Cyclooxygenase-derived proangiogenic metabolites of epoxyeicosatrienoic acids

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Arachidonic acid (ARA) is metabolized by cyclooxygenase (COX) and cytochrome P450 to produce proangiogenic metabolites. Specifically, epoxyeicosatrienoic acids (EETs) produced from the P450 pathway are angiogenic, inducing cancer tumor growth. A previous study showed that inhibiting soluble epoxide hydrolase (sEH) increased EET concentration and mildly promoted tumor growth. However, inhibiting both sEH and COX led to a dramatic decrease in tumor growth, suggesting that the contribution of EETs to angiogenesis and subsequent tumor growth may be attributed to downstream metabolites formed by COX. This study explores the fate of EETs with COX, the angiogenic activity of the primary metabolites formed, and their subsequent hydrolysis by sEH and microsomal EH. Three EET regiosomers were found to be substrates for COX, based on oxygen consumption and product formation. EET substrate preference for both COX-1 and COX-2 were estimated as 8,9-EET > 5,6-EET > 11,12-EET, whereas 14,15-EET was inactive. The structure of two major products formed from 8,9-EET in this COX pathway were confirmed by chemical synthesis: ct-8,9-epoxy-11-hydroxy-eicosatrienoic acid (ct-8,9-E-11-HET) and ct-8,9-epoxy-15-hydroxy-eicosatrienoic acid (ct-8,9-E-15-HET). ct-8,9-E-11-HET and ct-8,9-E-15-HET are further metabolized by sEH, with ct-8,9-E-11-HET being hydrolyzed much more slowly. Using an s.c. Matrigel assay, we showed that ct-8,9-E-11-HET is proangiogenic, whereas ct-8,9-E-15-HET is not active. This study identifies a functional link between EETs and COX and identifies ct-8,9-E-11-HET as an angiogenic lipid, suggesting a physiological role for COX metabolites of EETs.

omega-6 fatty acids | epoxyeicosatrienoic acids | metabolism | cyclooxygenase | angiogenesis

Arachidonic acid (ARA) is an omega-6 fatty acid that is metabolized by three major classes of enzymes, cyclooxygenases (COXs), lipooxygenases, and cytochrome P450s (CYPs), to produce an array of biologically active metabolites (1–3). The CYP pathway transforms ARA into four epoxyeicosatrienoic acids (EETs) in addition to hydroxylated metabolites (1). EETs have several biological actions, and are considered antiinflammatory, antiproteolytic, neuroprotective, cardioprotective, and analgesic (4). EETs also play a role in angiogenesis (5–10), the formation of new blood vessels from preexisting vessels that is important for many physiological and pathological processes, including cancer (11). EETs can enhance tumor growth and metastasis through their angiogenic activity (12); however, this activity is transient due to their metabolic instability. EETs are further metabolized by epoxide hydrolases (EHs), primarily soluble epoxide hydrolase (sEH), to their corresponding diols (4, 13), which are generally not biologically active (14) (Fig. 1B). Inhibition of sEH prolongs EET biological activity, potentiating their angiogenic activity, leading to increased tumor growth and metastasis in some systems (12, 15) (Fig. 1B).

ARA can also be metabolized by COX to form proangiogenic and proinflammatory prostaglandins (16). Angiogenesis stimulated from exposure to ARA can be mediated using COX inhibitors, which suppress the formation of prostaglandins (16). Compared with sEH inhibitors, COX inhibitors (i.e., the COX-2 selective inhibitor celecoxib) suppress angiogenesis and tumor growth, whereas sEH inhibitors alone tend to stimulate these same responses in animals on a high–omega-6 fat diet (15). It was therefore surprising that administration of a dual sEH and COX inhibitor led to an antiangiogenic and anticancer response more pronounced than administration of these inhibitors alone (15). Similarly, when a COX (celecoxib) and sEH inhibitor (trans-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid; t-AUCB) were administered simultaneously to mice, tumor growth was much more inhibited than administration of the same two inhibitors alone, as shown in Fig. 1B (15). These results indicate that co-circulation of a COX and sEH inhibitor produces a synergistic effect, better at inhibiting primary tumor growth and metastasis through the suppression of cancer angiogenesis. This suggests that EETs, whose levels are controlled by sEH, are metabolized by COX to form angiogenic, protumorigenic products that enhance the proangiogenic response of EETs. Inhibiting COX decreases their formation and activity, as observed in a previous study (16).

The link between the COX and sEH pathways that stimulates angiogenesis and tumor progression may be explained by a relatively unexplored pathway associated with EET metabolism. Although EETs are primarily metabolized by sEH, a few studies have observed that the 5,6-EET and 8,9-EET are substrates for COX-1 (17, 18) and COX-2 (19). The COX metabolites of EETs have largely undefined biological activity, yet Homma et al. determined that the ct-8,9-epoxy-11-hydroxy-eicosatrienoic acid (ct-8,9-E-11-HET) metabolite is a renal vasoconstrictor and potent mitogen, approximately three orders of magnitude more potent than its parent, 8,9-EET (20). This provides evidence that EETs undergo metabolism by COX, forming metabolites that have greater activity than their parent compounds. If these COX metabolites are also angiogenic, this activity may be mitigated with COX inhibitors.

