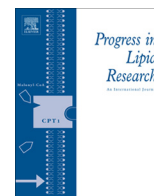




Contents lists available at ScienceDirect

Progress in Lipid Research

journal homepage: www.elsevier.com/locate/plipres

Review

Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer

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ARTICLE INFO

Article history:

Received 13 November 2013

Accepted 26 November 2013

Available online 15 December 2013

Keywords:

Cytochrome P450 epoxygenase

Soluble epoxide hydrolase

Epoxyeicosatrienoic acids

Epoxydocosapentaenoic acids

ABSTRACT

Epoxygenated fatty acids (EpFAs), which are lipid mediators produced by cytochrome P450 epoxygenases from polyunsaturated fatty acids, are important signaling molecules known to regulate various biological processes including inflammation, pain and angiogenesis. The EpFAs are further metabolized by soluble epoxide hydrolase (sEH) to form fatty acid diols which are usually less-active. Pharmacological inhibitors of sEH that stabilize endogenous EpFAs are being considered for human clinical uses. Here we review the biology of ω -3 and ω -6 EpFAs on inflammation, pain, angiogenesis and tumorigenesis.

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1. Introduction

Arachidonic acid (ARA, 20:4 ω -6) comprises a major component in the membrane phospholipids and plays a critical role in cell signaling [1–3]. Upon cellular stimulation, the incorporated ARA is released by several enzymes including diacylglycerol lipase and phospholipase A2 (PLA₂) to generate free intracellular ARA, which is rapidly metabolized by a series of enzymes to generate lipid mediators (LMs) in a process collectively termed the ARA cascade [1–3]. The lipid signaling in the ARA cascade is important because the LMs regulate many fundamental biological processes from inflammation to blood flow, and therefore are important therapeutic targets for multiple human disorders [1–3]. There are three major branches in the ARA cascade: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways. The COX and LOX pathways generate predominately but not exclusively pro-inflammatory LMs and a variety of approved drugs target these two branches [2]. In contrast, our knowledge of the CYP pathway, which is usually regarded as the third branch of the ARA cascade, is rather limited and has not yet been exploited therapeutically [3–6]. Lipid amides and other endocannabinoids are important chemical mediators [7]. However, they are usually not considered as part of the ARA cascade and only their epoxygenated metabolites are discussed here.

The CYP branch, which was first described in 1980s, converts ARA to two major classes of LMs: CYP ω / ω -1 hydroxylases (mainly CYP4A and CYP4F) catalyze the hydroxylation of ARA to generate 19-hydroxyeicosatetraenoic acid (19-HETE) and 20-HETE [8]. In the other branch of the CYP pathway, CYP epoxygenases (mainly CYP2C and CYP2J) catalyze the epoxidation of ARA to generate epoxygenated fatty acids (EpFAs) called epoxyeicosatrienoic acids (EETs) that include four regioisomers of 5,6-, 8,9-, 11,12- and 14,15-EET [3]. 20-HETE has been shown to have an array of largely detrimental effects, inducing hypertension, endothelial dysfunction, inflammation, cardiovascular diseases, angiogenesis and tumor growth [9–14]. EETs have been investigated as autocrine and paracrine signaling molecules which have anti-inflammatory, vasodilative, anti-hypertensive, cardio-protective, renal-protective, pro-angiogenic and analgesic effects [5]. As we simplistically discuss LMs with terms such as inflammatory or anti-inflammatory

and suggest beneficial or detrimental effects, it is important to remember most LMs have multiple effects that maintain a critical balance in normal physiology. Although chemically stable (other than the 5,6-EET regioisomer), EETs are highly unstable *in vivo* mainly due to the rapid metabolism by soluble epoxide hydrolase (sEH, encoded by *EPHX2*) to the less-active fatty acid diols termed dihydroxyeicosatrienoic acids (DHETs) (Fig. 1) [6]. Therefore, blocking the degradation of generally beneficial EETs by targeting sEH is pharmacologically attractive. During the past decade, pharmacological inhibitors of sEH (sEHIs) with IC₅₀ values in nM–pM range and good pharmacokinetic (PK) profiles *in vivo* have been developed [4,15]. The sEHIs, which stabilize endogenous EETs, are promising drug candidates for multiple human diseases and have been evaluated in phase II human trials [4,16].

Linoleic acid (18:2, ω -6), which is a biosynthetic precursor to generate ARA and is highly abundant in the western diet [17], is also a substrate of the CYP/sEH pathway [6]. The metabolism of linoleic acid by CYP epoxygenases generates the linoleic epoxides including 9,10-epoxyoctadecenoic acid (9,10-EpOME) and 12,13-epoxyoctadecenoic acid (12,13-EpOME), which are further metabolized by sEH to form the linoleic diols including 9,10-dihydroxyoctadecenoic acid (9,10-DiHOME) and 12,13-dihydroxyoctadecenoic acid (12,13-DiHOME) [6]. EpOMEs have been associated with multiple organ failure and adult respiratory distress syndrome in some severe burn patients [18–21]. We have shown that the sEH-mediated conversion of EpOMEs to DiHOMEs plays a critical role in the cellular toxicity of EpOMEs [22]. With a high consumption of linoleic acid in the western diet, it is critical to investigate the effects of linoleic acid metabolites on human health, in particular EpOMEs and DiHOMEs which have been demonstrated to have toxic effects.

Besides ω -6 polyunsaturated fatty acids (PUFAs), ω -3 PUFAs such as eicosapentaenoic acid (EPA, 20:5 ω -3) and docosahexaenoic acid (DHA, 22:6 ω -3) are also substrates of the enzymes in the ARA cascade, which convert them to the ω -3-series LMs [23–25]. A major theory to explain the health-promoting effects of ω -3 PUFAs is that they compete with ARA for the enzymatic metabolism, decreasing the formation of ω -6-series LMs that are predominately pro-angiogenic and pro-inflammatory and increasing ω -3-series LMs that have less detrimental and possibly beneficial effects [23–25]. Indeed, the metabolism of ω -3 PUFAs by COX and LOX

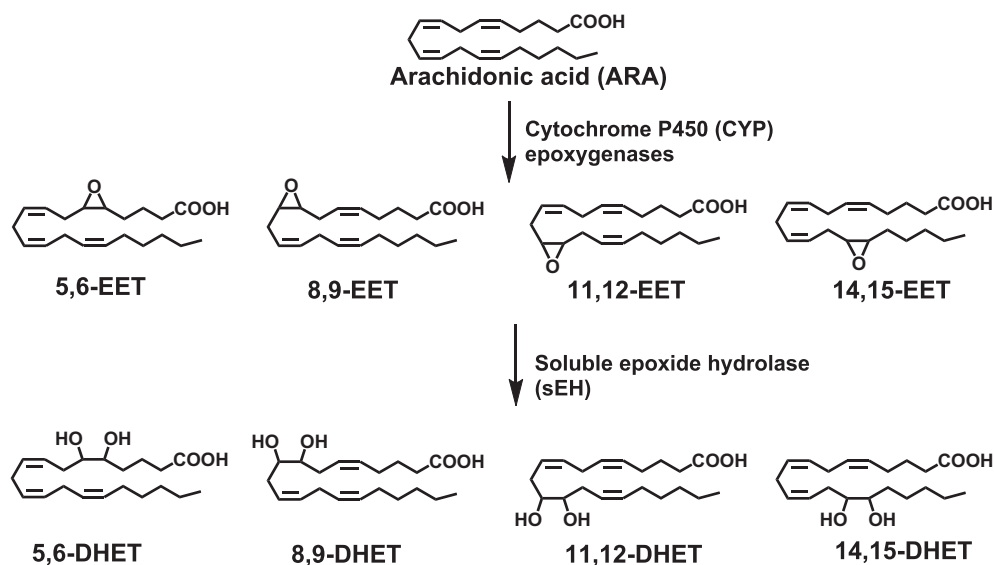


Fig. 1. The metabolism of arachidonic acid by cytochrome P450 (CYP) epoxygenases (largely CYP2C and CYP2J) leads to the formation of epoxyeicosatrienoic acids (EETs) including four regioisomers of 5,6-, 8,9-, 11,12- and 14,15-EET. EETs are further metabolized by soluble epoxide hydrolase (sEH) to form the fatty acid diols termed dihydroxyeicosatrienoic acids (DHETs) which are usually less-active or inactive.

enzymes generates ω -3-series prostaglandins [26,27] and leukotrienes [28], as well as unique ω -3 autacoids such as resolvins and protectins [25], which have anti-inflammatory or anti-angiogenic effects. EPA and DHA are believed to be poor substrates of COX and LOX enzymes [23], however they have been shown to be highly efficient alternative substrates of CYP epoxygenases, which convert them to the ω -3 EpFAs named epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs) respectively [29] (Fig. 2). Compared with EETs, the ω -3 EpFAs are generally better substrates of sEH which convert them to the corresponding ω -3-series fatty acid diols [30]. As expected from its structure, the 19,20-EDP is more slowly turned over by the sEH. Compared with EETs, the biological effects of the ω -3 EpFAs are less-studied. EEQs and EDPs have similar or more potent effects for vasodilation, anti-inflammation and analgesia than EETs [30,31], while EDPs and EETs have opposite activities on angiogenesis, tumor growth and

metastasis [32,33]. This offers us additional opportunities to manipulate profiles of EpFAs to improve human health.

EpFAs have been demonstrated to be involved in many human diseases and hold promise as novel therapeutic targets [5]. This review discusses the biological activities and mechanisms of actions of the ω -6 and ω -3 EpFAs including EETs, EEQs and EDPs on inflammation, pain, angiogenesis and cancer. EpFAs have also been shown to have anti-hypertensive, cardio-protective and organ protective effects. These topics have been covered in several recent reviews [5,34,35] and will not be discussed here.

