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Review Article

Epoxyeicosatrienoic acids and soluble epoxide hydrolase: potential therapeutic targets for inflammation and its induced carcinogenesis

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Abstract: Chronic inflammation is an important factor contributing to human carcinoma, and non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to have a preventive effect in the development of various types of carcinoma. However, NSAIDs also have adverse side effects including increased cardiovascular events, making them less than ideal for routine chemoprevention. Soluble epoxide hydrolase (sEH) is an enzyme that converts endogenous anti-inflammatory compounds, the epoxyeicosatrienoic acids (EETs), to the less anti-inflammatory dihydroxyeicosatrienoic acids (DHETs). Inhibition of sEH, by a highly selective and potent sEH inhibitor (sEHI), increases EETs leading to decreased inflammation. In our studies, administration of a sEHI in mouse colitis models led to decreased ulcer incidence and number of ulcers compared to controls, with no adverse side effects seen. In human tissue, sEH showed an increase in expression, as seen immunohistochemically, in ulcerative colitis (UC), UC-induced dysplasia, and UC-induced carcinoma. Thus, inhibition of sEH may be a novel biomarker and potential therapeutic target in inflammation and inflammation-induced carcinoma.

Keywords: Epoxyeicosatrienoic acids (EETs), soluble epoxide hydrolase (sEH), dihydroxyeicosatrienoic acids (DHETs), inflammation, non-steroidal anti-inflammatory drugs (NSAIDs), therapy, carcinogenesis, cancer

Introduction

The epoxyeicosatrienoic acids (EETs), products of arachidonic acid metabolism via the cytochrome P450 enzyme-mediated pathway, are key anti-inflammatory mediators. Soluble epoxide hydrolase (sEH) hydrolyzes EETs to dihydroxyeicosatrienoic acids (DHETs), which have dramatically reduced biological activity [1]. sEH inhibitors (sEHIs) are compounds that inhibit sEH activity and result in a variety of beneficial cardiovascular effects and anti-inflammatory effects with no toxicities seen in animal models to date. sEHIs have been shown to affect peroxisome proliferator-activated receptor activity, nuclear factor-kB (NF-kB), and key enzymes in arachidonic acid metabolism, including cyclooxygenase (COX) and lipoxygenase (LOX). Although many studies have illustrated a decreased risk of developing colorectal adenomas and carcinoma with the use of non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors, the risk of adverse cardiovascular events is a major concern. Our hypothesis is that sEH may potentially show the same benefits as NSAIDs and COX-2 inhibitors without the toxicities. In this review, we focus on linking the EET-sEH pathway to inflammation and carcinogenesis from experimental evidence to potential therapeutic application.

Soluble epoxide hydrolase (sEH)

Epoxide hydrolases are a family of enzymes that add water to the epoxide group of compounds to form the corresponding diols (**Figure 1**). The epoxide hydrolase family is divided into six main subtypes: soluble epoxide hydrolase (sEH), microsomal epoxide hydrolase (mEH), leukotriene A_4 hydrolase, hepoxilin A_3 hydrolase, and cholesterol 5,6-oxide hydrolase [2]. sEH (also known as cytosolic EH) is responsible for metabolizing

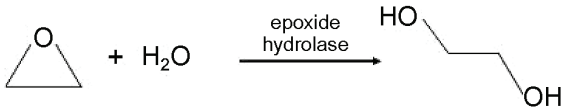

Figure 1. Epoxide hydrolase catalyzes diol formation by adding a water group to an epoxide.

