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

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*Running Title: Substrate-Selective Activation of COX-2 by AM-8138

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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.

ABSTRACT

Cyclooxygenase-2 (COX-2) oxygenates arachidonic acid (AA) and the endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide, to prostaglandins, prostaglandin glycercyl 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 an 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 (AEA) 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 physiological responses (6-8) (Fig. 1A). Similarly, 2-AG and AEA are oxygenated by COX-2 to PGH2-glycerol ester and ethanolamide respectively (5,9,10) (Fig. 1A). The PG-glycerol ester (PG-G) and ethanolamide (PG-EA) derivatives that result from COX-2-dependent 2-AG oxygenation exhibit a wide array of physiological 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 drugs (NSAIDs), such as aspirin, naproxen, and ibuprofen (18). The availability of so many COX inhibitors should provide useful tool compounds for the exploration of COX-2-mediated endocannabinoid oxygenation in vivo. However, under the conditions that these inhibitors are normally used, they block both AA and endocannabinoid oxygenation, making it impossible to differentiate the roles of these distinct pathways. These considerations 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 inhibition has been used to explore the role of COX-2 in modulating 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) (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 while the second subunit serves an allosteric function (27). Indeed, growing evidence indicates that various molecules, including non-substrate fatty acids as well as some NSAIDs, modulate COX activity by binding to the allosteric subunit (19,20,25,26). We have proposed that substrate-selective inhibition results from high affinity binding of inhibitors to the allosteric site, where they have little to no effect on AA oxygenation but profoundly inhibit 2-AG oxygenation. These same inhibitors 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 molecule 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 (St. Louis, MO) unless otherwise stated. Phenol was obtained from Fisher Scientific (Hanover Park, IL). 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 previously described (28) COX-1 was purified from sheep seminal 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 performed as previously described (30).

Oxygen Consumption Assays – To determine COX activity, pure mCOX-2 protein (50 nM) was reconstituted with 2 equivalents of hematin and then incubated in a 1 mL thermostatted cuvette at 37 °C in a solution containing 100 mM Tris-HCl,
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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 monitored using a Hansatech OXYG1 plus, connected to a DW1 oxygen electrode chamber controlled with Oxyview software (PP Systems Inc, 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 substrates. 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-compounds on COX activity, 50 nM of mCOX-2 or oCOX-1 was incubated with the desired compound for 15 min in a solution containing 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 of internal standards (PGE2-d4 and PGE2-G-d5). 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 content by LC-MS/MS. Potentiation assays were carried out in a similar manner as the inhibition assay; however AM-compounds 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,

\[ V_0 = \frac{V_{\text{max}}[S]}{K_M + [S]} \left( \frac{[S]}{K_I} + 1 \right) \] (1)

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 x 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 ABSCLIEX MS with the following transitions: m/z 370→317 for PGE2/D2; m/z 374→321 for PGE2-d6; m/z 444→391 for PGE2/D2-G; and m/z 449→396 for PGE2-G-d6. Peak areas were normalized 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 AM-8138’s effect on the potency of weak reversible inhibitors, mCOX-2 was pre-incubated 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 AM-8138’s effect on the potency of slow, tight-binding inhibitors, enzyme at 50 nM was first pre-incubated 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 one 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 previously described with modest modification (30). mCOX-
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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-OG, and 0.1% NaN₃. The concentration of β-OG 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 µL of the protein-inhibitor complex with 3.5 µL of a crystallization solution containing 50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) buffer, pH 8.0, containing 120 mM MgCl₂ and 22-26% polyethylene glycol monomethyl ether (PEG MME)-550. Crystals were transferred into a solution consisting of 50 mM EPPS, pH 8.0, containing 120 mM MgCl₂ and 28% PEG MME-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 (PDB: 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 AM8138 were generated through SMILES in COOT (34). Water molecules were added during the last cycles of refinement, and TLS 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 pre-incubation with the enzyme, AMG-313 weakly inhibited AA and 2-AG oxygenation, with 50% inhibition observed at ~50 µM of the 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. 3A and 3C) while exerting no effect on the enzyme’s ability to oxygenate AA (Fig. 3B and 3D). Since, 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. 4A and 4B). Total products in this assay include PG-Gs, the primary products of COX-2-mediated oxygenation of 2-AG, plus 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
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asymmetric unit were very similar, as indicated by RMSD 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 Å 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. 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 Å 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 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 ω-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 AM-8138’s interaction with the enzyme (Fig. 5). Hence, we evaluated the effect of mutation of these residues i.e., Y355F, Y355A, R120A, R120Q, and S530A, on AM-8138’s ability to potentiate 2-AG oxygenation. Of these, the R120A and Y355F mutations were moderately inhibitory to AA oxygenation while 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 AM-8138’s ability to effect an approximately 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, which 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 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. 6C and 6D. 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 while having 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 L531 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. 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 monooxygenated 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 approximately two-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 apparently 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, while 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).

