Epoxy metabolites of docosahexaenoic acid (DHA) inhibit angiogenesis, tumor growth, and metastasis


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Epidemiological and preclinical evidence supports that omega-3 dietary fatty acids (fish oil) reduce the risks of macular degeneration and cancers, but the mechanisms by which these omega-3 lipids inhibit angiogenesis and tumorigenesis are poorly understood. Here we show that epoxydocosapentaenoic acids (EDPs), which are lipid mediators produced by cytochrome P450 epoxygenases, inhibit angiogenesis, and suppress endothelial cell migration and protease production in vitro via a VEGF receptor 2-dependent mechanism. When EDPs (0.05 mg kg−1 d−1) are coadministered with a low-dose soluble epoxide hydrolase inhibitor, EDPs are stabilized in circulation, causing ∼70% inhibition of primary tumor growth and metastasis. In contrast to the effects of EDPs, the corresponding metabolites derived from omega-6 arachidonic acid, epoxyeicosatrienoic acids, increase angiogenesis and tumor progression. These results designate epoxyeicosatrienoic acids and EDPs as unique endogenous mediators of an angiogenic switch to regulate tumorigenesis and implicate a unique mechanistic linkage between omega-3 and omega-6 fatty acids and cancers.

Results

EDP Inhibits Angiogenesis in Vivo. To test the actions of EDPs on angiogenesis, we chemically synthesized all stable EDP regioisomers coadministered with a low-dose soluble epoxide hydrolase inhibitor, EDPs are stabilized in circulation, containing ∼70% inhibition of primary tumor growth and metastasis. In contrast to the effects of EDPs, the corresponding metabolites derived from omega-6 arachidonic acid, epoxyeicosatrienoic acids, increase angiogenesis and tumor progression. These results designate epoxyeicosatrienoic acids and EDPs as unique endogenous mediators of an angiogenic switch to regulate tumorigenesis and implicate a unique mechanistic linkage between omega-3 and omega-6 fatty acids and cancers.


Conflict of interest statement: University of California holds patents from the laboratory of B.D.H. on sEHI for the treatment of inflammation, hypertension, pain and other indications.

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and Fig. S2C). Endothelial cell adhesion to fibronectin and vitronectin is mediated by different integrins (32); thus, this result indicates that 19,20-EDP did not target a specific integrin to suppress endothelial cell migration. The 19,20-EDP also inhibited the activity of matrix metalloproteinase 2 (MMP-2) but with a weak activity (20% reduction at 1 μM and 45% reduction at 3 μM; Fig. 1E). It had no effect on endothelial cell proliferation in HUVECs 24 h after treatment (Fig. S2D and E). In comparison, EETs such as 11,12- and 14,15-EET at 1 μM increased HUVEC proliferation by ~82% (Fig. S2E). These results support that 19,20-EDP directly targets endothelial cells to suppress angiogenesis, primarily via suppression of endothelial cell migration.

**EDP Inhibits VEGF Receptor 2 Signaling.** We next asked whether 19,20-EDP inhibited angiogenesis via VEGF receptor 2 (VEGFR2) signaling (30). The 19,20-EDP at 1 μM dramatically inhibited VEGF-induced phosphorylation of VEGFR2 after a 10-min treatment in HUVECs. In contrast, 14,15-EET had no such effect (Fig. 1F). We further found that 19,20-EDP inhibited VEGF expression in HUVECs. An angiogenesis array (>80 genes) suggested that among all of the proangiogenic genes, 19,20-EDP had the most potent inhibitory effect on mRNA expression of VEGF-C (Table S1). This finding was confirmed by RT-PCR, which indicated that 19,20-EDP inhibited ~50% of VEGF-C expression at 1 μM and inhibited ~67% at 3 μM after a 6-h treatment in HUVECs, whereas it had no effect on VEGF-A expression (Fig. 1G). Together, these results indicate that 19,20-EDP inhibited angiogenesis via blocking VEGF–VEGFR2 signaling.