EETs to DHETs. The human sEH is encoded by the EPHX-2 gene [3]. EPHX-2 is located on the short arm of chromosome 8 (8p21-p12), is nearly 45kb in length and consists of 19 exons [4]. The sEH in mammals is a homodimer composed of two 60-kDa monomers. Each monomer has an N-terminal domain with lipid phosphatase activity and uncertain physiologic role [5,6], and a carboxy-terminal domain composed of an α/β hydrolase fold with epoxide hydrolase activity [7]. The amino-terminal domain and carboxy-terminal domain are functionally independent of one another, and inhibition of one domain has no effect on the enzymatic activity of the other domain [5].

sEH is distributed in various tissues with high levels of sEH in intestine, liver, kidney, brain and vasculature and lower levels of sEH in lung, spleen and testes [8-11]. Intracellularly, sEH is located mainly in the cytosol, but is also located in peroxisomes [12-14]. In human hepatocytes and renal proximal tubules, sEH localizes to both the cytosol and peroxisomes, but is exclusively cytosolic in other sEH containing cells such as pancreatic islet cells, intestinal epithelium, anterior pituitary cells, adrenal gland, endometrium, lymphoid follicles, prostate ductal epithelium, and blood vessels [15]. Functional significance of different subcellular locations of sEH is not known.

Cytochrome P450 epoxygenases mediate active eicosanoids

Arachidonic acid (AA) is metabolized by the Cyclooxygenase (COX), Lipoxygenase (LOX), and cytochrome P450 (CYP 450) epoxygenase pathways to generate biologically active mediators including prostaglandins, leukotrienes and eicosanoids that are involved in various biologic events as well as several disease processes including inflammation, as shown in **Figure 2**. The cytochrome P450 epoxygenase pathway leads to the formation of 19- and 20- hydroxyeicosatetraenoic acid (19- and 20- HETE) and the epoxyeicosatrienoic acids (EETs) [16,17]. The CYP2C and CYP2J enzymes convert arachidonic acid to four EET regioisomers, -EET [5,6], -EET [8,9], -EET [11,12], and –EET [14,15]. The epoxide group can attach to either side of the double bond in arachidonic acid, resulting in two differ-