1.1. Overview of the CYP/sEH pathway

CYP epoxygenases catalyze epoxidation of the double bonds of ARA to generate EETs. The epoxidation can occur at all of the four double bonds of ARA, leading to formation of four regioisomers

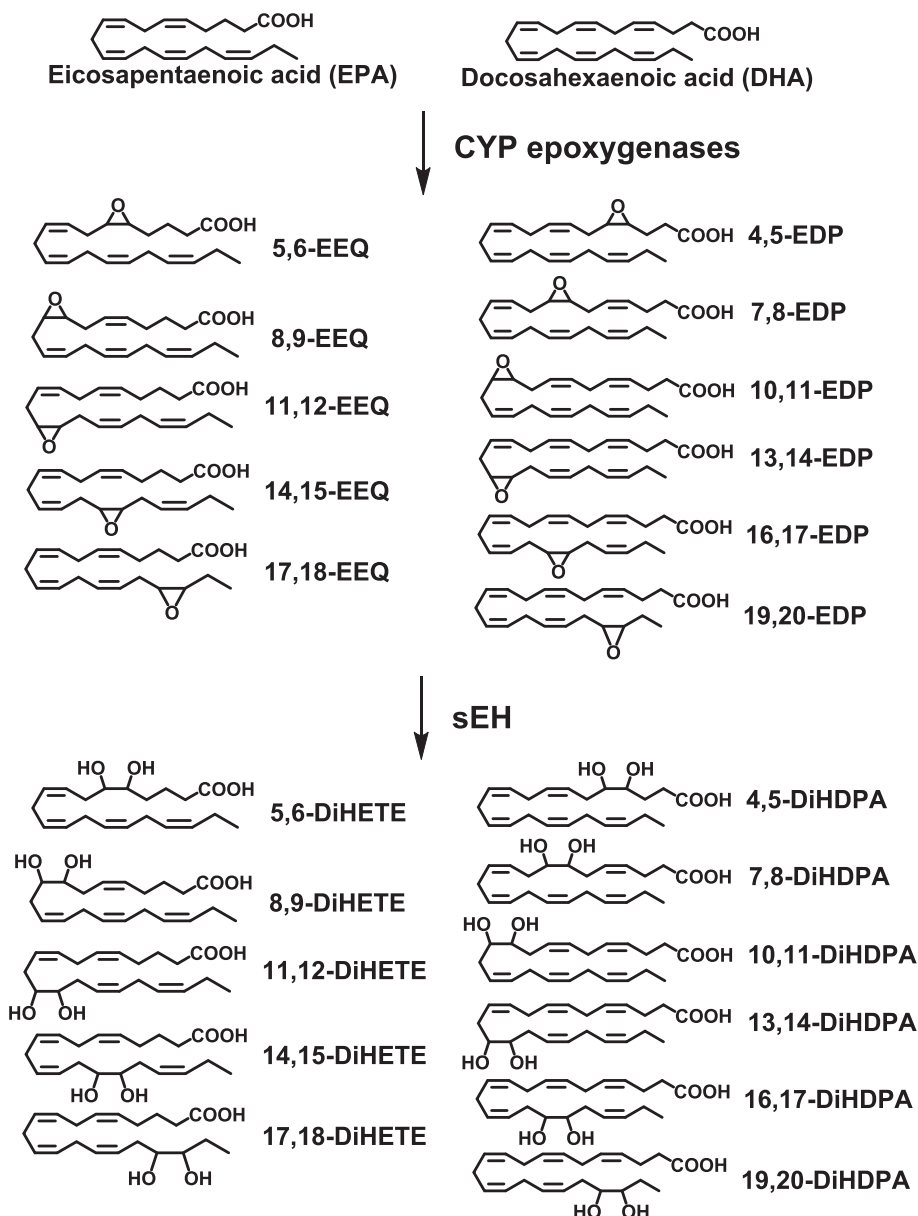


Fig. 2. The ω -3 PUFAs including EPA and DHA are highly efficient alternative substrates of the CYP/sEH pathway. The metabolism of EPA and DHA by CYP epoxygenases generates ω -3-series epoxygenated fatty acids (EpFAs), including 5 regioisomers of EEQs from EPA and 6 EDP isomers from DHA. Compared with ARA, EPA and DHA showed higher or similar activities toward the metabolism by CYP epoxygenases. Compared with EETs, EEQs and EDPs are also better substrates of sEH to be metabolized to the corresponding fatty acid diols (DiHETE and DiHDPA respectively) with the exception of 19,20-EDP.

(5,6-, 8,9-, 11,12- and 14,15-EET) [3]. Among these regioisomers, 5,6-EET is chemically unstable and undergoes rapid cyclization and hydrolysis, the other isomers are chemically stable except under acidic conditions. The CYPs referred to as epoxygenases are by no means specific, for example, they also oxidize reactive methylenes in PUFAs. The biochemistry of CYP epoxygenases in EETs biosynthesis have been discussed in several reviews [3,36,37]. A series of CYP enzymes such as CYP1A, CYP2B, CYP2C, CYP2D, CYP2G, CYP2J, CYP2N, and CYP4A are capable of converting ARA to EETs [3]. Generally each enzyme produces EETs with different profiles of optical- and regioisomers. In mammals CYP2C and CYP2J isoforms appear to be the predominate epoxygenases. For human and rat, CYP2C isoforms are the most abundant in liver and kidney, and CYP2J are the major ones in heart [3]. CYP2C and CYP2J are also the major epoxygenases in endothelium [36]. Similar to other CYP enzymes, the expression of CYP epoxygenases can be modulated by environmental factors and cellular stimuli [3,36,38]. Among these, the most physiologically relevant stimulator of CYP epoxygenase expression is hypoxia [39,40], which is a critical regulator to stimulate neovascularization [41], suggesting a role of CYP epoxygenases in angiogenesis.

Once formed, EETs are rapidly metabolized by sEH to generate the corresponding fatty acid diols called DHETs [6]. Compared with EETs, DHETs are widely believed to be inactive or less-active [5]; a recent study shows that opposite to the anti-inflammatory effects of EETs, DHETs are pro-inflammatory in stimulating monocyte migration [42]. Therefore the sEH-mediated metabolic step is generally regarded as a loss of beneficial biological activities. The biochemistry, expression and regulation of sEH have been discussed in several reviews [4–6]. The mammalian sEH is a homodimer composed of two ~62 kDa monomers. Each monomer has a ~35 kDa C-terminal domain which displays epoxide hydrolase activity and a ~25 kDa N-terminal domain which appears to have phosphatase activity [43]. sEH is highly expressed in many tissues including liver, kidney, lung, heart, brain, spleen, endothelium and mammary gland [6]. The highest sEH activity was observed in liver, followed with kidney. Even in organs with relatively low sEH activity, the enzymatic activity can be high in individual cell types. The expression of sEH is inducible by peroxisome proliferator-activated receptor α (PPAR α) and PPAR γ agonists [6]. PPAR γ agonists have been shown to inhibit tumor growth and metastasis by blocking angiogenesis [44,45], the anti-cancer effect of these compounds could be partially mediated by reduction of the amount of EETs via stimulation of sEH [32]. The expression of sEH is also up-regulated by angiotensin II [46] and homocysteine [47]. The sEH catalyzes hydrolysis of EpFAs by addition of water, in a two-step, base-catalyzed mechanism via the formation of a covalent intermediate [6]. Pharmacological inhibitors, many of which have a urea, amide, or carbamate group as the central pharmacophore to mimic the reaction transition states, were designed based on the catalytic mechanism of sEH enzyme [15]. A sEH inhibitor APAU has been evaluated in a Phase IIA trial targeting hypertension as the primary indication, recently more potent and metabolically stable sEHs have been developed and are being considered for evaluation in human trials. FDA-approved anti-cancer drugs sorafenib (Nexavar[®]) and regorafenib (Stivarga[®]) are also potent sEH inhibitors with IC₅₀ values in the low nM–pM range [48,49].

Besides the hydrolysis catalyzed by sEH, EpFAs are also metabolized by other pathways including β -oxidation, chain shortening and chain elongation [3]. EpFAs can be re-incorporated into the membrane phospholipids by acyl transferase causing a significant proportion of EpFAs to exist as esterified forms in the membrane phospholipids. In comparison, the fatty acid diols are less readily incorporated in the membrane phospholipids [3]. The biological significance of the membrane-incorporated EpFAs remains poorly studied. To address this question, more reliable analytical methods

are needed to quantify the membrane-incorporated EpFAs and diols and particularly to evaluate the kinetics of this process. EpFAs could also be further metabolized by COX, LOX and CYP enzymes to generate novel series of LMs, though little is known about the chemical structures and biological activities of these lipid metabolites. Both 5,6- and 8,9-EET have been shown to be substrates of COX enzymes, which convert them to metabolites which may have potent effects of vasodilation or stimulation of cell proliferation [50–52]. A COX metabolite of 8,9-EET, 11-hydroxy-8,9-epoxyeicosatrienoic acid, has been shown to be >1000-fold more potent than 8,9-EET in stimulating cell proliferation and c-fos expression in rat glomerular mesangial cells [52]. Previous research from our laboratory has shown that sEHs dramatically synergize with COX or LOX inhibitors to reduce inflammation [53,54]. It would be interesting to investigate whether the observed synergistic interactions are in part mediated by the LOX and COX-derived metabolites of EETs. It was recently shown that EPA-derived 17,18-EEQ is further metabolized to generate 12-OH-17,18-EEQ, which inhibited LTB₄-induced neutrophil chemotaxis and polarization *in vitro* with EC₅₀ = 0.6 nM [55]. Other pathways such as β -oxidation and chain elongation also participate in the metabolism of EETs. When sEH is inhibited, other metabolic pathways of EETs are up-regulated [56]. Since the fates of EETs are regulated by multiple metabolic pathways, pharmacological inhibition or genetic deletion of sEH only increases level of EETs in a limited range.