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 of the substrate-selective inhibitor (R)-flurbiprofen completely prevented inhibition of 2-AG oxygenation and also potentiated the oxygenation levels several-fold compared to 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 to 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, pre-incubation of mCOX-2 with AM-8138 blocked the inhibitory effects of 10 µM indomethacin and 100 nM diclofenac on AA oxygenation (Fig. 9C). For 2-AG oxygenation, the presence of AM-8138 not only blocked the time-dependent inhibition by these inhibitors, but oxygenation levels were also significantly increased beyond those of the uninhibited enzyme (Fig. 9D). The ability of AM-8138 to prevent inhibition of both AA and 2-AG oxygenation by either diclofenac or indomethacin suggests that
AM-8138 competes with these inhibitors for COX-2's allosteric site; however, it is also possible that binding of AM-8138 at the allosteric site causes a conformational change that prevents inhibitor binding 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- to 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, Table 2).

**Substrate-Selective Potentiation of 2-AG Oxygenation in RAW264.7 Cells** – To determine if allosteric potentiation of 2-AG oxygenation can occur within a cellular context, we pre-incubated RAW264.7 cells with AM-8138 for 24 h followed by a 6 h activation with the chemically defined lipopolysaccharide Kdo$_2$-lipid A (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 to 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 unaltered 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 inspection 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 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 (24,25). Kinetic studies indicated that palmitic acid potentiated AA oxygenation by ~2 fold by decreasing its $K_M$ from 8.5 µM to 3.5 µM; 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 provide the foundation for a model of allosteric regulation of COX-2 in which the heme-bound subunit catalyzes the COX and peroxidase reactions while 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 $k_{cat}$ while also preventing 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 $K_i$ (Table 2) is a reflection of competition between AM-8138 and 2-AG, as described by the Equation 2,

$$K_{iapp} = K_i \left(1 + \frac{[P]}{K_p}\right)$$

in which $K_{iapp}$ is the observed value of $K_i$ in the presence of the potentiator, AM-8138, $K_i$ 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 µM, which is in good agreement with the observed EC50 value of 0.7 µM (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 µM. 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 nonproductive conformation (38,39,41). These results suggest that the flexibility of Leu-531 is key to accommodation of bulkier ligands. Consistently, we find that mutations of Leu-531 to alanine or valine result in marked reduction of COX-2’s 2-AG oxygenation activity 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 $K_p$ 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 COX-1’s allosteric site, 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 isoforms.

The selective effects of AM-8138 on cyclooxygenase activity translated well to a cellular system. Our studies with KLA/ ionomycin-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 enzyme’s active site 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 towards 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.
REFERENCES


Substrate-selective activation of COX-2 by AM-8138


Acknowledgments - We thank James A. Wepy for his valuable input in developing the AM-8138 potentiation scheme. The authors would also like to thank William N. Beavers for helpful discussions and suggestions.