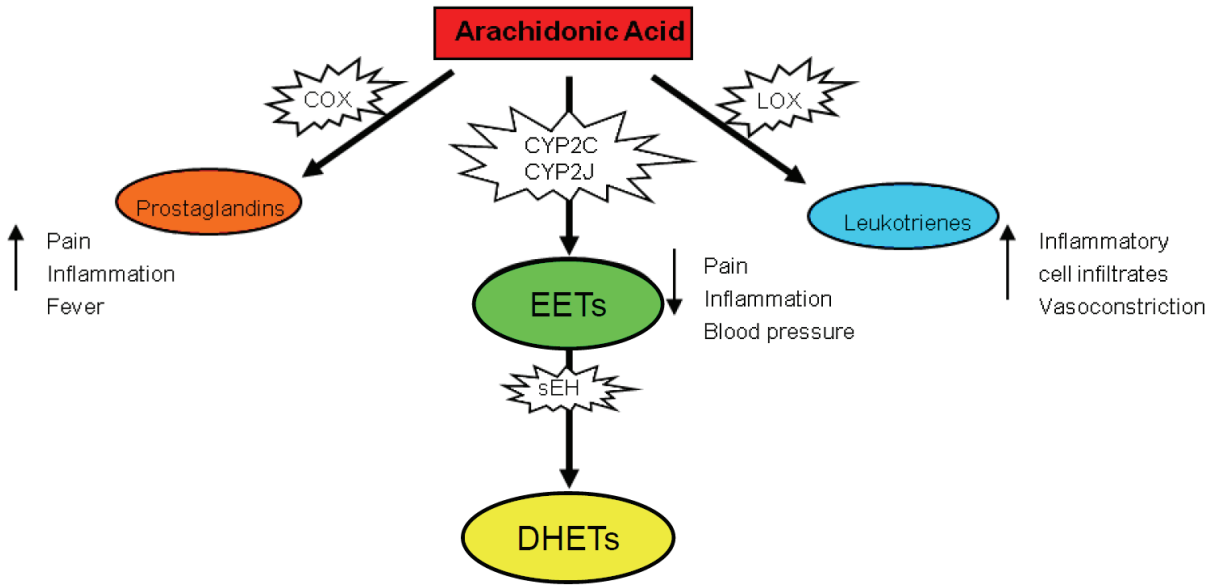

Figure 2. Arachidonic acid metabolism and the physiologic effects of arachidonic acid metabolites. COX = cyclooxygenase, CYP2C = cytochrome p450 2C, CYP2J = cytochrome p450 2J, DHETs = dihydroxyeicosatrienoic acids, EETs = epoxyeicosatrienoic acids, LOX = lipoxygenase, sEH = soluble epoxide hydrolase.

ent R/S and S/R enantiomers for each EET regioisomer[16]. The CYP 450 epoxygenases are capable of forming all four EET regioisomers, but -EET [11,12], and -EET [14,15] predominate [16]. EETs have a number of physiological functions including angiogenesis, apoptosis, fibrinolysis, mitogenesis, hormone secretion, vasodilation, bronchodilation and anti-inflammatory effects.

The EETs act in both an autocrine and paracrine manner and are located in various organs including the liver, kidney, and heart [18-20]. All EETs are incorporated into cellular phospholipids via a coenzyme A-dependent manner [18,21]. One possible mechanism for EET release is activation of a cell containing a CYP 450 epoxygenase. Upon activation, arachidonic acid is released from the phospholipids and the CYP 450 epoxygenase then synthesizes the EET [22]. Another possible mechanism is that the EETs are already preformed and stored in phospholipids and when a cell is "activated", the phospholipase releases the preformed EETs [23].

Once EETs are formed, it is thought that they can exert their autocrine and paracrine effects through two possible mechanisms. The first postulated mechanism is that EETs bind to a membrane receptor, which then activates an intracellular cascade affecting ion channels or DNA expression. Several studies support the idea of a G protein-coupled receptor [24-26] , while other studies propose tyrosine kinase and mitogen-activated protein kinase (MAPK) pathways [27-30]. The second postulated mechanism is through direct interaction of EETs with signal transduction pathways, transcription factors or ion channels.

EETs are metabolized through several different pathways including β-oxidation[31], chain elongation [9,32,33], hydration by soluble epoxide hydrolase (sEH), and to a lesser extent, EETs can be metabolized by CYP ω-oxidases [34], cyclooxygenase (COX) [35, 36], lipoxygenase (LOX) [37] and glutathione-*S*-transferase (GSH) [38]. The main catabolic pathway of EETs is the conversion to DHETs by sEH. EETs have increased anti-inflammatory properties compared to DHETs [1], but EETs are turned over so rapidly it is difficult to demonstrate this *in vivo*. However, the addition of a sEH inhibitor is an efficient way to increase EETs function physiologically.

EETs-sEH pathway and its anti-inflammation activity

The role of the EET-sEH pathway in inflammation has been assessed in rodent models [1, 39 -44]. The initial findings of the anti-inflammatory properties of EETs described by Node et al demonstrated that physiologic concentrations of EETs or overexpression of CYP2J2 (the CYP450 enzyme that transforms AA to EETs) inhibits inflammation through decreasing cytokine-induced endothelial cell adhesion molecule expression, and preventing leukocyte adhesion to vascular walls and tissues by a mechanism involving inhibition of (NF-kB) [45]. Furthermore, when EETs are stabilized by sEH inhibitors, they have dramatically increased anti-inflammatory properties [1, 39-44, 46]. Using rodent models, investigators have demonstrated that sEH inhibitors are effective in the treatment of inflammatory diseases [42]. Mice with lipopolysaccharide(LPS)-induced systemic inflammation, treated with the sEH inhibitor 12-(3-adamantane-1-yl-ureido)-dodecanoic acid n-butyl ester (AUDA-nBE), showed reduced production of nitric oxide, cytokines, and pro-inflammatory lipid mediators and significantly improved survival [42, 47]. In another study, daily subcutaneous injections of the sEH inhibitor AUDA-nBE were administered to spontaneously hypertensive rats that were either exposed to filtered air or tobacco smoke for a period of 3 days (6 h/day). The tobacco smoke-exposed rats treated with AUDA-nBE showed a significant decrease in bronchoalveolar inflammatory cells, including significant reductions in neutrophils, alveolar macrophages, and lymphocytes [43]; and a combination of sEH inhibitor and exogenously administered EETs is even more significant in reducing tobacco smoke-induced inflammation [43].

