism. Pharmacol. Rev. 65, 849–871
4. Hermanson, D. J., Gamble-George, J. C., Marnett, L. J., and Patel, S. (2014) Substrate-selective COX-2 inhibition as a novel strategy for therapeutic endocannabinoid augmentation. Trends Pharmacol. Sci. 35, 358–367
5. Rouzer, C. A., and Marnett, L. J. (2011) Endocannabinoid oxygenation by cyclooxygenases, lipooxygenases, and cytochromes P450: cross-talk between the eicosanoid and endocannabinoid signaling pathways. Chem. Rev. 111, 5899–5921
6. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. 69, 145–182
7. van der Donk, W. A., Tsai, A. L., and Kulmacz, R. J. (2002) The cyclooxygenase reaction mechanism. Biochemistry 41, 15451–15458