FOOTNOTES
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The abbreviations used are: mCOX-2, murine cyclooxygenase-2; oCOX-1, ovine cyclooxygenase-1; AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; PGs, prostaglandins; PG-Gs, prostaglandin glycerol ester
FIGURE LEGENDS

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 AEA to PGH₂, PGH₂-G, and PGH₂-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 while the other subunit exerts allosteric regulation. In the figure, heme (red) is shown in the catalytic site (orange), and the allosteric site is shown 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 of 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 in Experimental Procedures. The data are representative of different experiments involving triplicate determinations. The error bars indicate ± standard deviation. 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 ± standard deviation.

FIGURE 3. Effects of AM-8138, and AM-8125 on mCOX-2-dependent oxygenation of 2-AG and AA. Following co-incubation of 5 µM 2-AG (A&C) or AA (B&D) with AM-8138 (A&B) or AM-8125 (C&D) in a solution containing 50 nM of mCOX-2 at 37°C, PG-Gs (A&C) and PGs (B&D) were analyzed by LC-MS/MS as described in Experimental Procedures. The results are from three different experiments involving triplicate determinations. The error bars indicate ± standard deviation, and statistical significance of *, p < 0.05; **, p < 0.05; ****, p < 0.0001 relative to zero concentration of AM-8138 is indicated.

FIGURE 4. Comparison of maximum oxygen consumption to products formed upon oxygenation of 2-AG by mCOX-2 in the presence of AM 8138. O₂ uptake was measured by polarographic assay (A) or LC-MS/MS (B) during a 30 s incubation of 5 µM 2-AG with 50 nM mCOX-2 in the presence or absence of AM 8138 at concentrations of 1 µM and 20 µM. The data are representative of three experiments involving triplicate determinations. The error bars indicate ± standard deviation. C, Analysis of 2-AG oxygenation in the presence of varying concentrations of AM-8138 was carried out as described in Experimental Procedures. The data are from a single experiment involving triplicate determinations. The error bars indicate ± standard error of the mean and ****, p < 0.0001

FIGURE 5. Binding of AM-8138 in the mCOX-2 active site. Stereodrawing of the crystal structure of AM-8138 (cyan) bound in the active site of mCOX-2 (grey). The interacting residues are colored in yellow sticks; the two H-bonds are illustrated in dashes; the simulated annealing omit map (Fₒ-Fc) is contoured in blue at the level of 3 σ.

FIGURE 6. Effects of AM-8138 on 2-AG (A) and AA (B) oxygenation of mCOX-2 mutants. For each of the indicated mutant enzymes, 2-AG (A) and AA (B) oxygenation were measured in the presence (black bars) or absence (clear bars) of 5 µM AM-8138. Effects of AM-8138 on 2-AG (C) and AA (D) oxygenation of R120A/Y355A with (black bars) or without (clear bars) 5 µM AM-8138. Potentiation assays were performed as described in Experimental Procedures. The results are from three different experiments involving triplicate determinations. The error bars indicate ± standard deviation, and
statistical significance was determined using a one-way ANOVA analysis. Statistical significance at a level of p < 0.0001 was seen for all mutants except L531V, L531A, and V349A, where no significant difference was observed. ND indicates that the analyte was below the limits of detection of the assay.

FIGURE 7. Mass spectral characterization of products formed during oxygenation of 2-AG by mCOX-2 and V349A. A, extracted ion chromatogram of the ions representing PGGs, PGE$_2$-G-$d_5$, HETE-G, and 2-AG formed in the absence of AM-8138 by mCOX-2. B, extracted ion chromatogram of the ions representing PGGs, PGE$_2$-G-$d_5$, HETE-G, and 2-AG formed in the presence of AM-8138 by mCOX-2. C, extracted ion chromatogram of the ions representing PGGs, PGE$_2$-G-$d_5$, HETE-G, and 2-AG formed in the absence of AM-8138 by the V349A variant. D, extracted ion chromatogram of the ions representing PGGs, PGE$_2$-G-$d_5$, HETE-G, and 2-AG formed in the presence of AM-8138 by the V349A variant.

FIGURE 8. Steady-state kinetic analysis of potentiation by AM-8138. Analyses are provided for wild-type mCOX-2 (A), Y355F (B), S530A (C), and R120Q (D). The data points represent the amount of PGGs 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 ± standard error of the mean. The kinetic data were fit using Prism Software to a substrate-dependent inhibition model (Experimental Procedures).