EETs also increase peroxisome proliferator-activated receptor-gamma (PPAR-γ) transcription activity, which is a key molecular event involved in inhibiting NF-κB. Evidence from cell culture models shows that blocking PPARγ activity reduces the EET/sEH inhibitor-mediated anti-inflammatory effect, indicating PPARγ is an effector of EETs [48]. PPARγ exerts an anti-inflammatory effect by interfering with the activity of inflammatory transcription factors, such as Activator Protein-1 (AP-1), Signal Transducers

and Activators of Transcription (STAT), and nuclear factor κB (NF-κB) [49, 50]. Therefore, activation of PPARγ could be a key pathway to halt the inflammatory response[51]. Human peroxisome proliferator-activated receptor contains three isoforms, as a result of alternative splicing and differential promoter usage within the same gene. PPARγ1, the predominant isoform, is expressed in a broad range of tissues, including small bowel and colon [52, 53]. PPARγ ligands have also been shown to markedly reduce colonic inflammation in both dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid colitis models in mice, probably through inhibition of NF-κB activity and decrease of proinflammatory cytokine production [54-57]. Conditional knockout of PPARγ enhanced tumorigenesis in ApcMin/1 mice [55].

Our preliminary studies show that administration of the selective sEH inhibitors, AUDA-nBE and *trans*-4-[4-(3-adamantan-1-ylureido) cyclohexyloxy]benzoic acid (t-AUCB) significantly inhibits colitis and ulcer formation in both DSS-induced ulcerative colitis (UC) mice and IL-10 knockout mice with spontaneous colitis. Immunohistochemical analysis revealed that AUDA-nBE treatment markedly suppressed myeloperoxidase-labeled inflammatory cell infiltration. Further mechanistic studies showed that AUDA-nBE induced peroxisome proliferator-activated receptor-gamma (PPAR-gamma) activity and its downstream gene expression such as Aquaporin-7, and inhibited NF-kB signaling. In addition, long-term administration of *t*-AUCB markedly inhibited the development of spontaneous colitis-induced dysplasia and carcinoma in IL-10 knockout mice.

The recent data indicate that EETs or sEH inhibitors not only regulate cytokines and chemokines but also regulate other key enzymes in the arachidonic acid cascade [1]. An increase in EETs results in a dramatic down regulation of induced, but not constitutive, COX-2 and 5-LOX and their associated metabolites leading to a reduction in symptoms associated with severe inflammation [1]. Simplistically the sEH inhibitors mediate an increase in EETs, resulting in a shift from eicosanoids initiating and propagating inflammation to a pattern of resolution of inflammation [1]. Thus, cross talk among the pathways in the arachidonate and other lipid cascades is likely to be a general phenomenon. *Patterns* of eicosanoid profiles are far more informative than *individua*l biomarkers or arachidonic acid metabolites, indicating that the eicosanoid profile could be a valuable biomarker for testing the effect of a sEH inhibitor [1, 39]. A simplistic plasma biomarker of sEH inhibitor effectiveness, which has held in multiple species, is the dramatic increase in the ratio of fatty acid epoxides to their corresponding diols. A more complex marker, which also yields mechanistic understanding, is a reduction in inflammatory COX and LOX metabolites in inflammatory models following the administration of sEH inhibitor.