1.2. Metabolism of ω -3 PUFAs by CYP/sEH pathway

The ω -3 PUFAs including EPA and DHA were mainly derived from cold-water fish, and were of dramatically varying quality. Overall quality of fish oils is increasing, their composition is defined, and increasingly they may be derived from many sources including krill, tissues of marine mammals, algae and yeast. They are among the most popular dietary supplements in United States. In addition, major food companies are increasingly adding ω -3 PUFAs to food as value-added ingredients. Two ω -3 PUFA products have been approved by the FDA as prescription drugs to treat hypertriglyceridemia, including Lovaza[®] from GSK and Vascepa[®] from Amarin. Although epidemiological and pre-clinical data show a correlation between increases in ω -3 relative to ω -6 PUFAs in the diet and reduced risks of various chronic diseases such as cancers [57–63] and macular degeneration [64–67], the underlying mechanisms are largely unknown. A dominant theory to explain the effects of ω -3 PUFAs is that they suppress the metabolism of ARA which generates predominately pro-inflammatory and pro-angiogenic eicosanoids, or they serve as alternative substrates to generate ω -3 LMs which have unique biological actions [24–26,28]. Indeed, EPA effectively competes with ARA for metabolism by the COX enzymes, the COX-2 metabolite of EPA, prostaglandin E₃ (PGE₃), has less pro-angiogenic and pro-inflammatory effect than the ARA-derived metabolite PGE₂ [26,27]. 5-LOX plays a critical role in the anti-angiogenic effect of DHA, the 5-LOX metabolite of DHA, 4-hydroxy-docosahexaenoic acid (4-HDHA), has potent anti-angiogenic effect and genetic deletion of 5-LOX significantly attenuated the anti-angiogenic effect of DHA in a murine retinopathy model [28]. In a human study, Dwyer et al. showed that a diet rich in ω -3 PUFAs decreased the risks while a diet rich in ω -6 PUFAs increased the risks of atherosclerosis only in the sub-population with high 5-LOX activity [68]. These studies suggest that there is a strong gene-diet interaction. Understanding the molecular mechanism of ω -3 PUFAs could help to design better therapeutic paradigms and human trials to clarify their health benefits.

Besides the well studied COX and LOX pathways, recent research showed that ω -3 PUFAs are good alternative substrates of CYP epoxygenases [29]. Cell-free enzymatic assays showed that CYP epoxygenases have similar activities toward ARA, EPA and

DHA [29]. CYP epoxygenases selectively catalyze the epoxidation of the terminal double bond of ω -3 PUFAs, leading to predominate formation of 17,18-EEQ from EPA and 19,20-EDP from DHA, while no such selectivity was observed for biosynthesis of EETs from ARA [29,69–71]. Most of the ω -3 EpFAs are turned over by sEH more rapidly than EETs except for 19,20-EDP from DHA [30]. The high biosynthesis and low degradation of 19,20-EDP make it among the most abundant ω -3 EpFAs *in vivo* [29,72–74]. In endothelial cells, EPA has been shown to increase transcription of CYP2J2 in a time- and dose-dependent manner resulting in increased biosynthesis of EpFAs [75].

Because several PUFAs including linoleic acid, ARA, EPA and DHA are substrates of the CYP epoxygenases, the CYP pathway generates a large number of ω -6 and ω -3 EpFAs, whose levels are further regulated by the sEH enzyme [29,72–74]. Inhibition of sEH is thus a “shot-gun” approach, stabilizing multiple ω -3 and ω -6 EpFAs. Inhibition of sEH will stabilize EpFAs that are present in the tissue, but only will change the relative amounts of EpFAs based on the preference of the sEH for the EpFA substrates. In most tissues EETs are the major EpFAs because ARA is the most abundant PUFA in cell membrane, while DHA-derived EDPs could be the major EpFAs in DHA-rich tissues such as retina and brain [17]. In zebrafish which is DHA-rich, the most abundant EpFA is DHA-derived 19,20-EDP [76]. In mammals, the relative abundance of ω -6 ARA and ω -3 EPA and DHA in tissues are largely determined by dietary intake of PUFAs because mammals lack the enzymes for *de novo* biosynthesis of ω -3 PUFAs [17,23]. Studies on man and other mammals demonstrate that ω -3 supplementation increases levels of EEQs and EDPs in plasma and tissues. A 3-week feeding of ω -3 PUFA ethyl ester in rats reduced levels of EETs and increased levels of EEQs and EDPs in plasma, brain, heart, kidney, liver and lung [29]. Supplementation of 4 g/day of ω -3 PUFA ethyl ester (465 mg EPA and 375 mg DHA per 1 g capsule) in healthy volunteers for 4 weeks induced a respective \sim 5- and \sim 2-fold increase of EEQs and EDPs in human plasma, while the levels of EETs were not significantly changed [72,73]. Zivkovic et al. reported that supplementation of 4 g/d of fish oil (1.9 g/d EPA and 1.5 g/d DHA) in immunoglobulin A nephropathy patients for 24 months caused a \sim 2-fold increase of DHA epoxides and diols in plasma [74].

2. EETs on inflammation

Dysregulated inflammation is a common feature of most human diseases, therefore modulation or inhibition of inflammation has been proven to be an effective therapeutic strategy [77–79]. LMs play a central role to regulate inflammation. One of the most important LMs in inflammation is PGE₂ which is a COX-2 metabolite of ARA and has predominantly but not exclusively pro-inflammatory activity [80]. Aspirin has been used to inhibit inflammatory pain and fever for over a century. It suppresses inflammation via its action on COX-2 to inhibit the formation of PGE₂ [81]. This discovery resulted in the Nobel Prize in Physiology or Medicine in 1982. Other drugs targeting COX enzymes include non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (coxibs), which are among the most widely used drugs in the world to treat inflammation and pain [82]. However, chronic and high dose use of COX inhibitors can cause life-threatening cardiovascular risks, jeopardizing their clinical applications [83,84]. Most NSAIDs can also lead to organ specific damage and intestinal bleeding in addition to some apparently idiopathic problems in subpopulations. Novel therapeutic targets are urgently needed to treat inflammatory diseases. Recently EETs have been demonstrated to have potent anti-inflammatory effects *in vitro* and *in vivo* [85], suggesting that targeting sEH to stabilize EpFAs is a promising strategy to treat inflammatory disorders. In addition, dual inhibition of

COX-2 and sEH synergistically inhibits inflammation with reduced cardiovascular and gastrointestinal toxicity [53,54], indicating that sEHIs could be used as synergists with COX inhibitors to enhance the efficacy and reduce the adverse effects.

2.1. Biological activities of EETs on inflammation *in vitro*

Node et al. first reported the anti-inflammatory effects of EETs [85]. Synthetic EETs inhibited LPS-, IL-1 α - or TNF- α -induced vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells. The anti-inflammatory effects of EETs are regio-selective: 11,12-EET showing the most potent effect, followed with 8,9- and 5,6-EET, while 14,15-EET was inactive. 11,12-EET also suppressed other endothelial cell adhesion molecules such as E-selectin and intercellular adhesion molecule 1 (ICAM-1) in TNF- α -stimulated endothelial cells. Over-expression of CYP2J2 produced similar anti-inflammatory response, which was abolished by co-treatment with SKF525A, a broad pharmacological inhibitor of CYP enzymes including epoxygenases [85]. A recent study indicated that 14,15-EET inhibited TNF- α -stimulated inflammation in human bronchi, suggesting this EET regio-isomer is also biologically active to suppress inflammation in some systems [86]. In a recent study, Deng et al. studied LPS-induced inflammatory responses in primary lung endothelial cells isolated from transgenic mice with endothelial expression of human CYP2C8 and CYP2J2 [87]. Transgenic expression of CYP2C8 or CYP2J2, which has higher capacity to generate EETs, significantly inhibited LPS-induced expression of E-selectin and monocyte chemoattractant protein-1 (MCP-1) in isolated endothelial cells, and such anti-inflammatory effects were attenuated by a putative EET receptor antagonist 14,15-EEZE and a selective inhibitor of CYP epoxygenase MS-PPOH [87].

EETs were also shown to inhibit inflammation in inflammatory cells *in vitro*. The mRNA expression of CYP2J2 and CYP2C8 were detected in human peripheral leukocytes, monocytes (Mc) and the human monocyte THP-1 cell line, but not polymorphonuclear cells (PMNs) [88]. 11,12- and 8,9-EET inhibited basal TNF- α production in THP-1 cells; 11,12-EET abolished IL-1 β + PMA-induced COX-2 expression, while the CYP inhibitor SKF525A dose-dependently increased basal COX-2 expression in THP-1 cells [88]. In LPS-stimulated rat monocytes, CYP epoxygenase inhibitors SKF525A and 1-aminobenzotriazole (ABT) increased production of PGE₂, while 11,12-EET dose-dependently suppressed LPS-induced PGE₂ formation via a mechanism of inhibiting the enzymatic activity but not the expression of COX-2 [89]. The sEH inhibitor c-TUCB reduced levels of LPS-induced MCP-1 and TNF- α , but not IL-6 and macrophage inflammatory protein-1 α (MIP-1 α), in human monocytes [90].

The sEH metabolites of EETs, DHETs, are generally believed to be inactive or less-active in many assays [5]. However a recent study showed that opposite to the anti-inflammatory effects of EETs, DHETs are pro-inflammatory in stimulating monocyte migration *in vitro* and *in vivo* [42]. Pharmacological inhibition of COX and LOX pathways had no effect on MCP-1-induced monocyte chemotaxis; while inhibition of CYP or sEH enzymes dose-dependently blocked this process, which was rescued by DHETs, suggesting the contribution of DHETs in MCP-1-induced chemotaxis [42]. This study indicates that pharmacological inhibition of sEH not only stabilizes and increases level of EETs which are anti-inflammatory, but also reduces the formation of DHETs which are pro-inflammatory.