FIGURE 9. Allosteric modulation of substrate oxygenation by AM-8138. A, effect of 5 µM AM-8138 on COX-2-dependent 2-AG oxygenation in the presence or absence of 25 µM of (R)-flurbiprofen. B, effect of 5 µM AM-8138 on COX-2-dependent 2-AG oxygenation in the presence or absence of 0.1 µM of mfenamic acid. C & D, effect of 5 µM of AM-8138 on COX-2-dependent AA (C) or 2-AG (D) oxygenation in the presence or absence of 10 µM of indomethacin or 0.1 µM diclofenac. The assays were carried out as described in Experimental Procedures. The data are representative of three experiments involving triplicate determinations. The error bars indicate ± standard deviation and statistical significance at a level of p < 0.0001 was seen in the presence of AM-8138 (****).

FIGURE 10. Effects of AM-8138 on oCOX-1-dependent oxygenation of 2-AG and AA. Following co-incubation of 5 µM 2-AG (A&B) or AA (C) with AM-8138 in a solution containing 50 nM of mCOX-2 at 37°C, PG-Gs (A&B) and PGs (C) were analyzed by LC-MS/MS as described in Experimental Procedures. The concentration of AM-8138 used in (B) was 5 µM. The results are from three different experiments involving triplicate determinations. The error bars indicate ± standard deviation, and statistical significance at a level of p < 0.0001 was seen in the presence of AM-8138. (D) Steady-state kinetic analysis of potentiation by AM-8138 of oCOX-1-dependent 2-AG oxygenation. Analyses are provided for wild-type oCOX-1. 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 30 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 ± standard error of the mean. The kinetic data were fit using Prism Software to a substrate-dependent inhibition model (Experimental Procedures). Bars labeled **** indicate that the data were different from zero concentration of AM-8138 with a statistical significance of p < 0.0001. Bars labeled ns were not significantly different from zero concentration AM-8138.

FIGURE 11. Effect of AM-8138 on AA and 2-AG oxygenation in basal versus stimulated RAW264.7 cells. RAW264.7 cells were preincubated with or without 15 µM AM-8138 for 24 h and then with or without KLA (6 h) followed by ionomycin (45 min) prior to the analyses of substrates and products. The
KLA treated and un-treated cells were tested for expression levels of COX-1 and COX-2 (A) in the absence and presence of AM-8138. The levels of products, PGs (B), PG-Gs (C), and substrates, AA (D) and 2-AG (E) were also measured. The cells were also evaluated for the levels of AM-8138 (F). The data represent two independent experiments with triplicate determinations. The error bars indicate ± standard deviation, and statistical significance was determined using a one-way ANOVA analysis. Bars designated with asterisks indicate statistical significance at a level of p < 0.0001; ns, not significant.

FIGURE 12. Proposed mechanism for the allosteric regulation of COX by AM-8138. Homodimeric COX contains an allosteric (allo) and a catalytic (cat) site. Heme (red line) binds to the catalytic site. The high affinity binding of AM-8138 (blue) to the allosteric site results in a conformational change in the catalytic site via the dimer interface. This conformational change favors 2-AG (red) binding at the catalytic site potentiating its oxygenation by COX. The low affinity binding of 2-AG to the allosteric site in the absence of AM-8138, however, leads to a substrate-dependent inhibition and little to no product formation.
### TABLE 1