**EDP Inhibits Primary Tumor Growth.** To test the effects of EDPs on primary tumors, we studied a syngeneic Met-1 tumor, which is a highly aggressive triple-negative breast cancer (TNBC) model (33). Systematic administration of 0.05 mg·kg−1·d−1 19,20-EDP (1 μg/d) by osmotic minipumps had no effect on Met-1 tumor growth after 12 d of treatment (Fig. 2A). We reasoned that this was due to the rapid metabolism of 19,20-EDP by soluble epoxide hydrolase (sEH) in vivo (22, 34–36). This was supported by LC-MS/MS analysis that the continuous infusion of 19,20-EDP did not increase its concentration in plasma and tumors (Fig. 2B). To stabilize 19,20-EDP in circulation, a low-dose selective sEH inhibitor (sEHi), trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB, 1 mg·kg−1·d−1) (37), was coadministered with

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**Fig. 1.** EDPs inhibit angiogenesis. (A) The 19,20-EDP inhibited VEGF-induced angiogenesis in a Matrigel plug assay in C57BL/6 mice in a dose-dependent manner (n = 4–6 mice per group). Dose of VEGF is 100 ng per gel. (Left) Quantification of angiogenesis using hemoglobin assay. (Right) Image of representative gels and immunohistochemistry for CD31. (B) All EDP regioisomers inhibited VEGF-induced angiogenesis in mice (n = 6–10 mice per group). Dose of EDP regioisomer was 10 μg per gel. (C) The 19,20-EDP inhibited endothelial tube formation after 6-h treatment in HUVECs. (Left) Calcein AM-stained HUVEC microscopy. (Right) Quantification of migrated cells. (E) The 19,20-EDP inhibited MMP activity after 4-h treatment in HUVECs. (F) At a dose of 1 μM, 19,20-EDP not 14,15-EET, inhibited VEGF-induced VEGFR2 phosphorylation after 10-min treatment in HUVECs. (G) The 19,20-EDP inhibited VEGF-C mRNA expression after 6-h treatment in HUVECs. Results are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Met-1 tumor growth was reduced by 70 ± 20% (P < 0.001), whereas treatment with 19,20-EDP or t-AUCB alone had no effect on tumor growth (Fig. 2A and Fig. S3A–C), supporting the anticancer effect of 19,20-EDP. To further determine whether the anticancer effect is from 19,20-EDP or its sEH metabolite 19,20-dihydroxydocosapentaenoic acid (19,20-DiHDPA) (22), we tested the effect of 19,20-DiHDPA on Met-1 tumor growth. Continuous infusion of 19,20-DiHDPA (0.05 mg·kg⁻¹·d⁻¹) in mice had no effect on tumor growth (Fig. 2C), confirming the anticancer effect was not from this diol metabolite. Together, these results confirm that the combined treatment inhibited primary tumor growth via 19,20-EDP, which was stabilized by co-administration of t-AUCB. Contrary to the effects of 19,20-EDP, stabilized 14,15-EET increased Met-1 tumor growth by 66 ± 36% (P < 0.01) (Fig. 2D and Fig. S3D), demonstrating opposite effects of EETs and EDPs on tumor progression.

EDP Inhibits Tumor Angiogenesis. To determine whether the combined treatment (19,20-EDP + t-AUCB) inhibited tumor growth via suppressing tumor angiogenesis, we analyzed the endothelium in tumors by immunohistochemical detection of the endothelial cell marker CD31. Immunohistochemistry studies showed that the combined treatment decreased vascular density (CD31-positive vessels) by 46.8 ± 19.4% (P < 0.001) (Fig. 2E and Fig. S4). Cancer cell proliferation assays were carried out to test whether EDPs have direct antiproliferative effects. The 19,20-EDP at 1–3 μM had no effect on cell proliferation in multiple cancer cell lines, even when combined with t-AUCB to stabilize it in cancer cells (Fig. S5A). Together, these results indicate that the combined treatment inhibited tumor growth via inhibition of tumor angiogenesis, but not through a direct effect on cancer cell proliferation.

EDP Inhibits Tumor Metastasis. Tumor metastasis, the process by which tumor cells spread from the primary tumor site to other organs, causes 90% of human cancer deaths (38). Cancer cell invasion through the ECM is required to initiate tumor metastasis (38). Invasion was evaluated in vitro using a standard Matrigel-based Boyden chamber assay. With 1 μM of 16,17-EET or 19,20-EDP, FBS-induced cancer cell invasion was reduced ~40%. Contrary to the effects of EDPs, 11,12-EET at an equal dose approximately doubled cancer cell invasion (Fig. S5B). These results suggest that EETs and EDPs may have opposite effects on metastasis.

