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Soluble Epoxide Hydrolase as a Therapeutic Target for Cardiovascular Diseases

John D. Imig¹ and Bruce D. Hammock²

¹Department of Pharmacology & Toxicology and Cardiovascular Research Center, Medical College of Wisconsin

²Department of Entomology and Cancer Center, University of California at Davis

Preface

Cardiovascular effects of epoxyeicosatrienoic acids (EETs) include vasodilation, vascular smooth muscle cell anti-migratory actions, and anti-inflammatory actions. These endogenous lipid mediators are broken down to diols by soluble epoxide hydrolase (sEH), and so inhibiting this enzyme would be expected enhance the beneficial cardiovascular properties of EETs. The rapid development of 1,3-disubstituted urea based sEH inhibitors (sEHIs) has resulted in a number of studies demonstrating cardiovascular protection, and it has been shown that sEHIs are anti-hypertensive, anti-inflammatory, and protect the brain, heart and kidney from damage. Although challenges for the future exist — including improving the drug like properties of sEHIs and finding better ways to target sEHIs to specific tissues — the recent initiation of first in human clinical trials has highlighted the promise of sEHIs as a therapeutic target.

Many of the enzymes, receptors and the eicosanoid metabolites of the arachidonate cascade are major therapeutic targets, particularly for inflammatory disease. The first pathway to be targeted was cyclooxygenase (COX), which leads to the generation of prostaglandins (PG). Indeed, aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, are effective drugs that treat pain and inflammation.^{1,2} These drugs also may be useful for treating or preventing cardiovascular diseases —inhibition of blood clotting by aspirin has been touted as a preventative for ischemic events such as heart attacks and stroke¹ — and prostacyclin analogs are used for the treatment of pulmonary hypertension.^{3,4} On the other hand, enthusiasm for the COX pathway was greatly decreased because of the increased incidence of acute renal failure, myocardial infarction and thrombotic stroke in patients treated with COX-2 inhibitors.^{1,2,5,6} The second eicosanoid and inflammatory pathway targeted for therapeutics was the lipoxygenase (LOX) generation of leukotrienes (LT). 5-LOX and LT receptor antagonists have been developed for the treatment of asthma and seasonal allergies.^{7,8} These two eicosanoid pathways continue as important therapeutic targets as novel receptors and metabolites have been identified and their roles in a myriad of diseases are being better defined [figure 1].

A third eicosanoid pathway, the cytochrome P450 (P450) was first described in 1980 and is comprised of two enzymatic pathways^{9,10,11} - the hydroxylases and the epoxigenases. The hydroxylase P450 enzymes convert arachidonic acid into hydroxyeicosatetraenoic acids (HETEs). 20-HETE is the major metabolite of this pathway and has been determined to be pro-inflammatory and important to vascular function.^{12,13} This pathway and metabolite are currently being targeted for the treatment of cardiovascular diseases such as hypertension and stroke.¹³⁻¹⁶ The second pathway is the generation of epoxyeicosatrienoic acids (EETs) by P450 enzymes, which catalyze the epoxidation of arachidonic acid olefin bonds resulting in the production of four regioisomeric EETs: 5,6-EET; 8,9-EET; 11,12-EET; 14,15-EET. EETs or epoxyeicosanoids have been demonstrated to be endothelium-derived

hyperpolarizing factors (EDHFs), protect from ischemic injury and possess anti-inflammatory actions in canine and rodent disease models.¹⁷⁻²¹ Conversion of EET epoxides to their corresponding diols (DHETs) by soluble epoxide hydrolase (sEH) enzyme are responsible for decreasing EET levels and thus diminishing their beneficial cardiovascular properties,^{20,21} and so inhibition of this enzyme would be a target for cardiovascular disease. Recently, sEH inhibitors (sEHIs) have been developed to enhance the cardiovascular actions offered by EETs. This article will highlight the development of sEHIs as cardiovascular therapeutics and discuss the potential for this treatment and challenges that lie ahead.

Biological Aspects of Epoxyeicosanoids

Since the first descriptions of the biological actions of EETs, which included increases in epithelial transport in the kidney and dilation of small mesenteric resistance arteries, there has been growing interest in these eicosanoid metabolites.^{22,23} Interest in EETs was greatly increased in 1996 with the identification of EETs as an EDHF.¹⁷ Over the past decade it has become increasingly apparent that EETs have a myriad of cardiovascular actions, the overwhelming majority of which appear to be cardiovascular protective.