2.2. Biological activities of EETs on inflammation *in vivo*

Animal studies have shown that EETs inhibited inflammation in various disease models. Continuous infusion of 11,12-EET, but not

14,15-EET, inhibited TNF- α -induced endothelial VCAM-1 expression and the resulting mononuclear cells adhesion and rolling in murine carotid artery [85]. Pharmacological inhibition of sEH, which increased level of EETs, reduced LPS-induced mortality, systemic hypotension and tissue injury, as well as elevation of inflammatory cytokines in a LPS-induced sepsis model in mice [91]. Inhibition of sEH reduced tobacco smoke-induced lung inflammation in spontaneously hypertensive rats [92]. Treatment with sEHs decreased total bronchoalveolar lavage cell number with significant reductions noted in neutrophils, alveolar macrophages and lymphocytes in tobacco smoke-exposed rats. Co-administration of sEHs with EETs further enhanced the anti-inflammatory effect of sEHs [92]. In a streptozotocin-induced type 1 murine diabetic model, genetic deletion of sEH significantly reduced urinary MCP-1 excretion and NF- κ B activation in diabetic mice [93]. Tricloro-carban (TCC) is a widely used antimicrobial component in personal care products, which has been shown to be a potent sEH with IC₅₀ = 13 nM for human sEH. In a LPS-induced acute inflammation model, treatment with TCC increased plasma levels of EETs and attenuated LPS-induced hypotension and elevation of pro-inflammatory cytokines in plasma [94]. Inhibition of sEH also reduced inflammation related to cardiovascular diseases. The mRNA expression of pro-inflammatory cytokines in liver (TNF- α and IL-6) and adipose tissues (TNF- α , IL-6, IL-1 β and MCP-1) stimulated by high-fat diet were significantly reduced by treatment with the sEH *t*-AUCB [95]. In a deoxycorticosterone acetate plus high salt (DOCA-salt)-induced hypertensive model in mice, genetic deletion of sEH or treatment with *t*-AUCB reduced DOCA-salt-induced hypertension, renal inflammation and renal injury [96]. Deletion of sEH reduced DOCA-salt-induced production of pro-inflammatory cytokines, macrophage infiltration, NF- κ B signaling activation in renal tissues and elevation of urinary MCP-1 [96]. Inhibition or deletion of sEH reduces vascular remodeling and pro-inflammatory gene expression in an inflammatory model of neointima formation in mice [97]. IL-10 is an important anti-inflammatory cytokine, for example, genetic deletion of IL-10 induces inflammation in tissues and is used as a model for inflammatory bowel disease [98]. To study the role of sEH in inflammation caused by IL-10 deficiency, Zhang et al. created transgenic IL-10 (-/-) sEH (-/-) double knockout mice [99]. Deletion of sEH reduced the inflammatory cell infiltration, pro-inflammatory cytokine expression and NF- κ B signaling activation caused by IL-10 deficiency [99,100]. Beside manipulating sEH to modulate EETs, transgenic expression of CYP2C8 or CYP2J2 in mice significantly attenuated LPS-induced production of pro-inflammatory cytokines such as IL-6, MCP-1, E-selectin, IL-1 β and ENA-78, as well as NF- κ B signaling activation and inflammatory cell infiltration in lung tissues [87].

Together, these studies provide strong evidence to support the anti-inflammatory effects of EETs in various inflammatory models, suggesting that inhibiting sEH to stabilize EETs is a promising therapeutic strategy to treat inflammatory disorders. However it should be noted that there are inconsistent results of sEH inhibition in inflammation [101,102], more studies are thus needed to characterize the effects and mechanisms of EETs in inflammatory diseases and to determine what animal models are indicative of human conditions.

2.3. Mechanisms of EETs on inflammation

EETs have been shown to inhibit inflammation via blocking NF- κ B pathway, which is an important signaling cascade to regulate inflammation. Without cellular stimulation, the NF- κ B complex is sequestered in the cytoplasm through binding to the inhibitory protein I κ B α . When activated, I κ B α is phosphorylated and then degraded, resulting in release of the NF- κ B complex from I κ B α . The released NF- κ B translocates to the nucleus and activates the

expression of multiple pro-inflammatory genes [103]. Node et al. showed that 11,12-EET, but not 14,15-EET, inhibited TNF- α -induced I κ B α degradation and nuclear translocation of NF- κ B in endothelial cells, suggesting that EETs inhibit inflammation via blocking NF- κ B signaling activation [85]. Co-administration of the sEH AUDA with individual EET regioisomers inhibited TNF- α -induced I κ B α degradation in endothelial cells, which was attenuated by a PPAR- γ antagonist GW9662. These data suggest that EETs blocked NF- κ B signaling via a PPAR- γ -dependent mechanism [104]. In human bronchi, 14,15-EET inhibited TNF- α -stimulated inflammation via inhibition of I κ B α degradation [86]. In primary mouse neonatal cardiomyocytes, the sEH AEPU suppressed ascending aortic constriction-induced I κ B α phosphorylation and degradation, and nuclear accumulation of NF- κ B subunit p65 [105]. In primary endothelial cells isolated from Tie2-CYP2J2-Tr mice, LPS-induced I κ B α phosphorylation was attenuated compared with WT cells [87]. Animal experiments also support that EETs and CYP/sEH pathway modulate NF- κ B signaling. LPS-induced I κ B α phosphorylation in lung tissues was significantly attenuated in Tie2-CYP2C8-Tr, Tie2-CYP2J2-Tr and sEH-null mice compared with WT mice [87]. Genetic deletion of sEH suppressed activation of NF- κ B signaling in IL-10-knockout mice [99,100]. Inhibition or deletion of sEH suppressed DOCA-salt- and streptozotocin-induced NF- κ B activation in renal tissues [93,96].

Besides NF- κ B, other signaling pathways have also been investigated. For example, treatment with the sEH *c*-TUCB had no effect on LPS-induced NF- κ B nuclear translocation, while it abolished LPS-induced phosphorylation of JNK in adherent human monocytes, suggesting that JNK pathway is a potential target of sEHs or EETs in monocytes [90]. PPARs have also been shown to contribute to the anti-inflammatory effects of EETs [104,106–108].

2.4. Synergistic interactions of sEHs with other anti-inflammatory drugs

COX inhibitors such as NSAIDs and coxibs are the most widely used drugs in the world to treat inflammation. However, the dose-limiting cardiovascular side effects and gastrointestinal erosion of COX inhibitors have limited their clinical applications [83,84]. We discovered that dual inhibition of sEH and COX-2 pathways synergistically inhibited inflammation with reduced cardiovascular toxicity [53,54]. Co-administration of the sEH AUDA-BE or *t*-AUCB synergized with indomethacin, celecoxib, rofecoxib and aspirin to suppress LPS-induced inflammation in mice [53,54]. Genetic deletion of sEH also synergizes with aspirin to suppress inflammation [54]. Together, these results suggest a potent interaction of sEH and COX-2 pathways to regulate inflammation. The mechanisms for the synergistic interactions remain to be elucidated. Previous studies have shown that sEHs inhibit transcription of COX-2 [109] and COX inhibitors inhibit the enzymatic activity of COX-2 [82], therefore, a combination of these two drugs synergistically inhibit PGE₂ formation as we have demonstrated in animal experiments [53,54]. The synergistic reduction of PGE₂ in some tissues may partially explain the interaction of COX inhibitors with sEHs. The synergistic interactions of these two drugs suggest that the required doses of COX inhibitors could be reduced while producing equivalent effect, leading to attenuation of potential side effects.

A major theory to explain the cardiovascular risks of coxibs is that they block the formation of prostacyclin (PGI₂) which is a potent vasodilator, but not COX-1 derived thromboxane A₂ (TXA₂) which is a potent vasoconstrictor; therefore, a decrease of the PGI₂ to TXA₂ ratio may increase incidence of thrombotic cardiovascular events [110]. Therefore, the PGI₂-to-TXA₂ ratio is widely used as a biomarker to predict the COX-associated cardiovascular risks. Consistent with this theory, our previous study showed that a 6-h

treatment of celecoxib or rofecoxib significantly reduced the ratio of PGI₂ (stable metabolite 6-keto-PGF_{1α}) to TXA₂ (stable metabolite TXB₂) in plasma in mice. However, co-administration of coxibs with sEHs did not perturb the balance of PGI₂-to-TXA₂ ratio, suggesting the co-treatment may reduce cardiovascular toxicity compared to coxibs [53].

Relying upon co-administration of synergistic drugs may introduce problems such as poor patient compliance, complicated drug-drug interactions, or patient-dependent differences in the PK profiles of each drug [111]. Therefore, we took a poly-pharmacology approach to design and synthesize the first-in-class COX-2/sEH dual inhibitors which simultaneously antagonize both COX-2 and sEH [112]. Surprisingly, a COX-2/sEH dual inhibitor, PTUPB, has been shown to be far more efficacious in attenuating inflammatory pain *in vivo* than a coxib celecoxib, a selective sEHI *t*-AUCB, or a combination of celecoxib and *t*-AUCB [112]. Presumably the co-treatment regime or the dual inhibitor acts both to reduce production of inflammatory eicosanoids such as PGE₂ and to stabilize anti-inflammatory and cardio-protective eicosanoids such as EETs. Besides COX inhibitors, our study showed that sEHs also synergized with LOX inhibitors to inhibit inflammation [54]. The mechanisms for the synergistic interactions remain to be elucidated.

3. EETs on inflammatory and neuropathic pain

A potential therapeutic application for EpFAs, their mimics or sEHI is for alleviation of inflammatory and neuropathic pain. Based on a French survey, chronic pain persists within 30% of the population with 7% having characteristics of neuropathic pain [113]. The cost from this chronic pain, considering both health care costs and cost of lost productivity, is estimated to be up to 635 billion dollars for the United States [114]. Despite this expense, up to 40% of those with chronic pain say it is inadequately managed [115], indicating a need for new therapeutic strategies for treating pain. The use of sEHI and EpFAs as potential novel therapeutics for pain management has been previously reviewed by Inceoglu et al. [116] and Wagner et al. [117,118].

Much of the current research investigating the relationship between pain and EpFAs has used sEHs as the primary pharmacologic agent. The original basis for testing sEHs on pain stemmed from observations that sEHs reduce inflammation [91]. Based on this reduced inflammation, it was hypothesized that they may be effective at reducing inflammation-related pain. Consequently, it was shown that sEHs could attenuate hyperalgesia (increased sensitivity to painful stimuli) and allodynia (sensitivity to normally innocuous stimuli) in rodents with inflammation [53]. Observations that direct administration of EETs in absence of sEHI produced the same effect and the structural diversity of sEHI tested indicate EpFAs are the primary mediators of pain relief, rather than an off-target effect of a particular sEHI [119]. Other EpFAs including metabolites of DHA and EPA have similar activity on reducing inflammatory sensitization; however the effect is less potent for EPA metabolites [30]. Additionally, this effect was regioisomer selective, as illustrated by 13,14-EDP being more potent than other DHA EpFAs.