Significance

This study furthers our understanding of epoxyeicosatrienoic acid metabolism by cyclooxygenase (COX) enzymes as a physiologically relevant metabolic pathway, producing signaling molecules that are angiogenic. It explains, in part, why inhibiting the soluble epoxide hydrolase (sEH) in some systems is angiogenic whereas combining sEH inhibition with COX inhibition is dramatically antiangiogenic, which in turn may suppress tumor growth.


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ments for ARA reacting with COX-1 and COX-2 were similar to literature values (independent t test, $P > 0.05$) (21). Likewise, $K_M$ values for 8,9-EET with COX-1 and COX-2 are not significantly different from the ARA $K_M$s, indicating that both substrates have similar affinities for both enzymes. All values obtained for $V_m$ also showed no significant difference. 11,12-EET gave rates that were so low that the kinetic constants could not be calculated based on the response curves, and 14,15-EET showed no apparent reaction with COX-1 or COX-2 (SI Appendix, Figs. S3 and S4).

With respect to the enzymatic mechanism, epoxides placed at the 11,12 and 14,15 positions may alter the substrate orientation within the enzyme, limiting apparent reactivity. COX first catalyzes oxygenation by abstracting the substrate’s 13-proS-hydrogen, generating a radical with maximal electron density at C-11 and C-15, where oxygen then adds to form a peroxyl radical (22). Epoxides placed in either of these locations may limit the initial abstraction rate and addition of oxygen, compared with epoxides placed further from the initial catalytic C-13 (i.e., 5,6- and 8,9-EET). 14,15-EET was least active compared with the other regioisomers, possibly due to the epoxide proximity to the ω-end of the fatty acid. Because the ω-end resides within a channel at the top of the COX active site, substrates with substituents closer to the ω-end may further limit COX activity through added steric hindrance within this channel. For example, adding bulky substitutions within this channel has been shown to inhibit oxidation of ARA (23).

**Product Studies of EETs with COX.** It was evident from the oxygraph kinetics that the 5,6-, 8,9-, and less so the 11,12-EETs were active substrates for COX. This was subsequently confirmed by identifying the products (or lack thereof) formed by the kinetic reactions, using LC-QToF-MS. As expected, 5,6-EET metabolized to form its endoperoxide prostaglandin-like product (epoxy-PG$\Delta_2$, m/z 367.2121), confirming its activity with COX-1 and COX-2 (Fig. 3). Signal intensity of this product not only depended on the presence of enzyme in the reaction but also dramatically decreased in the presence of the COX inhibitor ibuprofen, indicating this product resulted from a functioning COX enzyme rather than a nonenzymatic-dependent reaction.

8,9-EET was metabolized by COX-1 and COX-2 to form two products having an accurate mass of m/z 335.2228, corresponding to the mass of the ct-8,9-E-HET products, as has previously been observed to form with COX-1 (17). Structure assignment for both was done by MS/MS fragmentation. (SI Appendix, Fig. S5 shows the fragment ions m/z 235.1425 and 127.0790 from the molecular ion (m/z 335.2218). This represented cleavage between the C–C bond both α and β from a 15-hydroxy group, corresponding to the ct-8,9-E-15-HET product. The MS/MS spectrum of the second 8,9-EET COX product supports the ct-8,9-E-11-HET structure, because the bond is fragmented α to an

### Results and Discussion

### Oxidation of ARA and EETs by Cyclooxygenases.

An oxygen electrode was used to measure oxygen uptake to determine whether the EET regioisomers were substrates for COX, and to compare their relative affinities with respect to the known COX-1 and COX-2 substrate ARA. Because oxygen is required as a cosubstrate for COX, we could determine whether or not each EET regioisomer is a COX substrate by examining the amount of oxygen consumption for EET incubations with COX-1 and COX-2. As shown in SI Appendix, Fig. S1, 5,6-, 8,9-, and to a lesser extent 11,12-EET were substrates in the presence of both enzymes, yielding measurable oxygen consumption over 25 s at 50 μM. 5,6-EET, reported to be a COX substrate (18, 19), noticeably reacted with COX-1 and COX-2 at concentrations ranging between 15 and 50 μM (SI Appendix, Fig. S2). Compared with 5,6-EET, which is metabolized by COX through the same mechanism as ARA to form an endoperoxide prostaglandin analog, the epoxide position in 8,9-EET blocks endoperoxide formation and instead forms monohydroxyl products on C-11 or C-15. These products are ct-8,9-E-11-HET or ct-8,9-E-15-HET (17). Kinetic plots for 8,9-EET with COX-1 and COX-2 are presented in Fig. 2, and $K_M$ and $V_m$ values for 8,9-EET and ARA can be found in Table 1. The $K_M$s obtained from these experiments for ARA reacting with COX-1 and COX-2 were similar to
This may be important for regulating turnover with sEH and mEH, whereas the 8,9-E-HETs are primarily substrates for sEH. In summary, 8,9-EET has similar having higher levels of mEH, such as in some regions in the brain 8,9-E-HET turnover by mEH may be important in tissues regardless, the slower kinetic turnover of 8,9-E-HETs by mEH is important in tissues having higher levels of mEH, such as in some regions in the brain where sEH is absent (24, 25). In summary, 8,9-EET has similar turnover with sEH and mEH, whereas the 8,9-E-HETs are primarily substrates for sEH. This may be important for regulating angiogenesis, because sEH is expressed in endothelial cells, unlike other EHs (26, 27). Inhibiting sEH has major effects on enhancing EET biological activity, including angiogenesis.