The cellular signaling mechanisms responsible for the various EET biological actions have been and continue to be intensively investigated. There is ample evidence that supports the possibility that EETs bind to receptors that are coupled by a G-protein to intracellular signaling cascades;^{24,25} however, an EET receptor has yet to be identified. EETs could also function inside the cell by coupling to and activating ion channels, signaling proteins or transcription factors. Experimental evidence supports an intracellular mechanism of action in that EETs are incorporated into cell membrane phospholipids, bind to fatty acid binding proteins, and peroxisome proliferators-activated receptor (PPAR) γ .^{21,24,26,27} Please see the following reviews for more comprehensive descriptions of EET biological activities and cellular signaling mechanisms.^{28,29}

As with other eicosanoid pathways, there is heterogeneity with respect to cellular signaling mechanisms and biological activities in various cell types and tissues. Other experimental issues have made investigation of EETs and the P450 enzymatic pathways difficult. One consistent concern is the quality and purity of regioisomeric EETs and their proper handling. Likewise, investigations in cell culture systems are limited by the fact that epoxigenase and epoxide hydrolase enzymes decrease rapidly following cell isolation. Experimental approaches to circumvent these issues include the generation of genetically manipulated mice, transfection of cell culture lines with P450 enzymes, and development of EET analogs and antagonists with better chemical properties and greater stability.^{18,30} Moreover, EET receptor identification could provide more clarity to the apparent biological heterogeneity much like the discovery of multiple PGE₂ (EP) receptors helped explain away the apparent contradictions in biological and cell-signaling mechanisms for that eicosanoid.^{31,32} Even with these experimental concerns there has been tremendous progress in determining EET biological actions and EETs remain an attractive therapeutic target for cardiovascular diseases.

Vascular Actions of EETs

EETs as vasodilators and EDHFs are the most extensively examined cardiovascular actions. Vasodilation in response to EETs has been observed in a number of organs including the heart, brain, kidney, skeletal muscle and intestine.^{13,17,23,33,34} In contrast, EETs cause vasoconstriction in the lung - a finding that was not unexpected since PGs also have opposite effects in this vasculature when compared to other organs.^{35,36} All regioisomeric EETs have been demonstrated to be vasodilators with 11,12-EET and 14,15-EET

consistently exhibiting the most vasodilator activity.^{21,34} These two regioisomeric EETs generated by endothelial cells dilate blood vessels by activating large-conductance calcium-activated K⁺ (K_{Ca}) channels on vascular smooth muscle cells,^{33,37,38,39} resulting in K⁺ efflux from the smooth muscle cell and subsequent membrane hyperpolarization.^{17,38} There is evidence for cAMP activation of protein kinase A (PKA) and ADP ribosylation of Gsα cell signaling mechanisms as being responsible for mediating EET activation of vascular smooth muscle cell K_{Ca} channels.³⁹⁻⁴² The ability of EETs to activate K_{Ca} channels and dilate blood vessels can be regulated by sEH-mediated conversion to DHETs that exhibit diminished or absent vasorelaxation.^{34,38,43} In this regard, sEH inhibition impedes the conversion of EETs to DHETs and improves dilator activity in human blood vessels.⁴³ EETs or sEHIs have been demonstrated to oppose the vasoconstrictor activities of the pro-hypertensive hormones endothelin-1 and angiotensin II.²⁰ Thus decreased endothelial EET conversion to DHETs could be one mechanism responsible for the anti-hypertensive actions observed with the administration of sEHIs as well as for other cardioprotective properties.