In addition to effects on inflammatory pain, sEHs have proven useful for decreasing diabetes-driven neuropathic pain. The efficacy of EpFAs on neuropathic pain was serendipitously discovered after models of neuropathic pain, used as a control for inflammatory pain, had an unexpected reduced sense of pain in response to noxious stimuli [109]. Although neuropathic pain has many etiologies, the model used for testing EpFAs on pain is destruction of peripheral nerves stemming from unregulated glucose toxicity seen in diabetics. In comparison to gabapentin, a currently prescribed treatment for neuropathic pain, sEHs were more effective and more potent with a more favorable PK profile in a

streptozocin model of type 1 diabetes [120]. Because many of the current therapies for treating chronic and neuropathic pain are associated with unwanted side effects, the development of an sEHI would provide relief for an unmet medical need.

3.1. Mechanisms of EETs on inflammatory pain

EpFA modulation of inflammatory pain has multiple effects including changes in COX-2 regulation and prostaglandin synthesis, interactions with TRP receptors and activity of CB₂ receptors. However, it is widely hypothesized that a yet undiscovered G-protein coupled receptor is the primary mechanism for modulating inflammatory pain [117].

Administration of sEHI during an inflamed state resulted in reduced COX-2 mRNA in the spinal cord [109] and protein [53] expression in the liver relative to inflamed controls. This change in COX-2 regulation is likely modulated by suppression of NF-κB stemming from EpFAs binding to PPARγ [53]. The combination of COX-2 and sEH inhibitors results in a synergistic effect on both reducing the pro-inflammatory prostaglandins and the nociception, perception of pain, associated with systemic inflammation [53]. From this synergistic effect, the development of COX-2/sEH dual inhibitors have resulted in particularly potent inhibitors of LPS-induced inflammatory pain with favorable PK profiles [112]. However, blocking pain is not completely dependent on modulation of the COX-2 pathway, as evidenced by sEHI-mediated analgesia during PGE₂ stimulated pain [121]. Interestingly, evidence indicates that EpFAs induce analgesia using a mechanism that requires prostaglandin activity, which explains why sEHI work only in an already inflamed state or a state of enhanced pain perception. The necessary component of this mechanism is cAMP, a downstream product of the prostanoid receptor, which can be mimicked by addition of phosphodiesterase inhibitors (PDEI) [121]. Thus co-administration of sEHI and PDEI results in reduction in pain-related response in the absence of an inflammatory state [121]. These data also suggest that a combination of sEHI and PDEI will enhance the potency, increase the therapeutic index, and broaden the activity of both drug classes. However, in the absence of inflammatory pain and upregulated cAMP, direct administration of ARA or EET increases sensitivity to pain briefly. A higher dose of EETs are required to enhance in a naive state than to control pain in a hyperalgesic state. The mechanism of this activity is not understood [119].

Paradoxically, when investigators have looked at sEH knockout mice, there does not seem to be the same regulation of COX-2 or modulation of pro-inflammatory prostaglandins seen with administration of sEHI [122]. This apparent discrepancy may be due to other genetic and physiological changes associated with strain of sEH knockout mice [123]. Alternatively each monomer of the sEH dimer has a catalytically active phosphatase domain active on lipophilic but not protein phosphatases [43]. The physiological role of this highly conserved domain is not known but is absent in the sEH knock out animals. In addition to *in vivo* administration to sEH knockout mice, adding 5,6-EET directly to dorsal root ganglia resulted in increased TRPA1-mediated sensitization [122]. Similar to the above results, direct addition of 5,6-EET to TRPV4 transfected cells resulted in generation of Ca²⁺ currents [124]. While these combined findings suggest that 5,6-EET could actually increase sensitivity to pain, the rapid cyclization of this regioisomer indicate a secondary metabolite is the active sensitizing component.

In addition to direct effects on inflammation and pain, mounting evidence suggests a component relating epoxygenated endocannabinoids to pain and inflammation. The best understood endocannabinoids, anandamide (an ethanolamine derivative) and 2-arachidonylglycerol (a glycerol derivative), are derivatives of ARA that bind to the cannabinoid (CB₁ and CB₂) receptors. These

receptors and ligands are named for their relation to the natural product Δ^9 -tetrahydrocannabinol – the active component of *Cannabis sativa* [125]. Changes in the endogenous cannabinoids are associated with a large spectrum of diseases including but not limited to obesity, inflammation, cardiovascular and metabolic disorders, cancer and pain [126]. Activation of CB₂ receptors inhibits nociception in multiple forms of sensitization [127,128], mediated by both inflammatory and non-inflammatory mechanisms [129]. An epoxide of anandamide, 5,6-epoxyeicosatrienoic acid ethanamide (5,6-EET-EA) binds to CB₂ receptor with high affinity ($K_i = 8.9$ nM) and increased stability relative to the parent anandamide. Compared to anandamide, which is hydrolyzed by fatty acid amide hydrolase (FAAH), 5,6-EET-EA can also be hydrolyzed by sEH to the respective diol, which can be inhibited by administration of sEHI [130]. Additionally, the 5,6-EET methyl ester is also a ligand for CB₂ receptor, although with significantly decreased potency ($IC_{50} = 19$ μ M) [116]. To test the possibility that an epoxide may be directly effecting inflammatory pain through the CB₂ receptor, Wagner et al. [118] used CB₂ antagonists to show the antinociceptive effects of sEHI were reduced when blocking CB₂ in an LPS model of localized inflammation. However, further experiments are necessary to determine the exact contribution of cannabinoid signaling to antinociception in both inflammatory and neuropathic pain.

3.2. Mechanisms of EETs on neuropathic pain

In addition to their effects on inflammatory pain, sEHI reduce neuropathic pain through multiple mechanisms that have still not been well characterized. In addition to the CB₂ receptor, a mixture of EETs can displace ligands for peripheral benzodiazepine receptor, neurokinin NK2 receptor and dopamine D₃ receptor with μ M affinity [116]; however, the physiologic significance of this remains to be determined. Other pain pathways which have been well characterized are the regulation opioid neuropeptide synthesis, regulation neurosteroid synthesis and increased somatostatin synthesis.

Multiple lines of evidence suggest that EETs interact with components of opioid receptors resulting in analgesia. 14,15-EET injected directly into the ventrolateral periaqueductal gray region of the brain produced effects similar to morphine [131]. 14,15-EET did not directly compete against radioligands for opioid receptors, but the effects of EETs were inhibited by antisera targeting the β -endorphin neuropeptides, Met-enkephalin and either μ -opioid or δ -opioid receptor antagonists. Furthermore, attenuated analgesic responses from morphine administration in CYP-null mice (which lack the ability to generate high levels of EETs) suggest this effect is essential for normal function of opioid receptor systems and is required for the analgesic effects of opioids [132]. Interestingly, inhibition of fatty acid oxidizing CYPs results in inhibited antinociception mediated by both morphine, an opioid, and imiprogan, a nonopioid analgesic with a poorly characterized mechanism of action [133,134]. It has been proposed that the EpFA mediates signaling by suppressing GABA terminals which mediate antinociception associated with the rostral ventromedial medulla, blocking convergent signals of the unknown imiprogan receptor, the opioid receptor and CB₁ receptor [133]. However, this hypothesis has not been confirmed and as of yet, still requires validation.

In addition to effects on opioid receptors, EETs are known to interact with neurohormone synthesis. As previously mentioned, EETs bind weakly to the peripheral benzodiazepine receptor, also known as TSPO, which is involved in the synthesis of analgesic neurosteroids in the central nervous system. Blocking the synthesis of these hormones with inhibitors resulted in abatement of sEHI-mediated analgesia, indicating that this weak binding is relevant physiologically [109]. Additionally, increased availability of either

sEHI or 5,6-, 8,9- or 11,12-EETs resulted in a cAMP dependent upregulation of steroidogenic acute regulatory protein (StARD1), a protein involved with and a general biomarker of neurosteroid synthesis, and progesterone, an analgesic compound and precursor for neurosteroids [109,135].

4. EETs on angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is critical for multiple physiological and pathological processes [136]. Blocking angiogenesis is a promising strategy to treat cancers and FDA has approved multiple anti-angiogenic drugs for cancer treatment [137]. Anti-angiogenic drugs are also used to treat macular degeneration [138]. On the other hand, pro-angiogenic drugs, which stimulate neovascularization to increase blood flow to tissues, could be useful for some human disorders such as hepatic insufficiency, immature lung development, burn and post surgical treatment and diabetic wound healing [139].

The process of angiogenesis is orchestrated by an array of angiogenic stimulators and inhibitors. While current research of angiogenic mediators has mainly focused on proteins such as VEGF, the non-proteinaceous LMs have received less attention. Emerging evidence demonstrates that the LMs potently regulate angiogenesis, tumor growth and metastasis and are thus therapeutic targets to treat cancers and angiogenic diseases [140]. COX- and LOX-derived LMs have been shown to stimulate angiogenesis and inflammation, playing critical roles in tumorigenesis. Epidemiological and pre-clinical evidence support that the COX and LOX inhibitors are effective to treat or prevent cancers [140]. In comparison, the roles of the CYP-derived EpFAs on angiogenesis and cancer are largely unknown [141].