To test the effect of EDPs on metastasis in vivo, we used a well-established Lewis lung carcinoma (LLC) model, in which resection of the primary s.c. tumor consistently stimulates growth of dormant metastases (28, 39). This spontaneous model of lung metastasis is believed to be triggered by reduced levels of circulating angiogenesis inhibitors that had been produced by the primary tumor (39) (Fig. 3A). Coadministration of either 16,17-EET or 19,20-EDP (0.05 mg·kg⁻¹·d⁻¹) combined with t-AUCB (1 mg·kg⁻¹·d⁻¹) dramatically inhibited LLC metastasis, with a ~70% reduction of lung metastasis foci and lung weight (P < 0.001) (Fig. 3B). Surprisingly, 16,17-EET alone significantly suppressed LLC metastasis by ~42% at day 17 after administration (P = 0.017) (Fig. 3B). In comparison, our previous study in the same model showed that systemic administration of 14,15-EET (0.015 mg·kg⁻¹·d⁻¹) caused an approximately three-fold increase of LLC metastasis (28), confirming the opposite effects of EDPs and EETs on tumor metastasis.

Discussion

The central finding of this study is that the DHA-derived lipid mediators EDPs inhibit angiogenesis, primary tumor growth, and metastasis. To the best of our knowledge, EDPs are unique lipid mediators to be discovered with such potent antiangiogenic, anticancer, and antimetastatic effects. In contrast, ARA-derived EETs increased angiogenesis and tumor progression, which is the central mechanism driving tumor angiogenesis and metastasis. The mechanism by which EDPs inhibit angiogenesis remains to be elucidated. The 19,20-EDP via the combined treatment,

19,20-EDP to block the sEH-mediated metabolism. LC-MS/MS analysis confirmed that coadministration of 19,20-EDP with t-AUCB significantly increased the level of 19,20-EDP, from a basal level of 10.12 ± 4.38 nM to 24.67 ± 8.52 nM in plasma (P < 0.05) and a basal level of 120.36 ± 12.91 pmol/mL to 353.4 ± 109.26 pmol/g in tumor tissues (P < 0.05), whereas treatment with 19,20-EDP or t-AUCB alone had little effect on 19,20-EDP level (Fig. 2B; complete lipid mediator profile is shown in Tables S2 and S3).

With an increased level of 19,20-EDP via the combined treatment,
EDPs could explain the anti-tumor metastasis. (Table S1) 5 mice per group. Results are presented as means ± SEM. *P < 0.05; **P < 0.001.

Fig. 3. EDPs inhibit tumor metastasis. (A) Lewis lung carcinoma (LLC) metastasis model in C57BL/6 mice. (B) Spontaneous LLC metastasis was decreased in EDP- and t-AUCB–treated mice relative to vehicle treatment 17 d after primary tumor removal (LLC resection). Images show representative lung metastasis in treated and control mice. (Scale bar, 1 cm.) n = 4–5 mice per group. Results are presented as means ± SEM. *P < 0.05; **P < 0.001.

consistent with previous reports (26, 28). Together, these results designate EDPs and EETs as unique mediators of an angiogenic switch to regulate tumorigenesis.

Previous research on omega-3 lipid signaling has mainly focused on the COX and LOX pathways (10–13), whereas the CYP pathway, which is the third branch of the lipid metabolic cascade (14–16), has received little attention (40). The present study implies that the previously unappreciated CYP epoxygenase pathway could play a critical role in mediating the opposite effects of omega-3 and omega-6 polyunsaturated fatty acids on angiogenesis and cancer. Omega-3 fatty acids have been shown to be poor substrates of COX and LOX enzymes (17), whereas they are highly efficient alternative substrates for numerous isoforms of CYP epoxygenases (16). Supplementation of DHA in vivo reduces the levels of EETs and increases the levels of EDPs in most organs (16). Thus, an exchange of proangiogenic EETs with antiangiogenic and anticancer EDPs could explain the antiangiogenic and anticancer effects of DHA. Increased formation of EDPs has also been observed in humans upon DHA supplementation (19, 20). Our findings may also be correlated with the effects of DHA in humans.