In addition, the synergistic effect of sEH inhibitors and common NSAIDs is further studied in a lipopolysaccharide (LPS)-induced acute systemic inflammation model and shows that the sEH inhibitors not only are more potent than common NSAIDs in reducing predominantly inflammatory eicosanoids like prostaglandin PGE2 but they synergize with NSAIDs in reducing inflammation [1]. These effects can be anticipated from the mechanism of action where 1) the NSAIDs inhibit COX 1 and/or 2 and shift the arachidonic acid substrate toward other pathways including the LOX and P450 pathway. 2) The EETs stabilized by sEH inhibitors are directly anti-inflammatory, and transcriptionally reduce the COX 2 and 5-LOX induced by inflammation [58]. The sEH inhibitors also alter the eicosanoid profile associated with rapid blood clotting following administration of COX-2 inhibitors toward a more normal clotting response. Thus the sEH inhibitors should reduce the dose needed of some COX and LOX inhibitors for targeted therapy.

Development of highly potent and selective sEH inhibitors (sEHI)

The development of stable, highly potent, and highly selective inhibitors for sEH is a key and important step not only for the elucidation of the biology associated with EETs and sEH but also for developing a practical and efficient anti-inflammatory therapeutic agent. The sEHIs first identified were epoxides of either the substituted chalcone oxide or *trans*-3-phenylglycidol classes. Substituted chalcone oxides inhibit sEH through electronic stabilization of the covalent enzyme-inhibitor intermediate. *trans*-3-phenylglycidols bind sEH in an enantioselective manner and are slow sEHIs. Although these epoxide sEHIs are potent and selective, they are

unstable with transient sEH inhibitory effects [59, 60].

The newer class of sEHIs, including urea- and carbamate-based compounds, are much more potent and selective for sEH[60]. An example of this class of sEHIs is *N,N'*-dicyclohexylurea (DCU). DCU is weakly soluble in water, thus several urea-based sEHIs with increased water solubility and increased potency have been developed [61]. X-Ray crystal structure determinations show that the urea inhibitors establish hydrogen bonding and salt bridges between the urea portion of the inhibitor and residues on the sEH active site [7,62,63].

The most well studied sEH inhibitors are 12-(3-adamantyl-ureido)-dodecanoic acid (AUDA) based compounds. AUDA and its esters and salts have been widely used to evaluate the biological role of sEH. However, its rapid metabolism, moderate oral availability, and poor physical properties made this difficult. A typical modified agent in the AUDA family is 12-(3-adamantyl-ureido)-dodecanoic acid *n*-butyl ester (AUDA-*n*BE). AUDA was followed by the development of several sEHI compounds that bind within the catalytic site of sEH via hydrogen bonding. These compounds show dramatically improved water solubility, good oral bioavailability in multiple species, and have a lower melting point facilitating formulation [61, 62, 64-68]. Many structures with improved physical properties have resulted from placing hydroxyl, carbonyl, sulfonyl, ether, ester, carbamate, and other functionalities at the so-called secondary pharmacophore. Several N,N'-disubstituted urea compounds having a conformationally restricted *cis*- or *trans*-1,4-cyclohexane R group to the urea have been developed as sEH inhibitors with good oral bioavailability [61,62,64-68]. *Trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyl oxy]benzoic acid (*t*-AUCB) is an example of this category of potent sEH inhibitors with a greater *in vivo* metabolic stability, low nanomolar activity (IC_{50} = 1.3 +/- 0.05nM) and increased oral bioavailability [64].