4.1. Biological activities of EETs on angiogenesis *in vitro*

The first data to suggest the pro-angiogenic effect of EETs was that endogenous EETs released from astrocytes stimulated cellular proliferation and tube formation of co-cultured endothelial cells [142]. Further studies showed that treatment with synthetic EETs stimulated angiogenesis in endothelial cells [36,143,144]. 11,12- and 14,15-EET are the most studied EET regioisomers and they have been shown to increase endothelial cell proliferation, migration and invasion, which are critical cellular steps involved in angiogenesis [145–149]. 11,12-EET also increased activity of matrix metalloproteases (MMPs), though the identity of the MMP enzymes involved remain to be confirmed [39]. Besides 11,12- and 14,15-EET, 5,6- and 8,9-EET have also been shown to stimulate cellular proliferation and tube formation in pulmonary murine microvascular endothelial cells, while the diols (5,6-, 8,9-, 11,12- and 14,15-DHET) lacked such effects [150]. Overexpression of CYP epoxygenases or pharmacological inhibition of sEH produced similar pro-angiogenic effects in endothelial cells [39,40,148, 150–152], while pharmacological inhibition of CYP epoxygenases suppressed angiogenesis [148]. Over-expression of CYP2C9 or treatment with 11,12-EET increased the expression of COX-2 in endothelial cells, consistent with the pro-angiogenic effect of EETs [153]. CYP2C in endothelial cells is inducible by hypoxia which is a key regulator to stimulate angiogenesis, suggesting that EETs are involved in the hypoxia-induced angiogenic responses [39]. Together, these results support the pro-angiogenic effects of EETs *in vitro*.

4.2. Biological activities of EETs on angiogenesis *in vivo*

Animal studies were followed up to support the pro-angiogenic effects of EETs *in vivo*. Treatment with 11,12- or 14,15-EET

stimulated neovascularization in a Matrigel plug assay and a chick chorioallantoic membrane (CAM) assay [148,151,154]. 5,6- and 8,9-EET stimulated angiogenesis in a subcutaneous sponge assay in mice [150]. Local treatment with 11,12-EET, 14,15-EET or the sEH *t*-AUCB accelerated wound epithelialization and neovascularization in mice [155]. Recently Panigrahy et al. demonstrated that endothelium-derived EETs accelerated organ and tissue regeneration by stimulation of angiogenesis [156]. This study used three transgenic mouse models which have high level of EETs: endothelial expression of human CYP2C8 and human CYP2J2 (Tie2-CYP2C8-Tr and Tie2-CYP2J2-Tr) and genetic knockout of sEH (sEH-null). In these high-EETs transgenic mouse models, accelerated liver regeneration, kidney compensatory growth, lung compensatory growth, wound healing, corneal neovascularization and retinal vascularization were observed. Continuous infusion of 14,15-EET or administration of sEH also stimulated tissue regeneration, while transgenic over-expression of sEH which reduced EETs levels delayed normal organ and tissue growth. This study suggests that sEHs could be novel therapeutics for a number of human diseases which requires stimulation of angiogenesis [156].

4.3. Mechanisms of EETs on angiogenesis

VEGF is an important signal protein that stimulates angiogenesis [157] and EETs have been shown to stimulate angiogenesis via a VEGF-dependent mechanism. 14,15-EET stimulated mRNA and protein expression of VEGF in human dermal microvascular endothelial cells [154]. EETs also increased production of VEGF *in vivo*, the plasma level of VEGF was significantly elevated in tumor-bearing Tie2-CYP2C8-Tr and sEH-null mice compared with WT mice [32]. Interestingly, VEGF, which can be stimulated by EETs, can also increase the expression of CYP epoxygenases and EETs biosynthesis [158,159]. These results suggest that there is a positive feedback loop between EETs and VEGF. Depletion of VEGF using VEGF antibody abolished the pro-angiogenic effects of 14,15-EET *in vitro* and *in vivo* [154]. Neither the sEH *t*-AUCB nor TUPS stimulated endothelial cell migration in endothelial basal medium without VEGF, while they significantly increased endothelial cell migration with the presence of VEGF [32]. These results indicate that the pro-angiogenic effects of EET require VEGF. The biology of VEGF is largely mediated by its receptor VEGF receptor 2 (VEGFR2) [157]. The putative EET receptor antagonist 14,15-EEZE suppressed VEGF-mediated angiogenesis via a VEGFR2-independent mechanism [158]. Our previous study showed that 11,12-EET had no effect on VEGF-induced VEGFR2 phosphorylation, while DHA-derived 19,20-EDP abolished VEGFR2 activation in endothelial cells [33]. More studies are needed to characterize the biology of EETs on VEGFR2.

Besides VEGF signaling, another major pathway involved in the pro-angiogenic effects of EETs involves activation of the epidermal growth factor (EGF) receptor. EETs were first shown to activate EGF receptor in renal epithelial cells [160,161], such effects were also observed later in endothelial cells [148,149] and cancer cells [162]. 11,12-EET increased the supernatant level of EGF/heparin binding EGF (HB-EGF), via a mechanism involving activation of MMPs to liberate cell surface bound EGF/HB-EGF, resulting in activation of the EGF receptor [148]. Inhibition of the EGF receptor using a pharmacological inhibitor or EGF receptor-neutralizing antibody attenuated 11,12-EET-induced angiogenesis in a CAM assay *in vivo* [148]. These results support that 11,12-EET stimulates angiogenesis via an EGF receptor-dependent mechanism. Besides EGF receptor signaling, EETs activated multiple signaling pathways related with angiogenesis, including Src-STAT-3 [154], sphingosine kinase-1 [149] and PI3K/Akt [147,163].

5. EETs on cancer

5.1. Biological activities of EETs on tumorigenesis *in vitro*

Angiogenesis is required for tumor growth and metastasis of almost all types of cancers. The pro-angiogenic effects of EETs indicate their potential roles in tumor progression. The Wang group first showed that over-expression of CYP2J2 or treatment with synthetic EETs in cancer cells stimulated cancer cell proliferation, migration and invasion [164–166]. Follow-up studies showed that EETs stimulated proliferation, migration and invasion in certain cancer cell lines. Nithipatikom et al. showed that 11,12-EET stimulated cancer cell migration and invasion in human prostate cancer cells (PC-3, LnCaP and Du145), while pharmacological inhibitors of CYP epoxygenase or EET antagonist 14,15-EEZE inhibited such effects [162]. 11,12-EET induced cell stretching and myosin-actin microfilament formation via a mechanism involving activation of EGF receptor and Akt in prostate cancer cells [162]. Our recent study also repeated that 11,12-EET stimulated PC-3 invasion through Matrigel [33]. Mitra et al. showed that 14,15-EET, but not other EET regioisomers, increased cell proliferation of the breast cancer cell line MCF7 with a high threshold of 1–3 μ M [167]. 14(S),15(R)- and 14(R),15(S)-EET showed similar efficacy to induce MCF7 proliferation, indicating the effect of EETs on MCF7 cell proliferation was regio- but not stereospecific [167]. PPAR α and PPAR γ agonists induce sEH expression [6], which could contribute to the anti-cancer effects of these compounds [44,150].

5.2. Biological activities of EETs on tumorigenesis *in vivo*

Over-expression of CYP2J2 in cancer cells has been shown to stimulate tumor growth and metastasis *in vivo* [164–166]. The endothelium is a major site for biosynthesis of EETs. Recently Panigrahy et al. demonstrated that endothelium-derived EETs stimulated tumor growth and metastasis in multiple tumor models [32]. High levels of EETs in transgenic Tie2-CYP2C8-Tr, Tie2-CYP2J2-Tr and sEH-null mice slightly increased primary tumor growth but dramatically stimulated tumor metastasis. Continuous infusion with 14,15-EET via osmotic mini-pumps or treatment with a high-dose sEH *t*-AUCB (10 mg/kg/day) also increased tumor progression, while the corresponding diols DHETs have no such effects. Lowering EETs by transgenic over-expression of sEH or using the EET antagonist 14,15-EEZE reduced tumor progression. The biological effects of EETs are at least partially mediated by VEGF. The plasma levels of VEGF were significantly elevated in Tie2-CYP2C8-Tr and sEH-null mice compared with WT mice. Continuous infusion of 14,15-EET also increased VEGF expression in tumor endothelium and stromal fibroblasts of LLC tumors. Depletion of VEGF using Ad-sFlt dramatically suppressed B16F10 tumor growth in Tie2-CYP2J2-Tr and sEH-null but not in WT mice [32]. This study demonstrates that EETs play a central role in tumor growth and metastasis by stimulation of tumor angiogenesis.

This study raises questions on the biological implication from the sEH inhibition regarding cancer risks. Currently sEHs are considered for human clinical trials for multiple disorders such as pain and inflammation [5], thus this study raised potential safety concerns of sEHs in cancer patients. However it should be noted that in animal experiments the doses of sEHs to treat hypertension, pain, and inflammation are in low mg/kg range (0.1–1 mg/kg), while the active dose used in the tumor experiment was as high as 10 mg/kg/day [32]. Our study showed that at 1 mg/kg/day, *t*-AUCB had no effect on primary tumor growth and metastasis in mice [33]; while at 10 mg/kg/day, *t*-AUCB significantly increased tumor growth and metastasis [32]. These results indicate that the biological effects of sEHs (or EETs) on tumorigenesis have a high

threshold. This still leaves the question if the therapeutic index of sEH is sufficiently high to justify long term chronic use as pharmaceuticals.

5.3. Expression of CYP and sEH in tumor tissues

CYP2J2 expression was increased in carcinoma cell lines (LS-174, ScaBER, SiHa, U251, A549, Tca-8113, Ncl-H446 and HepG2) compared with non-cancer cell lines (HT-1080 and HEK293) [164]. The mRNA and proteins of CYP2C8, CYP2C9 and CYP2J2 were detected in human prostate cancer cells, producing predominately 11,12-EET *in vitro* [162]. CYP3A4 is up-regulated in 80% of breast cancer tissues and is correlated with poor prognosis [168]. Recently CYP3A4 was shown to catalyze epoxidation of ARA to generate EETs in the breast cancer MCF7 line [167]. CYP epoxygenases such as CYP2J2 have been reported to be highly expressed in human tumor tissues [164,166]. Guengerich and Turvy analyzed expression of CYP in a group of 100 liver samples and discovered that the expression of CYP2C was elevated in samples from metastatic cancer patients, though the expression levels varied by two orders of magnitude [169]. However, other studies demonstrate a more complicated expression pattern of CYP epoxygenase in tumor tissues. CYP epoxygenases were underexpressed or undetectable in adenocarcinoma and squamous cell carcinoma tissues [170], breast tumor tissues [168] and renal tumor tissues [171]. The expression of sEH also varied greatly in tumor tissues [143]. Compared with matched benign tissues, the sEH expression was elevated in colon, renal and testicular cancer, but decreased in liver and pancreatic cancer [172]. The complicated expression pattern of CYP epoxygenases and sEH makes it difficult to investigate the significance of CYP/sEH pathway in tumorigenesis. Besides CYP2C and CYP2J, a large number of other CYP isoforms could also contribute to biosynthesis of EETs, which are metabolized by multiple pathways beside sEH [3]. Therefore, analyzing the tissue or plasma level of EETs, rather than just the expression levels of selected CYP epoxygenases or sEH, may provide more consistent predictions regarding the roles of EETs and other EpFAs in tumorigenesis.