8,9-E-HET Hydrolysis. Based on kinetic and product studies, 8,9-EET formed the most abundant COX products compared with other EET regioisomers. Therefore, we synthesized these products to understand their metabolic fate with EH enzymes. To confirm that 8,9-E-HET products are substrates for sEH and microsomal EH (mEH), we developed an analytical method for their hydrolytic products to determine kinetic constants (kcat and KM) for sEH and mEH (SI Appendix, Fig. S9 for mEH). These constants were compared with the known EH substrate 8,9-EET (Table 2 for sEH and SI Appendix, Table S2 for mEH). All 8,9-E-HETs were substrates of both EHs, with comparable kcat/KM values for each enzyme. For sEH, the KM and Vmax constants for 8,9-EET were higher than for the 8,9-E-HET products. However, the resulting kcat/KM values for all four compounds were similar, indicating that at physiological concentrations, sEH hydrolyzes these four oxylipids in a similar fashion regardless of the presence and location of the hydroxyl group. For mEH, an enzyme shown primarily to act on xenobiotic epoxides but that also can metabolize EETs, especially 8,9-EET (4), the kcat/KM value for 8,9-EET was at least two orders of magnitude higher than for the 8,9-E-HETs, with ct-8,9-E-15-HET being catalyzed 10-fold more than both 8,9-E-11-HET isomers. In this case, the hydroxyl group drastically lowers the enzymatic conversion rate so that the substrates may actually be binding to the enzyme, resulting in slow turnover. However, preliminary inhibition data showed that at high concentration (100 μM) the 8,9-E-HETs are no better at inhibiting mEH activity on a reporting substrate (cis-stilbene oxide 50 μM) than 8,9-EET (98% inhibition); ct-8,9-E-11-HET, ct-8,9-E-15-HET, and ct-8,9-E-15-HET gave 65, 71, and 95% mEH inhibition, respectively. Regardless, the slower kinetic turnover of 8,9-E-HETs by mEH is important in tissues having higher levels of mEH, such as in some regions in the brain where sEH is absent (24, 25)

![Fig. 3. EET products formed after reaction with COX-1 and COX-2, measured by HPLC-QToF-MS. (A) Intensity of the ion selective for 5,6-epoxy-PGH2 (m/z 367.2121). (B) Intensity of the ion selective for 8,9-EET COX-1 and COX-2 products (m/z 335.2228). The larger peak is identified as ct-8,9-E-11-HET and the smaller as ct-8,9-E-15-HET. (C) Intensity of the ion selective for 8,9-EET COX-1 and COX-2 products (m/z 335.2228). Products were only observed in the chromatographs associated with substrate in the presence of either COX enzyme, inhibited with either ibuprofen or celecoxib, and absent in the blank chromatographs (without substrate).](image-url)
angiogenesis, including arthritis, diabetic retinopathy, cutaneous gastric ulcers, and cancer (28). EETs are endothelial mitogens and activate several signaling pathways that promote angiogenesis (9, 10, 29), tumor growth, and metastasis (12). Because the tumorigenic activity attributed to EETs is suppressed by COX inhibitors, it raises the possibility that COX metabolites of EETs may also be angiogenic.

We determined whether the ct-8,9-E-11-HET and ct-8,9-E-15-HET products of 8,9-EET were angiogenic using the in vivo Matrigel plug assay, which measures angiogenesis within an s.c. implanted plug rather than the whole animal. Using this assay, we could compare the effect of EETs and their 8,9-E-HET COX products on cell infiltration and the total number of cells within each plug, extent of extravascular red blood cells (hemorrhage), and number of microvessels (Fig. 5). The VEGF/FGF positive control group had the most effect at producing a large amount of cell infiltration and number of cells, which led to the formation of intact capillaries observed from CD31 staining (SI Appendix, Fig. S10). VEGF and FGF are both crucial factors in the development of blood vessels and induce angiogenesis through distinct pathways (30) yet synergize to enhance vessel formation (31). The presence of these growth factors also is associated with EET mitogenesis and angiogenesis. VEGF induces CYP2C8 expression, resulting in increased EET levels in endothelial cells (29), and incubation of 14,15-EET induces expression of FGF-2 in a Src/P13K-Akt–dependent manner in human dermal microvascular endothelial cells (32). In this study, the EET mixture increased the amount of cell infiltration, fourfold more than the vehicle control (Fig. 5A), but the change induced by the mixture was not significant for the other parameters. 8,9-EET had a significant amount of cell infiltration, total cells (primarily endothelial cells and fibroblasts), and microvessels, three-, two-, and sixfold more than the vehicle control, respectively (Fig. 5A, C, and D). The hemorrhage variation of 8,9-EET was high (Fig. 5B), therefore its level was not significant with respect to the control.