EETs and EDPs are best described as regulators of inflammation and vascular tone (21–24). Compared with EETs, EDPs are more potent than the EETs for vasodilation (~1,000 times more potent than EETs) (24) and anti-inflammation (22). These results further argue that a replacement of EETs with EDPs upon omega-3 supplementation causes multiple beneficial effects. Previous studies showed that EETs stimulate angiogenesis via up-regulation of VEGF (VEGF-A) in vitro and in vivo (25, 28). Here we found that EDP had no effect on VEGF-A expression, whereas it potently inhibited the expression of VEGF-C in vitro (Fig. 1G and Table S1). VEGF-C is a critical mediator of lymphangiogenesis (41) and is an important therapeutic target for cancer. Currently an anti-VEGF-C monoclonal antibody VGX-100 is in phase I cancer clinical trials. Further studies are needed to test whether EDP suppresses VEGF-C and the resulting lymphangiogenesis in vivo. In addition, we demonstrate VEGFR2 as a potential cellular target for the antiangiogenic effect of EDPs. A 10-min treatment of 1 μM 19,20-EDP dramatically inhibited VEGF-induced VEGFR2 phosphorylation in endothelial cells (Fig. 1F), supporting 19,20-EDP inhibition of angiogenesis via a VEGFR2-dependent mechanism. This is consistent with our findings that 19,20-EDP inhibited VEGF-induced angiogenesis in vitro and in vivo (Fig. 1). VEGFR2 is the most important VEGF receptor, mediating almost all known cellular responses of VEGF and is the therapeutic target of numerous angiogenesis inhibitors on the market (30). However, a common side effect of angiogenesis inhibitors that target the VEGF–VEGFR2 pathway is the induction of hypertension (42). Due to the extremely potent vasodilatory effects of EDPs (24), EDPs may have unique advantages in antiangiogenic cancer therapy by avoiding hypertension, which is a side effect associated with all current antiangiogenic drugs. Further studies are needed to investigate the effects of EDPs on blood pressure and other cardiovascular functions.

The tissue levels of endogenous EETs and EDPs are determined by the ARA and DHA released from membrane phospholipids, CYP epoxygenases, and sEH. Among the most abundant epoxy lipid mediators in omega-6 fatty acid-rich and omega-3-rich tissues are EETs and EDPs, respectively, which are further increased by genetic deletion or pharmacological inhibition of sEH (16, 43). For example, in zebrafish, 19,20-EDP was reported to be the most abundant epoxy lipid mediator; the other epoxy
lipids were very minor, and inhibition of sEH caused an approximately fivefold increase of 19,20-EDP level (43). The findings in this paper suggest that inhibition of sEH will cause opposite effects on angiogenesis in omega-6–rich and omega-3–rich tissues. Indeed, sEH inhibition increased angiogenesis in omega-6–rich tissues or cells (27, 28), whereas it suppressed angiogenesis in omega-3–rich tissues such as zebra fish and the retina (43, 44). These conflicting results can at least partially be explained by our findings of the opposite effects of EETs and EPDs on angiogenesis. Our findings further indicate that sEH could be a potential therapeutic target to treat retinal neovascularization, which is a major cause of blindness in humans (4). DHA is most enriched in retinal tissues, comprising ~60% of polyunsaturated fatty acids in retina (18). Based on our findings here, inhibition of sEH would stabilize and increase the levels of DHA-derived EPDs, attenuating retinal angiogenesis (44). Further studies are needed to test the effect of sEH inhibitors on retinal neovascularization.

A significant finding of this paper is that high levels of systemic EPDs cause dramatic inhibition of primary tumor growth and tumor metastasis by inhibiting tumor angiogenesis (Figs. 2 and 3). Based on the potent anticancer and antimetastatic effects of EPDs, EPDs are potential structural targets to develop stable analogs that mimic EPDs as anticancer agents. Previous studies have shown that fatty acid epoxides are highly unstable in vivo (34). A major pathway to metabolize the epoxides involves the sEH enzyme to generate fatty acid diols, which are usually less active (35, 36). The sEH enzyme is abundantly expressed in numerous tissues (45) and we have shown that EPDs are highly efficient substrates for sEH (22). Therefore, coadministration of a low-dose sEHi was required to stabilize EPDs in circulation, leading to dramatic inhibition of tumor growth and metastasis (Figs. 2 and 3). In the present study, administration of sEHi t-AUCB (1 mg·kg−1·d−1) had no effect on tumor growth and metastasis (Figs. 2A and 3B), and our previous study showed that t-AUCB at 10 mg·kg−1·d−1 significantly increased tumor progression (28). These results suggest that the effect of sEHi (or EETs) on tumor progression is dose dependent; more studies are needed to characterize the threshold for the effects of sEHi (or EETs) on cancer.