Vascular homeostasis is controlled by endothelial cell and vascular smooth muscle cell proliferation and migration, and EETs and sEH appear to be important regulators of these cellular processes.^{18,30,44-49} EETs promote endothelial cell proliferation and migration and are angiogenic, and it has been demonstrated that the epoxides and not the corresponding diols resulted in proliferative effects.⁴⁵ In murine and human cell lines, EETs or overexpression of CYP2C epoxygenases leads to proliferative responses,^{30,46} that have been attributed to activation of two cell-signaling pathways; the p38 mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase-Akt (PI3K/Akt) pathway.³⁰ 11,12-EET activates MAPK, which upregulates cyclin D, and Akt, which phosphorylates forkhead factors (FOXO) and decreases cyclin-dependent kinase inhibitor p27^{kip1} in endothelial cells.^{46,47} More recently, 11,12-EET-mediated proliferation, migration and tube formation in human umbilical vein cells (HUVECs) was demonstrated to be dependent on activation of sphingosine kinase 1 (SK1) that phosphorylates sphingosine to generate sphingosine-1-phosphate (S1P).⁴⁹ On the other hand, EETs exhibit antimigratory actions in vascular smooth muscle cells. 11,12-EET and 14,15-EET moderately attenuated the migration of aortic smooth muscle cells in response to platelet-derived growth factor,⁵⁰ and CYP2J epoxygenase overexpression or inhibition of sEH also reduced smooth muscle cell proliferation and migration.⁵⁰ Activation of the cAMP-dependent PKA pathway and decreased cyclin D levels have been implicated in the antimigratory actions of EETs and sEHIs in vascular smooth muscle cells.³⁰ Although these findings suggest a vascular smooth muscle cell effect of EETs and sEHIs, other reports have failed to demonstrate that EETs or sEHIs result in vascular smooth muscle proliferation.^{51,52} More importantly, *in vivo* angiogenesis is stimulated by EETs in a subcutaneous sponge model and inhibition of sEH enhanced these pro-angiogenic and neo-vascularization responses.⁴⁸ Effects of EETs and sEHIs on proliferation and migration of endothelial and vascular smooth muscle cells reveal the possible importance of targeting this pathway in angiogenesis, atherosclerosis and other cardiovascular diseases.

Anti-inflammatory Actions of EETs

Inflammation and inflammatory diseases contribute significantly to vascular and end organ damage and cardiovascular disease progression.^{53,54} Likewise, interactions between inflammation and the epoxygenase pathway, that can affect cardiovascular function in disease states, have been clearly established.⁵⁵⁻⁵⁸ Cytokines can decrease CYP2C expression and oppose epoxygenase-mediated vasodilation.^{18,59} Conversely, TNFα and CCR2 inhibition result in an increase in kidney CYP2C expression and decrease renal injury in hypertension.^{55,56} Experimental evidence supports the postulate that EETs interfere with activation of the transcription factor, nuclear factor κB (NF-κB) to exert their vascular anti-

inflammatory effects.^{57,58} 11,12-EET but not other regioisomeric EETs prevent TNF α induced activation of NF- κ B and increased vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells.⁵⁷ Likewise, CYP2J epoxygenase overexpression in endothelial cells also decreases NF- κ B activation.⁵⁷ Although additional studies will be required to determine the exact cellular signaling mechanisms responsible for the anti-inflammatory actions of EETs, there is considerable evidence that EETs decrease inflammation. Further anti-inflammatory actions attributed to EETs include decreased aggregation of human polymorphonuclear leukocytes and leukocyte adhesion to endothelial cells,⁵⁸⁻⁶¹ and attenuation of IL-1 β induced fever. In the latter case 11,12-EET (administered to the brain) had a greater antipyretic action than other EETs.^{62,63} Studies using sEHs also support the notion that EETs have anti-inflammatory actions.⁶⁴⁻⁶⁷ Inhibition of sEH decreased cigarette smoke-induced lung inflammation and significantly reduces neutrophils, alveolar macrophages and lymphocytes in the bronchial fluid.⁶⁸ These findings have provided sufficient evidence that sEHs could be protective against the deleterious effects of inflammation associated with cardiovascular diseases as well as a treatment for other inflammatory diseases.

Epoxygenase Pathway Polymorphisms in Human Disease

The notion that both the EETs and the sEH enzyme are therapeutic targets for human disease is supported by genetic studies. There are a number of polymorphisms in *EPHX2* — the gene responsible for sEH production — that result in amino acid substitutions that influence sEH enzymatic activity.⁶⁹⁻⁷³ Two studies have linked genetic variation in *EPHX2* to increased risk for coronary artery disease, and smoking further increased the risk associated with this genetic variation.^{69,72,74} Other cardiovascular diseases associated with genetic variation in *EPHX2* include ischemic stroke and hypercholesterolemia.^{71,75} Genetic variations in the epoxygenase CYP2J2, CYP2C8 and CYP2C9 can effect transcription or enzymatic activity.^{73,76,77} CYP2C8 and CYP2C9 variants have been associated with myocardial infarction and cardiovascular disease.^{76,77} Taken together, these findings in the patient population provide evidence that sEHs could have potential therapeutic value in a large variety of cardiovascular diseases and that they may be of particular benefit for patients of certain genotypes.