The lack of significance in the hemorrhage area (Fig. 5B) for 8,9-EET may be due to the angiogenic activity of the EETs. Although previous studies have never quantified the amount of hemorrhage with respect to EETs, a few studies have measured increased total hemoglobin after 14,15-EET (9, 32) and 11,12-EET (33) administration. In these cases, there was less variability than the hemorrhage response in this study. One major factor controlling the red blood cell infiltration within the gel plugs is the concentration of heparin, a proangiogenic agent required to stabilize FGF (34). The magnitude of angiogenesis increases with heparin concentration, but heparin may also lead to greater hemorrhage. In these previous studies measuring total hemoglobin after EET administration, the heparin concentration was not reported. It is possible that reducing the amount of heparin may provide less variable results, although it has been reported that heparin at 40 U/mL produced the most consistent results with respect to the FGF angiogenic response (34). In this study, all groups had equal heparin concentration (40 U/mL). Therefore, the large hemorrhage variability may be due to 8,9-EET alone (although interactions between EETs and heparin are not well-understood).

Similar to the EETs, ct-8,9-E-11-HET significantly enhanced the amount of cell infiltration and total cell number within each Matrigel plug relative to the vehicle control (Fig. 5). Although the ct-8,9-E-11-HET and ct-8,9-E-15-HET angiogenic activity may synergize with the activity observed for the EETs, COX inhibition with celecoxib may reduce angiogenesis and the protumorigenic effects of EETs, at least in part, by blocking formation of ct-8,9-E-11-HET.

Although EET angiogenic activity may be enhanced through metabolism by COX to form ct-8,9-E-11-HET, the cell signaling pathways that promote the angiogenic activity of ct-8,9-E-11-HET are currently unknown. There were notable differences between the ct-8,9-E-11-HET group and others in the gross morphology of the Matrigel plugs, all of which were very fragile and had large areas of hemorrhage (Fig. 5G). H&E staining of Matrigel sections showed marked presence of extravascular RBCs and many hematomas (SI Appendix, Fig. S11). The extensive hemorrhaging, 17-fold more than the vehicle control, suggests formation of unstable neovessels. Unstable neovessels resulting in excessive hemorrhage in Matrigel plugs was also previously observed from platelet inhibition (35). Platelets preferentially adhere and stabilize newly formed angiogenic vessels (35), ct-8,9-E-11-HET may inhibit platelets or other stabilizing factors, leading to an angiogenic response that includes formation of weak blood vessels. EETs, particularly 11,12- and 14,15-EET, reduce platelet aggregation through membrane hyperpolarization and enhanced expression and activity of endothelial fibrolytic enzymes (36), suggesting a similar and potentially greater interaction between ct-8,9-E-11-HET and platelets, reducing endothelial stability that leads to hemorrhage, although elucidating this relationship obviously warrants further research.

The hemorrhaging was also coupled with an inflammatory infiltrate made up of leukocytes in plugs treated with ct-8,9-E-11-HET (SI Appendix, Fig. S12). ct-8,9-E-11-HET may be a stimulator of inflammatory angiogenesis, due to the presence of these
inflammatory cells. Leukocyte infiltration has been observed in lipopolysaccharide-conditioned Matrigel, a model for inducing inflammatory angiogenesis (37). In this model, neutrophils invade and degrade the gel by creating clefs, followed by migrational macrophages. Growth factors such as VEGF and bFGF released by neutrophils and macrophages subsequently induce endothelial cell migration and tube formation, and the resulting angiogenesis is directly dependent upon leukocyte migration. This may explain why, despite the presence of hemorrhage, ct-8,9-E-11-HET still produced intact vessels, which formed through the presence of additional growth factors from the leukocytes.

By contrast to ct-8,9-E-11-HET, ct-8,9-E-15-HET and the combination of ct-8,9-E-11-HET and ct-8,9-E-15-HET were not significantly different from the vehicle control, suggesting some biological dependency on the position of the hydroxyl group, that ct-8,9-E-15-HET may not be a critical component for stimulating angiogenesis (Fig. 5 A–D). Using the purified enzymes described above, ct-8,9-E-11-HET forms 2- to 10-fold more than ct-8,9-E-15-HET, although formation of these products may be dependent on species, tissue, and cell type. Although the combination of ct-8,9-E-11-HET and ct-8,9-E-15-HET was expected to be angiogenic, given that ct-8,9-E-11-HET gave an effect on its own, this group did not produce any significant activity. ct-8,9-E-15-HET may be an antagonist to ct-8,9-E-11-HET, which acts to block or reduce the activity of ct-8,9-E-11-HET. Alternatively, because ct-8,9-E-11-HET and ct-8,9-E-15-HET together produced a total concentration of 100 μM and the level of ct-8,9-E-11-HET in this treatment was lower than the individual treatment, the activity of ct-8,9-E-11-HET might be below the detection limit of this assay.