CYP epoxynogenases such as CYP2J2 have been reported to be highly expressed in some human tumors (46). CYP3A4, which is expressed in breast cancer, is also induced by increased overall survival in breast cancer (47), which was recently identified as an epoxynogenase that catalyzes the conversion of ARA to EETs (48). Overexpression of CYP epoxynogenases in cancer cells or endothelial cells accelerates tumor growth and metastasis (28, 46, 49), which are largely attributed to ARA-derived EETs (18). Based on our findings, coadministration of DHA and sEHi may be an effective strategy to reduce risks of cancers with high CYP epoxynogenase expression, by not only suppressing EETs but also increasing EPDs. Some anticancer drugs on the market, such as Sorafenib, are also potent sEH inhibitors (50). Analyzing the levels of EETs and EPDs in plasma or tumors, or the expression of CYP epoxynogenases in tumor samples, may help to screen the patients who will most likely benefit from the omega-3 intervention.

Together, the present study and our previous report (28) demonstrate a central role of EPDs and EETs in angiogenesis and tumorigenesis, demonstrating that the CYP/sEH pathway plays a critical role in mediating the antiangiogenic and anticancer effects of omega-3 fatty acids (4, 9). These findings also illustrate unique opportunities to treat pathological angiogenesis and cancers using omega-3 lipids.

Materials and Methods

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

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. Briefly, 0.5 ml growth-factor-reduced Matrigel (BD Biosciences) was mixed with 100 ng mouse VEGF 164 or 500 ng mouse FGF-2 (R&D Systems), 20 units heparin (APP Pharmaceuticals), with or without EPDs. Then the gel was s.c. injected into C57BL/6 mice in the abdominal area. After 4 d, the animals were euthanized to dissect the implanted Matrigel plugs. The gel plugs were weighed, homogenized in 1 mL PBS buffer, and centrifuged; the content of hemoglobin in the supernatant was analyzed by Drabkin’s reagent (Sigma-Aldrich) and normalized to the gel weights. Angiogenesis was also characterized by immunohistochemistry of CD31 staining (29).

Primary Tumor Growth. Met-1 tumor pieces (1 mm3) were transplanted into the fourth inguinal mammary fat pads of FVB female mice (Charles River) (33). All of the mice in the tumor experiments were maintained on a standard mouse chow, which contains ~1.2% (wt/vwt) omega-6 and ~0.2% omega-3 fatty acids. When the tumors reached the size of 2–3 mm in diameter (takes around 2 wk), the mice were s.c. implanted with Alzet osmotic minipumps (model 1002) loaded with 19,20-EDP and sEHi t-AUCB, which was dissolved in a mixed solvent of polyethylene glycol 400 (PEG400; 50%, vol/vol) and DMSO (50%, vol/vol). The dose of 19,20-EDP was 0.05 mg·kg−1·d−1 and the dose of t-AUCB was 1 mg·kg−1·d−1. During this period, animals were checked by ultrasound imaging (Acuson Sequoia 512; Siemens) to mark changes in tumor growth. At the end of the experiment, the tumors were dissected to measure tumor weight. The plasma and tumors were also collected for lipidomics analysis as described below (51). Tumor angiogenesis was analyzed by immunohistochemistry using CD31 and H&E staining.

Tumor Metastasis. Tumor metastasis was studied using an LLC model as described before (28, 39). Briefly, 14 d after s.c. injection of the LLC cells into C57BL/6 mice, the primary LLC tumors were resected to trigger spontaneous lung metastasis. On the same day of tumor resection, the mice were implanted with Alzet osmotic minipumps loaded with t-AUCB and 16,17- or 19,20-EDP, which was dissolved in a mixed solvent of PEG400 (50%, vol/vol) and DMSO (50%, vol/vol). The dose of 16,17- or 19,20-EDP was 0.05 mg·kg−1·d−1 and the dose of t-AUCB was 1 mg·kg−1·d−1. After 17 d of treatment, the mice were euthanized to dissect the lung tissues; surface metastasis foci were counted by means of a stereomicroscope.

Statistics. Group comparisons were carried out using one-way analysis of variance or Student t test. P values less than 0.05 were considered statistically significant. For tumor metastasis, data are presented as mean ± SEM; in other experiments, data are presented as mean ± SD.

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