Design of Soluble Epoxide Hydrolase Inhibitors

The rapid development of sEHs for *in vivo* use and clinical testing in the past decade is quite remarkable. A landmark study published in 2000⁷⁸ demonstrated that injection of a sEHI to a spontaneously hypertensive rats (SHR) lowered blood pressure. This study was followed up by the first demonstration that chronic inhibition of sEH lowered blood pressure in angiotensin-induced hypertension.⁷⁹ Another breakthrough came in 2005 when it was shown that an orally administered sEHI was anti-hypertensive and slowed the progression of renal damage.⁸⁰ Following this, a number of studies have provided exciting findings on the broad potential for sEHs as a cardiovascular therapeutic, and as further evidence of the rapid development of this therapeutic, a first in class sEHI began clinical phase IIa testing in humans this year (<http://www.aretetherapeutics.com/news/2009/021009.html>). In this section we will describe the evolution of selective sEHs from enzyme inhibition *in vitro* to oral administration into rodents and subsequently humans.

There are two well studied α/β -hydrolase fold epoxide hydrolase enzymes that differ by subcellular localization and substrate selectivity.^{56,81,82} The microsomal epoxide hydrolase (mEH) is involved in the metabolism of environmental contaminants, and it has been extensively studied in this role,^{81,82} whereas the sEH was first discovered while studying the metabolism of terpenoid epoxide that mimicked insect juvenile hormone.^{81,82} At the same

time EETs were being established as endogenous lipid mediators with biological activity. Subsequently it was discovered that arachidonic acid and linoleic acid epoxides are metabolized by sEH and sEH converts these epoxides to diols with high V_m and low K_m .^{81,82,83} Since this finding some three decades ago, the message and gene have been cloned and the sEH catalytic mechanism determined. The mammalian sEH is a homodimer with monomers arranged in an anti-parallel form.^{81,82,83} Each monomer is composed of two domains; the C-terminal domain contains epoxide hydrolase activity while the N-terminal domain hydrolyzes phosphates on lipophilic backbones.^{84,85,86} This highly conserved enzyme is widely distributed in tissues including the liver and kidney where sEH specific activity is highest.^{85,87,88,89} The functional significance of the N-terminal domain of the mammalian sEH remains highly speculative. The N-terminal domain possesses phosphatase activities that dephosphorylate polyisoprenyl phosphates known to regulate cholesterol levels.^{84,86,90} It has been speculated that the N-terminal domain could stabilize the epoxide hydrolase activity because expression of the human sEH C-terminal domain alone has reduced activity.⁹⁰ The N-terminal domain might promote dimerization of the sEH enzyme.^{89,91} To date, there are no known selective inhibitors of this N-terminal domain, so determination of the functions of the N-terminal domain awaits further experimental studies. Current sEHIs inhibit the epoxide hydrolase activity of the C-terminal domain without affecting the phosphatase activity of the N-terminal domain.

The first generation sEHIs were potent inhibitors acting as alternative substrates and included chalcone oxides and glycidols.^{21,85,92} Unfortunately, these alternative substrates were quickly inactivated by glutathione and glutathione transferases making them difficult to use in tissues and *in vivo*.^{21,85} A major breakthrough came when amides, ureas and carbamates were found to be potent and stable transition state inhibitors of sEH, because these tools facilitated experiments to search for endogenous roles for this enzyme.^{85,93} The design of these transition state mimics was based on the knowledge of the catalytic mechanism of the enzyme.^{85,93} X-ray structures of the murine and human enzyme with these urea sEHIs suggested that the urea is the central pharmacophore and that hydrogen bond stabilized salt bridges between urea and residues of the C-terminal sEH active site were formed.^{85,93} This supports the hypothesis that ureas are imitating features present in transient intermediates or transition states encountered along the reaction coordinate of the epoxide ring opening by the sEH. These 1,3-disubstituted ureas, carbamates and amides inhibit the C-terminal epoxide hydrolase activity of the sEH enzyme with nanomolar K_i 's but do not dramatically alter phosphatase activity of the N-terminal domain.^{85,93} The urea pharmacophore appeared to be the most potent, however with adequate substituents amides and carbamates of equal potencies can be obtained. Subsequent modifications to improve *in vivo* stability allowed evaluation of the role of the sEH enzyme in cardiovascular diseases.^{65,85,93,94} Although the mEH has the same catalytic mechanism as the sEH, selection of substituents on the amides and ureas allow one to design inhibitors with greater than $1000 \times$ selectivity for one hydrolase over the other.^{85,95} The sEHIs tested appear to be very selective for the sEH and the 300+ positive hits from a NIH screen on sEH have no consistent inhibition of other enzymes.⁹⁶ The anticancer drug sorafenib — a potent inhibitor of several kinases is a very potent sEH inhibitor. This joint inhibition appears limited to sEHI closely related to sorafenib and possibly the sEH inhibition of sorafenib reduces some of the side effects associated with this drug class when used at high doses.⁹⁷