Lipid treatments were at levels much higher than their reported nanomolar endogenous levels (38). The 100 μM concentration was chosen because EETs and 8,9-E-HETs are labile, and only dosed once within the 7-d treatment period. However, ct-8,9-E-11-HET may be angiogenic at endogenous levels based on its expected presence within endothelial cells. These cells are known to express high levels of CYPs, which form EETs that have diverse effects on the vasculature, including angiogenesis. For example, overexpression of CYP2J2 in bovine aortic endothelial cells promotes cell proliferation, migration, and tube formation (39). COX-1 is constitutively expressed in most tissues, and therefore we might expect the formation of the 8,9-E-HET metabolites, because their formation by COX-1 was demonstrated in this study and others (17). The combination of COX-1 and COX-2 may lead to the highest levels of E-HETs formed, as we show EETs to be substrates for both enzymes. Under normal conditions, COX-2 is expressed at low or undetectable levels but becomes up-regulated by inflammatory, mitogenic, and physical stimuli (40). In addition, the expression of VEGF, a critical factor for angiogenesis, up-regulates COX-2, which may in turn increase endogenous levels of ct-8,9-E-11-HET that will promote angiogenesis (41).

In summary, we have explored the fate of EETs with COX, with particular attention to 8,9-EEt and the angiogenic activity of its products. Although these products are substrates for sEH, inhibition of sEH may stabilize 8,9-E-HETs in circulation, leading to angiogenesis from increased levels of ct-8,9-E-11-HET, which may in turn promote tumor growth and metastasis.

Materials and Methods
Details of the experimental protocols are given in SI Appendix, Materials and Methods.

COX Activity Assay by Oxygen Consumption. A Hansatech Oxygraph Plus oxygen electrode system was used to measure the oxygen consumption involved in the activity of COX-1 and COX-2 with EETs (5,6- and 14,15-EET). Tris HCl buffer [100 mM, pH 8.0, with 5 mM EDTA (USB)] was first added to the electrode chamber. Bovine hematin and phenol were then added to give final concentrations of 2 μM and 1 mM, respectively. This solution was warmed to 37 °C before adding COX-1 or COX-2 ([COX]_{final}=5 μg/mL, determined by the bicinchoninic acid assay). Reactions were initiated by adding a solution of the epoxy fatty acid in 1:1 ethanol/Tris HCl buffer through the central bore of the Oxygraph plunger, with final concentrations ranging from 0.1 to 80 μM. Oxygen consumption was followed until the rate before addition of substrate returned. The amount of ethanol was 0.8% of the total volume (300 μL). Km and Vm for COX-1 and COX-2 with each substrate were
estimated by nonlinear regression to the Michaelis–Menten equation (SigmaPlot 11.0, Systat Software). Reactions were done with n = 2 to 4 replicates. Products from these reactions were analyzed using LC-QTOF-MS, described in SI Appendix, Materials and Methods.

**EH Kinetic Assay Conditions.** Kinetic parameters for 8,9-EET, tr-8,9-E-11,9-HET, ct-8,9-E-11,9-HET, and ct-8,9-E-15-HET were determined under steady-state conditions using recombinant human SEH (95% pure) and mEH (80% pure). The E-HETs were first converted from their methyl ester to their carboxylic acid form using recombinant partially purified carboxylesterase 2 (CES 2) free of epoxide hydrolase activity. In glass tubes (10 × 75 mm), to 90 μL of a solution of CES 2 (27 μg/mL in Na3PO4 buffer (0.1 M, pH 7.4, containing 0.1 mg/mL BSA)), 1 μL substrates in DMSO (S1 = 2.5 to 50 μM) was added and the mixture was incubated for 30 min at 37 °C. Each enzyme in Na3PO4 buffer (SEH = 0.10 μg per tube; mEH = 2.96 μg per tube) was then added (10 μL). The mixture was incubated for an additional 30 min at 37 °C. Reactions were quenched by adding 100 μL of methanol containing 0.4 μM N-cyclohexyl-N′-dodecanic acid urea as an internal standard, used for its similar structural to the measured analytes rather than its ability to inhibit SEH. The quantity of products formed, either 8,9-dihydroxyeicosatetraenoic acid (8,9-DHET), 8,9,11-trihydroxyeicosatetraenoic acid (8,9,11-THET), or 8,9,15-THET was determined by LC-MS/MS. Kinetic constants (Km and Vmax) were calculated by non-linear fitting of the Michaelis–Menten equation (SigmaPlot 11.0). Reactions were done in triplicate (n = 6).

**Matrigel Plug Assay.** All procedures and animal care were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of California. Growth factor-reduced Matrigel (0.5 mL) at 37 °C was mixed with Matrigel (0.5 mL) at 37 °C. Reactions were quenched by adding 100 μL of methanol containing 0.4 μM N-cyclohexyl-N′-dodecanic acid urea as an internal standard, used for its similar structural to the measured analytes rather than its ability to inhibit SEH. The quantity of products formed, either 8,9-dihydroxyeicosatetraenoic acid (8,9-DHET), 8,9,11-trihydroxyeicosatetraenoic acid (8,9,11-THET), or 8,9,15-THET was determined by LC-MS/MS. Kinetic constants (Km and Vmax) were calculated by non-linear fitting of the Michaelis–Menten equation (SigmaPlot 11.0). Reactions were done in triplicate (n = 6).