**X-ray Data Collection and Refinement Statistics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mCOX-2•AM-8138</th>
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</thead>
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<tr>
<td><strong>Data Collection</strong></td>
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</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.9792</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>112.4 – 2.16 (2.24 – 2.16)</td>
</tr>
<tr>
<td>Space group</td>
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<tr>
<td>Unit cell</td>
<td>215.8, 121.5, 134.8, 90, 123.6, 90</td>
</tr>
<tr>
<td>Total reflections</td>
<td>867,679 (86,519)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>152,390 (15,303)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.7 (5.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.12 (95.94)</td>
</tr>
<tr>
<td>Mean I/sigma(I)</td>
<td>10.12 (1.48)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>39.46</td>
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<tr>
<td>R-merge</td>
<td>0.136 (1.35)</td>
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<tr>
<td><strong>Refinement</strong></td>
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</tr>
<tr>
<td>R-work/R-free (%)</td>
<td>17.3/21.9 (27.6/29.9)</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>19,544/17,905/528/1,111</td>
</tr>
<tr>
<td>(all/protein/ligands/water)</td>
<td></td>
</tr>
<tr>
<td>Protein residues</td>
<td>2232</td>
</tr>
<tr>
<td>RMS bonds/angles(Å/o)</td>
<td>0.009/1.41</td>
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<tr>
<td>Ramachandran favored/outliers (%)</td>
<td>97.0/0.05</td>
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<tr>
<td>B-factor (All/protein/ligands/solvent)</td>
<td>45.5/45.1/57.9/44.6</td>
</tr>
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</table>

*The values in parentheses are for the highest resolution shell; Rsym = \left(\frac{\sum_{hkl} \sum_{i} |I_i(hkl) - \langle I_i(hkl) \rangle|}{\sum_{hkl} \sum_{i} \langle I_i(hkl) \rangle}\right) \times 100\% and R = \left(\frac{\sum_{hkl} \sum_{i} |F_i(hkl) - |F_i(hkl)|}{\sum_{hkl} |F_i(hkl)|}\right) \times 100\% ,

where Fo and Fe are the observed and calculated structure factors, R_free = test set 3.0%.
### TABLE 2
Steady-state kinetic parameters of 2-AG oxygenation by wild-type mCOX-2, S530A, Y355F, R120Q, and wild-type oCOX-1 with or without AM-8138.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>-AM-8138</th>
<th>+AM-8138</th>
<th>-AM-8138</th>
<th>+AM-8138</th>
<th>-AM-8138</th>
<th>+AM-8138</th>
<th>-AM-8138</th>
<th>+AM-8138</th>
<th>-AM-8138</th>
<th>+AM-8138</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (µM)</td>
<td>( k_{cat} ) (s(^{-1}))</td>
<td>( K_i ) (µM)</td>
<td>( k_{cat} / K_m ) (µM(^{-1})s(^{-1}))</td>
<td>( K_m ) (µM)</td>
<td>( k_{cat} ) (s(^{-1}))</td>
<td>( K_i ) (µM)</td>
<td>( k_{cat} / K_m ) (µM(^{-1})s(^{-1}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WTmCOX-2</td>
<td>1.3 ± 0.3</td>
<td>0.61 ± 0.04</td>
<td>45 ± 11</td>
<td>0.47</td>
<td>3.2 ± 0.6</td>
<td>1.4 ± 0.1</td>
<td>250 ± 140</td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S530A</td>
<td>N.D.(^c)</td>
<td>N.D.(^c)</td>
<td>N.D.(^c)</td>
<td>0.25(^c)</td>
<td>4.7 ± 0.8</td>
<td>1.3 ± 0.1</td>
<td>130 ± 40</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y335F</td>
<td>1.3 ± 0.4</td>
<td>0.33 ± 0.04</td>
<td>12 ± 3</td>
<td>0.25</td>
<td>3.9 ± 0.5</td>
<td>1.2 ± 0.1</td>
<td>65 ± 12</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R120Q</td>
<td>N.D.(^d)</td>
<td>N.D.(^d)</td>
<td>N.D.(^d)</td>
<td>N.D.(^d)</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>22 ± 4</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT oCOX-1</td>
<td>1.2 ± 0.3</td>
<td>0.18 ± 0.02</td>
<td>14 ± 4</td>
<td>0.15</td>
<td>1.3 ± 0.3</td>
<td>0.77 ± 0.06</td>
<td>33 ± 8</td>
<td>0.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)AM-8138 (5 µM) was added to 50 nM of 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 was normalized to DMSO control values. Data are the mean ± S.E.M. of triplicate determinations.