Potential role of sEH in inflammation-driven carcinogenesis

Non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors have been shown to significantly reduce adenoma formation in patients with a high risk for the development of colorectal cancer. In 1988, Kune et al reported that aspirin use results in a decreased risk of developing colorectal carcinoma[69] and subsequent studies showed a decreased risk for the development of adenomatous polyps with aspirin use [70-73]. The development of COX-2 specific inhibitors seemed a promising option for the prevention of colorectal carcinoma without the toxicities seen with aspirin use, namely gastrointestinal bleeding. In the first randomized controlled clinical trial using a COX-2 inhibitor as chemoprevention for colorectal cancer, celecoxib was given to 83 patients with familial adenomatous polyposis (FAP) over a period of 6 months with a significant reduction in adenoma size and number and no recorded toxicities [74]. On the heels of this initial study, three major clinical trials were started investigating the effects of selective COX-2 inhibitors in patients at high-risk for colorectal carcinoma. The Adenoma Prevention with Celecoxib trial (APC) and the Prevention of Sporadic Adenomatous Polyps (PreSAP) trial both used celecoxib, while the Adenomatous Polyp Prevention on Vioxx (APPROVe) trial used rofecoxib [75-77]. All three trials found significant reductions in the development of adenomas, however all three trials were halted before completion due to an increased cardiovascular risk noted in patients participating in both the APPROVe and APC trials. The adverse endpoints in these two trials included myocardial infarction, stroke, congestive heart failure and death from cardiovascular disease [77-79]. In addition, the APPROVe trial showed that rofecoxib use was associated with an increased incidence of ulcers, bleeding, perforations and obstruction of the upper gastrointestinal tract [77].

Toxicity is a major concern in the development of anti-inflammatory agents, as is illustrated by the side effects of COX-2 inhibitors, such as rofecoxib. The thrombotic events associated with the use of rofecoxib and other NSAIDs have been attributed to a high dose but also to the destabilization of platelets due to the increased ratio of thromboxane A2 (TXA2) to prostacyclin (PGI2). However, sEH inhibitors have demonstrated cardiovascular protective effects in animal models of hypertension, and a phase I clinical trial is completed. For a phase I trial, one needs an investigational new drug approval (IND), which requires extensive toxicological studies that, in the case of SEHIs, to date are clean. In the academic laboratory, AUDA has