Supporting Information

Cyclooxygenase derived proangiogenic metabolites of epoxyeicosatrienoic acids

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SI Materials and Methods

Materials. The 8,9-E-HET methyl ester regioisomers, including the trans,trans(tt)-8,9-E-11-HET, ct-8,9-E-11-HET, and ct-8,9-E-15-HET, were chemically synthesized in our lab. The EET methyl ester regioisomers were also synthesized in our lab, including the 8,9-; 11,12-; 14,15-EET. All 8,9-E-HETs and EETs were dissolved in ethanol stock solutions (10-100 mM), and stored at -80 °C until use. ARA, 5,6-EET, ovine COX-1, and human recombinant COX-2 were purchased from Cayman Chemical Company (Ann Arbor, MI). The sEH inhibitor 1471 (trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid) was synthesized in house. Mouse recombinant FGF-2 was purchased from Sigma Aldrich (Saint Louis, MO), reconstituted in sterile ddH2O to 100 µg/mL and stored at -20 °C. Heparin sodium salt (212 units/mg) was also obtained from Sigma Aldrich. Mouse recombinant VEGF 164 was purchased from R&D Systems (Minneapolis, MN), reconstituted in phosphate buffer to 50 µg/mL and stored at -20 °C. Optima™ grade methanol and water were purchased from Fisher Scientific (Waltham, MA). Growth Factor Reduced Matrigel was purchased from Corning (Bedford, MA). All other chemical reagents were purchased from Sigma Aldrich unless indicated.

Elucidating COX product structures. COX reactions with EETs were analyzed for products using LC-MS. Samples were prepared using the oxygraph system as described above, with either 40 or 50 µM substrate concentrations. Three controls were also used, as follows: one control containing substrate only, a second containing enzyme only, and a third containing a COX inhibitor. For the inhibitor control, celecoxib or ibuprofen in 1:1 ethanol/Tris-HCl buffer was added subsequent to enzyme incubation to give a final concentration of 100 µM and was allowed to equilibrate for at least 2 min. The reaction was initiated by addition of epoxy fatty acid. Each reaction mixture was then quenched with 600 µL ice-cold methanol, centrifuged at 13,200 g for 10 minutes, followed by LC-QToF-MS analysis.

COX activity assay by LC-QToF-MS product analysis. Analysis was performed using an Agilent (Santa Clara, CA) 1290 Infinity LC system coupled to an Agilent 6530 QToF-mass spectrometer in negative electrospray ionization mode. For the analysis of the EET COX products, samples were injected (3 µL) and separated using an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 µm) coupled to an Acquity CSH C18 VanGuard pre-column (5 × 2.1 mm; 1.7 µM) at 40 °C with the following gradient method at 0.5 mL/min using methanol and water mobile phases, each containing 0.1% formic acid: the initial solvent composition at t = 0 min was 50:50 water/MeOH, which changed to 100% methanol over 14 min and held for 3 min to t = 17 min, before returning to 50:50 water/MeOH at 17.1 min. The column was allowed to re-equilibrate for 2.9 min for a total run time of 20 min. The scanning mass range was m/z 60-1700, with an acquisition speed of 2 spectra/s. MS/MS fragmentation (CE = -15 eV) was done to identify the structures corresponding to the two peaks (m/z 335.2228) that formed after 8,9-EET
oxygenation with COX, using the same LC conditions described above. MS/MS spectra for the products of enzymatic transformation were also compared to synthetic ct-8,9-E-11-HET and ct-8,9-E-15-HET.

**Structural elucidation of COX-metabolites for 11,12-EET.** To further define the products resulting from reaction of 11,12-EET with COX-1 and -2, the reaction was altered to increase the amount of product formed. The EETs (10 µM) were incubated within the oxygraph chamber with 25 µg/mL COX-1 or COX-2, and the reaction was carried out for 6 min. Following this, either mouse sEH (25 µg/mL), sulfuric acid (final concentration: 1–200 mM), or acetic acid (50 mM) were added to hydrolyze any epoxides to diols. Incubations with either sEH or acid were carried out for 12 min. For these reactions, the following controls were used: substrate only, COX only, EET and COX enzyme only, EET and sEH only. Samples were then removed from the oxygraph chamber and quenched with methanol before LC-QToF-MS analysis as described in the main methods section. Fragmentation was carried out for the 11,12-EET products, however the signals were not intense enough for any confident structural elucidation based on the fragmentation pattern.

**LC-MS/MS analysis.** LC-MS/MS analysis of the enzymatic hydration products were performed with a Agilent 1200 Series HPLC equipped with a 4.6 × 30 mm, 3 µm Gemini C18 column (Phenomenex, Torrence, CA) held at 45 °C. The sample chamber was held at 4 °C. The HPLC was interfaced to the electrospray ionization probe of an AB Sciex 4000 Qtrap tandem quadrupole mass spectrometer (Framingham, MA), operating in MRM mode. Solvent flow rates were fixed at 350 µL/min with a curtain gas flow of 35 L/h, an ion source 1 and 2 gas flow of 40 L/h, an ion spray of -4500 V, and a desolvation temperature of 450 °C. Electrospray ionization was accomplished in the negative mode. For all analytes analyzed for the EH kinetic assays, the following LC method was used: initial conditions of 35:65 acetonitrile:water, increasing to 95:5 over 3 min (t = 3 min), holding at 95:5 for 3.9 min (t = 6.9 min), reverting to initial conditions of 35:65 over 1.1 min (t = 8 min) and re-equilibrating for 2 min (t = 10 min). Both mobile phases contained 0.1% acetic acid.