\(^b\)Data were analyzed using a kinetic model for substrate inhibition.

\(^c\)Curve fitting of the data was successful, but large errors were observed.

\(^d\)Enzyme activity was too low for evaluation of kinetic constants.
FIGURE 1.

A

\[
\text{Substrates (R)}
\]

\[
\text{Arachidonic Acid (AA)}
\]

\[
\text{2-Arachidonylethanolamide (AEA)}
\]

\[
\text{Arachidonylethanolamide (AEA)}
\]

B

C

AM-8138

AM-8125

AMG-313

AM-8138
FIGURE 2.

A

B

Substrate-selective activation of COX-2 by AM-8138

[AMG-313] (µM)

% Activity remaining

Oxygen Consumption (µM)

AA  2-AG  AM-8138  AM-8125  AMG-313
FIGURE 3.

(A) Substrate-selective activation of COX-2 by AM-8138. The graph shows the concentration of 2-AG (µM) in response to different concentrations of AM-8138 (µM). The y-axis represents the concentration of PG-Gs (µM), and the x-axis represents the concentration of AM-8138 (µM).

(B) shows the concentration of AA (µM) in response to different concentrations of AM-8138 (µM).

(C) Substrate-selective activation of COX-2 by AM-8125. The graph shows the concentration of 2-AG (µM) in response to different concentrations of AM-8125 (µM).

(D) shows the concentration of AA (µM) in response to different concentrations of AM-8125 (µM).
FIGURE 4.
FIGURE 5.
FIGURE 6.
FIGURE 7.

A. XIC 2-AG + mCOX-2

B. XIC 2-AG + mCOX-2 + AM-8138

C. XIC 2-AG + V349A

D. XIC 2-AG + V349A + AM-8138

Substrate-selective activation of COX-2 by AM-8138
Substrate-selective activation of COX-2 by AM-8138

FIGURE 8.

A

B

C

D

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FIGURE 9.

A. Substrate-selective activation of COX-2 by AM-8138 with (R)-flurbiprofen. 

B. Substrate-selective activation of COX-2 by AM-8138 with Mefenamic Acid.

C. Comparison of PGs production in the presence and absence of AM-8138 with Indomethacin and Diclofenac.

D. Comparison of PGs production in the presence and absence of AM-8138 with Indomethacin and Diclofenac.
FIGURE 10.

A  0.75  0.50  0.25  0.00
PG-Gs (µM)  0.00  0.05  0.10  0.15  0.20  0.25  0.30  0.35
[AM-8138] (µM)  0.00  0.01  0.10  1.00  5.00  10.00  50.00  100.00

B  1.00  0.75  0.50  0.25  0.00
PG-Gs (µM)  0.00  0.05  0.10  0.15  0.20  0.25  0.30  0.35
[AM-8138] (µM)  0.00  0.01  0.10  1.00  5.00  10.00  50.00  100.00

C  0.35  0.30  0.25  0.20  0.15  0.10  0.05  0.00
PGs (µM)  0.00  0.01  0.10  1.00  5.00  10.00  50.00  100.00
[AM-8138] (µM)  0.00  0.01  0.10  1.00  5.00  10.00  50.00  100.00

D  0.035  0.030  0.025  0.020  0.015  0.010  0.005  0.000
V_0 (µM/s)  0.00  10  20  30  40  50
[2-Arachidonylglycerol] (µM)  0.00  0.05  0.10  0.15  0.20  0.25  0.30  0.35

- AM-8138  + AM-8138

2-AGA

AAC

oCOX-1

mCOX-2

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FIGURE 11.

A. % of Vehicle Control

B. PGs

C. PGs

D. AA

E. 2-AG

F. AM-8138
FIGURE 12.

Substrate-selective activation of COX-2 by AM-8138

- Increased PG-Gs
- Decreased PG-Gs
- PG-Gs

Potentiation

Substrate-Dependent Inhibition

Resting Enzyme
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, Lipin Ji, Marsha R. Eno, Alexandros Makriyannis and Lawrence J. Marnett

*J. Biol. Chem.* published online February 2, 2015

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