6. Biological activities of ω -3 EpFAs

6.1. Biological activities of ω -3 EpFAs on vascular tone and inflammation

EDPs have been shown to be the most potent EpFAs in the dilation of blood vessels [31]. EDP regioisomers (except 5,6-EDP which is chemically unstable) had EC_{50} values ranging from 0.5 to 24 μ M for dilation of porcine coronary arterioles precontracted with endothelin, while the corresponding diol 13,14-DiHDPa was >1000-fold less active with an EC_{50} value of 30 ± 22 nM, and the parent fatty acid DHA only dilated vessels at ≥ 1 μ M. EDPs also potently activated BKCa channels with a 1000-fold higher potency than EETs [31]. Animal experiments also support the anti-hypertensive effects of ω -3 EpFAs. A combination of the sEH TPPU and a ω -3-rich diet caused a more potent reduction of Ang-II-induced hypertension than treatment with sEH or ω -3 diet alone. Stabilized 19,20-EDP also suppressed hypertension, suggesting that the anti-hypertensive effect of the co-treatment was at least partially mediated by the formation of EDPs [173].

The EPA epoxide 17,18-EEQ has been shown to inhibit TNF- α -induced inflammation in human bronchi via NF- κ B- and PPAR- γ -related mechanisms [174]. 17,18-EEQ reduced TNF- α -induced hyperresponsiveness, COX-2 expression, I κ B α degradation and phosphorylation of p38 mitogen-activated protein kinase (p38-MAPK) in human bronchi [174]. In a previous study, 14,15-EET has also been shown to inhibit TNF- α -induced inflammation in a similar system [86]. In a carrageenan-induced

inflammatory pain model in rats, EDPs and EETs had similar efficacy on reduction of inflammatory pain, while the effects of EEQs were less potent [30]. The parent fatty acids and the corresponding fatty acid diols lacked such effects. The effects of EDPs on pain were region-specific, 13,14-EDP was the most potent regioisomer, followed with 16,17- and 19,20-EDP [30]. These studies demonstrate that similar to EETs, the ω -3 EpFAs also have potent effects to reduce inflammation and pain.

6.2. Biological activities of ω -3 EpFAs on angiogenesis

Opposite to the effects of EETs in stimulation of angiogenesis, our recent study demonstrated that DHA-derived EDPs are strongly anti-angiogenic [33]. In a Matrigel plug assay in mice, treatment with synthetic EDP regioisomers (7,8-, 10,11-, 13,14-, 16,17- and 19,20-EDP) inhibited VEGF-induced neovascularization in a dose-dependent manner with EC_{50} = 0.3 μ g per mouse. The 5,6-EDP regioisomer was chemically unstable, thus it was not tested. 19,20-EDP also inhibited fibroblast growth factor-2 (FGF-2)-induced angiogenesis using the Matrigel plug assay, supporting the potential broad-spectrum anti-angiogenic effects of EDPs [33]. In contrary, previous studies in Matrigel plug assays showed that EETs stimulated angiogenesis when treated alone [148,154], and further up-regulated VEGF-induced angiogenesis in the presence of VEGF [156]. Together, these results support the opposite effects of ω -6 and ω -3 EpFAs on angiogenesis. To test whether EDPs inhibits angiogenesis via targeting endothelial cells, we tested the effects of EDPs on angiogenesis in the primary endothelial cell line HUVECs. 19,20-EDP inhibited endothelial tube formation after 6-h treatment in HUVECs, supporting its anti-angiogenic effect *in vitro*. The process of angiogenesis composes of multiple cellular steps including cell proliferation, migration and production of MMPs. We found that 19,20-EDP potently inhibited VEGF-stimulated endothelial cell migration, with no effect on cell proliferation and weak inhibitory effect on MMP-2 activity in HUVECs, suggesting that EDPs inhibited angiogenesis primarily via suppressing endothelial cell migration [33]. The detailed mechanism by which EDPs suppressed endothelial cell migration requires more study.

19,20-EDP at 1 μ M almost abolished VEGF-induced VEGFR2 activation after a 10-min treatment in HUVECs, supporting that 19,20-EDP inhibits angiogenesis via a VEGFR2-dependent mechanism [33]. VEGFR2 is a critical mediator of angiogenesis, regulating all known biology of VEGF [157]. Most anti-angiogenic drugs on the market target VEGFR2/VEGF pathway to inhibit angiogenesis. However, VEGF is also critical for cardiovascular system [157]; therefore, all of currently available anti-angiogenic drugs cause cardiovascular side effects such as hypertension [175]. Human studies support that ω -3 PUFA supplementation reduced hypertension [176–178], and DHA-derived EDPs are among the most potent vessel dilators ever discovered [31]. In this aspect, pharmacological or nutritional manipulation of tissue level of EDPs may have unique advantages in anti-angiogenic therapy with reduced adverse effects on pain, blood pressure and other cardiovascular functions than current therapies.

Interestingly, our study also suggested potential roles of EDPs in lymphangiogenesis, an important process involved in tumor metastasis and other human diseases [179]. An angiogenesis assay (>80 genes correlated with angiogenesis) showed that the most down-regulated gene by 19,20-EDP is VEGF-C. RT-PCR analysis further confirmed that treatment of 19,20-EDP in HUVECs inhibited transcription of VEGF-C in a dose-dependent manner, while it has no effect on mRNA expression of VEGF-A [33]. VEGF-C is a critical mediator to regulate formation of new lymphatic vessels [179], suggesting a potential role of EDPs in inhibiting lymphangiogenesis. Currently no lymphangiogenesis inhibitors

have been approved in therapy and a VEGF-C antibody is in Phase I human trials to treat cancers. More studies are needed to characterize the effects of EDPs on lymphangiogenesis and associated diseases.

The opposite effect of EETs and EDPs may explain away some inconsistent results in previous studies. Substantial studies have demonstrated that pharmacological inhibition or genetic deletion of sEH increases angiogenesis. For example, Panigrahy et al. showed that genetic deletion of sEH increased tissue regenerations in liver, lung and kidney via stimulation of angiogenesis [156]. However, recent studies showed that inhibition or deletion of sEH suppresses angiogenesis in zebrafish [76] and retina [180]. These “inconsistent” results could be partially explained by our findings of the opposite effects of EETs and EDPs on angiogenesis (Fig. 3). The ω -6 ARA is the most abundant PUFA in membrane phospholipids in most tissues, pharmacological inhibition or genetic deletion of sEH would thus mainly accumulate ARA-derived EETs to stimulate angiogenesis. On the other hand, the ω -3 DHA is highly enriched in retina and brain tissues [17], inhibition or deletion of sEH is likely to mainly accumulate DHA-derived EDPs, leading to suppression of angiogenesis in these tissues. Currently there is no report of the tissue levels of EpFAs in retinal or brain tissues, while previous study has shown that EDPs are the most abundant EpFAs in zebrafish which is rich in ω -3 PUFAs, inhibition of sEH causes a 5-fold increase of the level of EDPs in zebrafish [76]. Since endogenous DHA is highly enriched in retinal tissues [17], pharmacological inhibition of sEH may be a potential strategy to suppress retinal angiogenesis and associated diseases such as macular degeneration. These studies emphasize that sEH inhibition is a “shot-gun” approach, modulating the levels of multiple EpFAs from several ω -3 and ω -6 PUFAs including ARA, EPA and DHA. The profiles of the EpFAs are tissue specific and can be modulated by nutrition. This modulation of lipid stores through nutrition offers more therapeutic opportunities by modulating sEH.

Besides our study, Cui et al. showed that 17,18-EEQ, but not other EEQ regioisomers derived from EPA, inhibited cell proliferation in the immortalized endothelial cell line bEND.3 at a dose of 10 μ M, while EETs at the same dose showed opposite effects to increase cell proliferation in bEND.3 cells [181]. This study suggests that EPA epoxides may also suppress angiogenesis.

6.3. Biological activities of ω -3 EpFAs on cancer

Cell culture experiments showed that EDPs, treated alone or combined with a sEHI, had no effect on cancer cell proliferation; while they potently inhibited cancer cell (prostate cancer PC-3

cells) invasion, opposite to the effect of 11,12-EET [33,162]. Animal experiments support that EDPs inhibit tumor growth and metastasis by suppressing tumor angiogenesis [33]. EDPs are highly unstable *in vivo* due to their rapid metabolism by sEH, continuous infusion of even the relatively stable 19,20-EDP (toward sEH metabolism) by osmotic mini pumps failed to elevate the level of 19,20-EDP in plasma and tumor tissues. We found that co-administration of 19,20-EDP with a low-dose sEHI t-AUCB caused a \sim 2-fold increase of 19,20-EDP level in circulation, while treatment with 19,20-EDP or sEHI alone had no effect on the systematic level of 19,20-EDP. The elevated level of 19,20-EDP by the co-treatment resulted in \sim 70% inhibition of Met-1 breast tumor growth (a highly aggressive triple-negative breast cancer model) in FVB female mice by blocking tumor angiogenesis, while treatment with 19,20-EDP or sEHI alone had no such effect. In addition, the sEH metabolite of 19,20-EDP, 19,20-DiHDPA, had no effect on Met-1 tumor progression. Together, these results support that 19,20-EDP had potent anti-cancer effect, which was stabilized by co-administration of a sEHI to block the sEH-mediated metabolism. The co-treatment reduced vessel density in Met-1 tumors, supporting anti-cancer effect was in part mediated by inhibition of tumor angiogenesis. In an angiogenesis-dependent LLC metastasis model [182], the co-treatment inhibited \sim 70% of LLC metastasis toward lung tissues, supporting that EDPs had potent anti-metastatic effects. These data suggest utility for stabilized mimics of EpFAs in medicine as well as a combination of sEHI with careful nutritional intervention.