**Quantification in Matrigel plug assay.** Angiogenesis was characterized by histological H&E and immunohistochemistry CD31 staining. Cell number was measured by using systematic random sampling after H&E staining. Cells were counted in every fourth 100x100 µm square after taking 1x1 mm images spanning over each gel. Counting was done using Adobe Photoshop CC 2015.5 (Adobe Systems, Inc, San Jose, CA). We also quantified the extent of cell infiltration into each plug using the Aperio Imagescope software (Leica Biosystems, Buffalo Grove, IL), subtracting the area of cell infiltration from the total area of the gel. We quantified the area of extravascular red blood cells, which indicate hemorrhage. All plugs were normalized to the size of the largest plug. Microvessel formation was quantified after CD31 staining using the Aperio Imagescope Microvessel Algorithm. All data are the average of 4-6 independent Matrigel plugs.

**mEH Inhibition Assay.** 8,9-EET, tt-8,9-E-11-HET, ct-8,9-E-11-HET, and ct-8,9-15-HET were tested as inhibitors for mEH using an assay previously described (1). Inhibitors (100 µM) were pre-incubated with mEH (2 µg/mL) in Tris/HCl (0.1 M, pH 9.0) buffer with BSA (0.1 mg/mL) for 3 min at 37 °C. The substrate [³H]-cis-stilbene oxide (c-SO, 50 µM) was then introduced and incubated for 10 min. Reactions were quenched by addition of isooctane (250 µL) and the remaining activity was measured by quantifying the radioactive diol formed in the aqueous phase.
using a liquid scintillation counter. The positive control 2-nonylthio-propionamide (NPTA) (2) gave 100% inhibition at 100 µM.

References
**Figure S1.** Comparison of the oxygen consumption between COX-1 and COX-2 reactions ([COX]$_{\text{final}}$ = 5 µg/mL) with AA and the 5,6-; 8,9-; 11,12-; 14,15-EET as substrates ([Substrate]$_{\text{final}}$ = 50 µM). This figure shows a typical run, and was repeated with similar results at least n = 3-4 times on 2 different days. The control contained active enzyme but lacked the lipid substrate.
Figure S2. Rate of oxygen consumption (nmol O₂/min) measured by reaction with 5,6-EET (0 – 50 µM) with COX-1 and COX-2 ([COX]ₘₚₐₓ = 5 µmol/mL). Standard error bars reflect n=2.