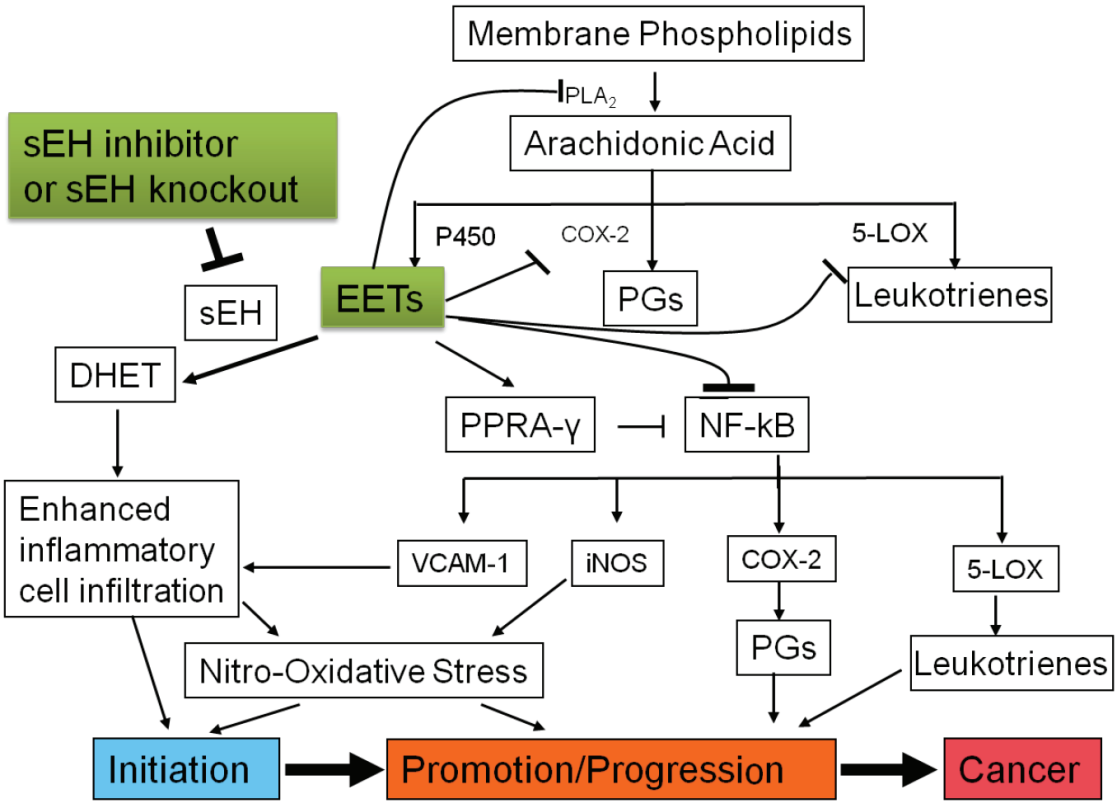

Figure 3. Multiple pathways of inflammation inhibition through targeting sEH. COX-2 = cyclooxygenase-2, DHETs = dihydroxyeicosatrienoic acids, EETs = epoxyeicosatrienoic acids, iNOS = inducible nitric oxide synthase, 5-LOX = 5-lipoxygenase, NF-κβ = nuclear factor- κβ, P450 = cytochrome P450, PGs = prostaglandins, PLA = phospholipase A, PPAR-γ = peroxisome proliferator-activated receptor-γ, VCAM-1 = vascular cell adhesion molecule-1.

shown high selectivity for sEH enzyme inhibition in a commercial screen of pharmacological and toxicological targets including COX and LOX enzymes. AUDA and *t*-AUCB have shown no obvious toxicity when tested at high oral doses in rodents.

There is limited information on the role of sEH in inflammation-induced carcinogenesis. Our preliminary study showed that up-regulation of sEH was identified in human ulcerative colitis (UC) and UC-induced dysplasia and carcinoma. Tissue microarrays of 180 patients with UC (n=72), colitis-induced dysplasia (n=54), and colitis-induced carcinoma (n=54) were established. Adjacent normal colonic tissue was also identified in these patients (n=79). Avidin-biotin-peroxidase approach was used with anti-sEH antibody and proper +/- controls. sEH staining positivity was evaluated for its intensity on a scale of 0-3+ and compared with positive controls(liver & kidney). sEH showed distinct expression in the microarray samples. Normal colon displayed positivity in 39.1% (n=79), mainly in the focally reactive epithelia. In UC, 74.6% (n=72) displayed positivity, extensively expressed in the hyperplastic epithelia. 88.2% (n=54) of colitis-induced dysplasia and 93.9% (n=54) of colitis-induced carcinoma displayed positivity. Markedly increased sEH staining intensity was observed in UC, UC-induced dysplasia and carcinoma and the average staining intensity of sEH was 0.43 in normal glands, 0.89 in UC, 1.29 in dysplasia, and 1.31 in carcinoma.

Conclusion

We believe that inhibition of sEH significantly reduces the conversion of EETs to their corresponding DHETs, and thus is a novel target for pharmacologic therapies of inflammation and

carcinogenesis. The mechanism of inflammation inhibition is by suppressing inflammatory cell recruitment, modulating the arachidonic acid metabolite profile and further targeting the PPAR-gamma and NF-kB pathways, which leads to an inhibition of COX-2, 5-LOX, iNOS, as well as VCAM-1 (**Figure 3**). Targeting of sEH with specific pharmacological inhibitors, as we have shown in animal models, provides direct experimental evidence for the role of sEH in inflammation and cancer development without the toxicities seen with the use of NSAIDs and selective COX-2 inhibitors. We believe sEH could be a novel biomarker for inflammation and inflammation-induced carcinogenesis and that sEH inhibitors could be used for cancer prevention with the same preventive effects of NSAIDs but without the adverse effects.

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