As far as we know, EDPs are the first class of lipid mediators which have such potent anti-cancer and anti-metastatic effects. Tumor metastasis results in 90% of human cancer death [183], therefore, drugs which inhibit tumor metastasis and increase survival are useful in cancer therapy. It is likely that EDPs also target other pathways or targets related with metastasis, more studies are thus needed to characterize the effects and mechanisms of EDPs on metastasis. Due to the potent effects of EDPs on tumor growth and metastasis, EDPs are potential structural targets that can be used to develop stable analogs as anticancer agents. Previous research has shown medicinal chemistry approaches to design and synthesize stable mimics of ω -3 EpFAs, similar approaches could be used to generate EDP mimics to treat cancers [184].

The opposing effects between EpFAs from ω -3 and ω -6 PUFAs on angiogenesis and tumorigenesis suggest the biological effects of sEH inhibition could be diet-dependent. In mammals, the relative abundance of ω -6 ARA and ω -3 fatty acids (EPA and DHA) in tissues is largely determined by dietary intake of PUFAs [17]. DHA is the most abundant ω -3 fatty acid in most tissues, the levels of EPA in tissues are usually very low [17,23]. It is expected that under a ω -3-rich diet, sEH inhibition would mainly accumulate DHA-derived EDPs that are anti-angiogenic, while under a ω -6-rich diet, sEH inhibition would mainly accumulate pro-angiogenic EETs from ARA. Co-administration of sEHI and ω -3-rich diet could be beneficial for cancer treatment, while a combination of sEHI and ω -6-rich diet could be useful for some human diseases or injuries which require stimulation of angiogenesis. For example, a stimulation of tissue repair and angiogenesis with ω -6 PUFAs and sEHI could be of therapeutic benefit following severe burns, surgery or other dermal injuries. The stimulation of tissue repair and angiogenesis probably contributes to the success of sEHI in treating equine laminitis [185]. The sEHs are promising therapeutics to various human disorders, while the pro-angiogenic and pro-metastatic side effects have raised some safety concerns of sEHs in cancer patients. The discovery of the anti-angiogenic effect of EDPs suggests that a combination of a ω -3-rich diet and sEHI could enhance the efficacy with reduced or eliminated safety concerns for cancers with sEHs.

Sorafenib (Nexavar[®]) is a multikinase inhibitor of tumor cell proliferation and angiogenesis approved to treat advanced renal

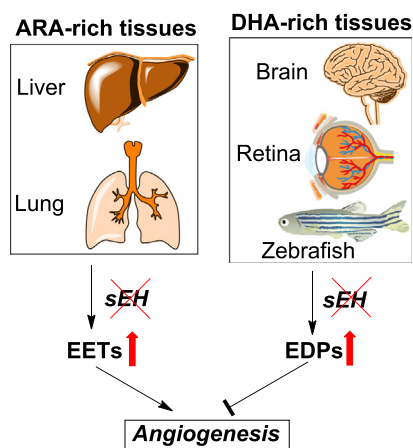


Fig. 3. Genetic deletion or pharmacological inhibition of sEH in ω -6-rich and ω -3-rich tissues leads to accumulation of EETs and EDPs respectively, leading to different effects on angiogenesis.

and liver cancers [186,187]. A similar compound, regorafenib (Stivarga®), has recently been approved to treat advanced gastrointestinal stromal tumors and metastatic colorectal cancer [188,189]. However, both drugs are reported to cause severe adverse effects such as fatigue, hypertension and hand-foot skin reaction, thus limiting their therapeutic applications [186–189]. In addition to sEH inhibition, we have found that both drugs are also potent inhibitors of sEH (sorafenib IC₅₀ = 12 nM, regorafenib IC₅₀ = 0.5 nM) [48,49], much more potent than their kinase inhibitory effects [190]. The hypothesis has been advanced that sorafenib is tolerated by cancer patients due, in part, to its inhibition of sEH and the resulting increase in analgesic, anti-hypertensive and anti-inflammatory EpFAs such as EETs. Therefore, based on our data of the anti-angiogenic, anti-cancer and anti-metastatic effects of EDPs, a combination of sorafenib or regorafenib with a diet high in ω -3 PUFAs (particularly DHA) and low in ω -6 PUFAs could boost EDPs and reduce EETs, leading to enhanced anti-cancer efficacy and reduced adverse effects [33]. EDPs have potent vasodilative and analgesic effects [30,31], thus the co-administration could also reduce the side effects, such as hypertension, that are associated with this type of drugs.

7. Conclusion and future work

Multiple approaches were carried out to investigate the biological activities of EpFAs in cell cultures and animal experiments. These approaches include (1) treatment with synthetic EpFAs, (2) pharmacological inhibition of CYP epoxygenases, (3) inhibition of sEH using sEHIs with diverse structural features, (4) transgenic expression of CYP epoxygenases, and (5) transgenic over-expression or knockout of sEH. Substantial evidence obtained from these studies support that EpFAs are important signaling molecules to regulate inflammation, pain, angiogenesis and cancer [5]. The sEHIs have been shown to have beneficial effects in various *in vivo* disease models [4,5,15]. These compounds have been in Phase II human clinical trials targeting hypertension and novel classes of sEHIs are being considered for human trials targeting other disorders [4,15,16]. In addition, the sEHIs have been shown to synergize with various drugs to enhance the efficacy and reduce the adverse effects [53,54,191,192]; therefore, these compounds hold promise as therapeutics for disorders in humans and companion animals.

The discovery of the receptor(s) for EpFAs could help to clarify some inconsistent results [101,102]. Many classes of LMs such as prostaglandins and leukotrienes exert their biological activities via G-protein coupled receptors (GPCRs) [2]. EETs have been shown to specifically bind specific membrane proteins, supporting the presence of putative EET receptor(s) [193,194]. Until now the identities of the EET receptors remain unknown. The biological activities of EETs appear to be cell type- and tissue-selective. For example, EETs stimulated VEGF expression in endothelial cells, but not in smooth muscle cells [154]. EETs have been consistently shown to stimulate cellular proliferation in primary endothelial cells, but the effects in cancer cells or other cells were greatly varied [164,167]. The discovery of the receptors could help to explain away the contradictions in biological and cell signaling of EETs. Other classes of EpFAs such as EEQs and EDPs likely also act through cellular receptor(s). Certainly an understanding of how these lipid mediators regulate so many diverse biological processes would advance the field.

Compared with EETs, the ω -3 EpFAs (EEQs and EDPs) are even less-studied. Until now several studies have suggested that the ω -3 EpFAs, in particular DHA-derived EDPs, inhibit inflammation, hypertension, pain, angiogenesis, tumor growth and metastasis [30,31,33,173]. More studies are needed to investigate their biological activities and mechanisms of actions. These promising

discoveries suggest that stabilizing or mimicking ω -3 EpFAs represent therapeutic targets. On the basis of our research, sEHIs (including anti-cancer drugs sorafenib and regorafenib) may synergize with ω -3 PUFAs to boost the levels of EDPs, leading to enhanced anti-angiogenic and anti-cancer effects. Investigation of the drug-nutrition interactions in pre-clinical models could lead to rapid human clinical trials in cancer patients. Endogenous DHA is highly enriched in retinal and brain tissues, pharmacological inhibition of sEH is thus promising to suppress pathological angiogenesis in these tissues.

Pre-clinical and epidemiological studies support the potential health benefits of ω -3 PUFAs on various chronic diseases, including but not limited to cancers [57–62,195], macular degeneration [64–67], hypertension [176–178], cardiovascular diseases [196,197] and inflammatory diseases [198]. There are large pools of fatty acids in man, and some of these pools are refractory to turnover. Thus, the effects of altering dietary intake of PUFAs may be delayed. Thus, we can anticipate that altering lipid composition and relative flux can be a long term flux effect relative to the relatively rapid effects from many pharmaceuticals. However, the underlying molecular mechanisms are largely unknown and the biological efficacy of ω -3 PUFAs in humans remains controversial [199–201], which greatly limits the effective utilization of ω -3 PUFAs for disease prevention. A major theory for health-promoting effects of ω -3 PUFAs is that they suppress the formation of ARA-derived LMs and generate ω -3-series of LMs with beneficial actions [24,25]. It is likely that people with different genetic or biochemical background will metabolize ω -3 PUFAs to bioactive lipid mediators in distinct manners, leading to different biological responses. Indeed, it was recently shown that there is a high degree of inter-individual variability in lipid metabolism upon supplementation of ω -3 PUFAs [202]. Understanding the specific metabolic pathways and the metabolites involved in the bioactivities of ω -3 PUFAs is thus critical to develop effective therapeutic paradigms and human trials to clarify their health benefits [24,28,68,203]. The opposite effects of EETs and EDPs on tumor growth and metastasis suggest a novel mechanistic linkage between ω -3 and ω -6 PUFAs and cancer. Our findings demonstrate that the previously unappreciated CYP/sEH pathway could play a critical role to mediate the opposite effects of ω -3 and ω -6 PUFAs on cancer [32,33]. The polymorphisms in the genes encoding CYP [204,205] and sEH [206–210] could affect the metabolism of DHA and alter the response to DHA supplementation. For example, based on our findings, people carrying Lys55Arg and Cys154Tyr mutations of sEH, which lead to higher sEH enzymatic activities [209], are expected to have lower tissue levels of EDPs and thus poorer anti-cancer responses upon dietary DHA supplementation. Such knowledge, together with utilization of nutrigenomic and metabolomic methods, could lead to targeted human trials to better understand the metabolic individuality and nutrition effects of ω -3 PUFAs on human health [211].

Acknowledgments

We acknowledge support from National Institute on Environmental Health Sciences R01 ES02710 and P42 ES04699, Research Investments in the Sciences and Engineering (RISE) Program of University of California Davis. B.D.H. is a George and Judy Marcus Senior Fellow of the American Asthma Society.

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