Figure S3. Rate of oxygen consumption (nmol O₂/min) measured by reaction with 11,12-EET (0 – 300 µM) with COX-1 and COX-2 ([COX]ₖᵣₐᵢᵢₚ = 5 µmol/mL). Standard error bars reflect n=2.
Figure S4. Rate of oxygen consumption (nmol O₂/min) measured by reaction with 14,15-EET (0 – 300 µM) with COX-1 and COX-2 ([COX]_{final} = 5 µmol/mL). Standard error bars reflect \( n=2 \). The low oxygen consumption observed with 14,15-EET as a substrate correlates with lack of significant product formation determined by LC-QToF-MS.
Figure S5. MS/MS fragmentation of the two products formed from the 8,9-EET reaction with COX-1 and COX-2. (A) HPLC-ToF-MS analysis of 8,9-EET oxidation by COX-1, intensity of selected ion selective for the ct-8,9-E-HET products (m/z 335.2228 [M–H]). 1. MS/MS spectrum of ct-8,9-E-15-HET 2. MS/MS spectrum of the ct-8,9-E-11-HET, with identified fragment ion masses shown. Spectra were obtained using a collision energy of –15 eV.
Figure S6. Chemically and biologically produced ct-8,9-E-15-HET gave apparently identical retention times and MS/MS fragmentation for both COX-1 and COX-2. Chromatograph of the ct-8,9-E-HET isomers (m/z 335.2228) (A) and MS/MS fragmentation for the labeled peak at 7.8 min (B) of the biologically synthesized (above) and chemically synthesized (below) ct-8,9-E-15-HET. Peak at 8.2 min in the above chromatograph corresponds to the ct-8,9-E-11-HET isomer. All spectra were obtained using a collision energy of −15 eV.
Figure S7. Chemically and biologically produced \textit{ct}-8,9-E-11-HET gave apparently identical retention times and MS/MS fragmentation for both COX-1 and COX-2. Chromatograph of the \textit{ct}-8,9-E-HET isomers (m/z 335.2228) (A) and MS/MS fragmentation for the labeled peak at 8.2 min (B) of the biologically synthesized (above) and chemically synthesized (below) \textit{ct}-8,9-E-11-HET. Peak at 7.8 min in the above chromatograph corresponds to the \textit{ct}-8,9-E-15-HET isomer. All spectra were obtained using a collision energy of –15 eV.
**Figure S8.** 11,12-EET reacts with COX-2 to form hydroxy-11,12-EETs that can be hydrolyzed by sEH to trihydroxy products, as observed from LC-QToF-MS analysis. (A) Intensity of the selected ion specific for the hydroxy-11,12 EET products (m/z 335.2228). The chromatograph in red correspond to the products formed after 11,12-EET reacts with COX-2. The black chromatograph corresponds to the remaining COX products after hydrolysis with sEH (25 µg/mL). (B) Intensity of the selected ion specific for the 11,12-trihydroxy-EETs resulting from sEH hydrolysis (m/z 353.2328). The chromatograph in black corresponds to the trihydroxy-11,12-EET products formed after reaction with COX-2 and sEH. The chromatograph in red shows lack of trihydroxy-11,12-EET products, in the absence of sEH. (C) Metabolism of 11,12-EET by COX-2 yields hydroxy-11,12-EETs. Further metabolism by sEH produces trihydroxy products (THETs). The location of the hydroxy group in these products has not been proven, and is represented here by the bracketed OH on C15.
**Figure S9.** Determination of the kinetic constants for 8,9-EET, \( \overline{tt} \)-8,9-E-11-HET, \( ct \)-8,9-E-11-HET, and \( ct \)-8,9-E-15-HET with the human mEH ([mEH] final 2.96 µM, in Na\(_3\)PO\(_4\) buffer (0.1 M pH 7.4 containing 0.1 mg/mL of BSA at 37 °C. The angiogenic \( ct \)-8,9-E-11-HET is relatively resistant to metabolism by human mEH. The kinetic constants (\( K_M \) and \( V_m \)) were calculated by non-linear fitting of the Michaelis equation using the enzyme kinetic module of SigmaPlot version 11 (Systat. Software Inc., Chicago IL). Results are values ± standard error, \( n=6 \).
Figure S10. VEGF and FGF-2 produce intact capillaries lined by endothelial cells, given their role as fundamental factors that regulate angiogenesis. This representative image is of a Matrigel plug section from mice injected with VEGF and FGF-2 and stained with CD31 to show contribution of endothelial cells (green arrow) to microvessel formation (dark brown). Matrigels containing 200 ng/mL mouse VEGF 164 and 100 ng/mL mouse FGF-2 were implanted s.c. and recovered on day 6 after implantation. Fibroblasts are marked with blue arrows. Image was taken at 20× magnification. Scale bar: 200 µm.
Figure S11. Matrigel plugs dosed with ct-8,9-E-11-HET appeared to have extensive hemorrhaging, and were further processed by staining with hematoxylin and eosin to measure hemorrhage area. Matrigels containing 100 µM of ct-8,9-E-11-HET were implanted s.c. and recovered on day 6 after implantation. A notable amount of hemorrhage is shown here by the numerous undefined borders of the vessels and extravascular RBCs. Inflammatory eosinophils and lymphocytes are marked as yellow and red arrows, respectively, endothelial cells with green arrows, and fibroblasts with blue arrows. Image was taken at 20× magnification. Scale bar: 200 µm.
Figure S12. *ct*-8,9-E-11-HET may stimulate inflammatory angiogenesis in addition to angiogenesis. This is a representative image of hematoxylin and eosin staining of a Matrigel section from mice injected with *ct*-8,9-E-11-HET. Matrigels containing 100 µM of *ct*-8,9-E-11-HET were implanted s.c. and recovered on day 6 after implantation. Inflammatory eosinophil infiltrate and lymphocytes are marked as yellow and red arrows, respectively, endothelial cells with green arrows, and fibroblasts with blue arrows. Presence of these inflammatory cells suggest that *ct*-8,9-E-11-HET may stimulate inflammatory angiogenesis. Image was taken at 20× magnification. Scale bar: 200 µm.
Table S1. Analytes of interest, HPLC retention times, multiple reaction monitoring (MRM) transitions, declustering potentials, and collision energies. CUDA was used as an internal standard for the quantification of all analytes. Analytes listed as alternative (alt) were used for further product identification (qualifying transitions).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>MRM transition</th>
<th>Declustering potential (V)</th>
<th>Collision energy (V)</th>
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</thead>
<tbody>
<tr>
<td>CUDA</td>
<td>3.9</td>
<td>339 &gt; 214</td>
<td>-65</td>
<td>-32</td>
</tr>
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<td>8,9,11-THET</td>
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<td>353 &gt; 139</td>
<td>-50</td>
<td>-25</td>
</tr>
<tr>
<td>8,9,11-THET alt</td>
<td>3.6</td>
<td>353 &gt; 185</td>
<td>-50</td>
<td>-18</td>
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<tr>
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<td>-35</td>
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<tr>
<td>8,9,15-THET alt</td>
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Table S2. Kinetic constants of recombinant purified human mEH for 8,9-EET, tt-8,9-E-11-HET, ct-8,9-E-11-HET, and ct-8,9-E-15-HET. Results are average ± standard deviation (n= 6). Statistically significant differences were determined by the independent t-test. * indicates significance at p ≤ 0.05