The first report of a sEHI to demonstrate *in vivo* biological effects used a single bolus dose *N,N'*-dicyclohexylurea (DCU), which lowered blood pressure in hypertensive rats.⁷⁸ Chronic administration was first achieved with 1-cyclohexyl-3-dodecyl-urea (CDU), which when injected intraperitoneally for four consecutive days had anti-hypertensive actions.⁷⁹

These high melting ureas have limited solubility in either water or organic solvents so careful formulation is needed to demonstrate *in vivo* efficacy.^{81,85,98} Incorporation of functional polar groups into one of the alkyl chains of 1,3-disubstituted urea sEHs resulted in compounds that were weak structural mimics of EETs with improved physical properties.^{94,99} One example was 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA, figure 2) that has been widely used in cultured cells and animals.^{21,65,81,85} Although AUDA can be orally administered, it requires DMSO for *in vitro* experiments or a sizable amount of 2-hydroxypropyl β -cyclodextrin for it to be administered in drinking water for *in vivo* studies.^{21,65,81} If lipophilic compounds fall out of solution bioavailability decreases dramatically. As expected for an enzyme with a largely hydrophobic catalytic tunnel, addition of polar groups in general results in a dramatic reduction in potency. However, a polar group — termed — a secondary pharmacophore — such as an ether, ester, amide, sulfonamide, alcohol or ketone roughly 7-8Å from the polar group of the central pharmacophore increased water solubility without sacrificing potency.^{81,94,98,100} The application of this concept resulted in more drug-like sEH molecules including *t*-AUCB, TPAU (figure 2), and others that have both excellent potency and efficacy in many species.

In parallel to the development of sEHs better suited for experimental studies, development of sEHs for use in humans has advanced. These sEHs are initially being developed for the treatment of hypertension. Arête Therapeutics commenced phase 1 clinical trials in healthy volunteers with the first in class sEH inhibitor AR9281 in October 2007 and phase 2 trials are in progress (<http://www.aretetherapeutics.com/news/2009/021009.html> and <http://www.aretetherapeutics.com/news/2008/121808.html>). Amazingly, in less than a decade the sEH has gone from obscurity to a recognized therapeutic target, and sEHs have gone from their first demonstration of anti-hypertensive actions to being tested for the treatment of diseases in humans.

Cardiovascular Therapeutic Effects of Soluble Epoxide Hydrolase Inhibitors

sEHs have cardiovascular protective effects in hypertension, cerebral ischemia, cardiac ischemia, cardiac hypertrophy and atherosclerosis,^{79,101-105} suggesting that these agents have broad potential for the treatment of many cardiovascular diseases and associated morbidity.^{21,65,106} The progression of end organ damage, inflammation, and endothelial dysfunction associated with cardiovascular disease are also attenuated by sEH inhibition.^{67,103,104,107} Studies conducted in mice with *Ephx2* gene deficiency support the notion that sEH effects are a consequence of inhibition of the C-terminal epoxide hydrolase domain.^{35,101,103,104,108,109,110} Although these *Ephx2* $-/-$ mice have the potential to unveil the function of the N-terminal domain of the sEH enzyme, a role for this domain has remained elusive.

Antihypertensive effects

sEHs have hypertensive properties in numerous animal models of hypertension.^{20,21,65} (figure 3) In the SHR, the urea DCU lowered blood pressure and decreased urinary DHET excretion,⁷⁸ and CDU (given once daily) lowered blood pressure in hypertension driven by angiotensin infusion in the rat.⁷⁹ The first sEH to be successfully administered orally to hypertensive animals,⁸⁰ AUDA, lowered blood pressure in rat and mouse models of hypertension.^{80,102,111} Blood pressure was consistently lowered by 25 to 30 mmHg in the rat models of hypertension, however; in mice that had angiotensin dependent hypertension, blood pressure was lowered to that of control mice.^{80,102,111} The mechanism for lowering blood pressure appears to be dependent on decreased vascular resistance and enhanced sodium excretion by the kidney.^{67,80,102} These findings are in line with the biological

actions of EETs to dilate blood vessels and inhibit renal tubular sodium reabsorption.^{20,21,22,111} It was also in these initial hypertension studies that the first evidence for end organ protection by sEHs was recognized.^{65,67}

There have been some conflicting reports on sEH-mediated blood pressure lowering in rats and mice. These species differences may be because many tissues in some rat strains including liver and kidney have very low sEH activity and rat strains vary dramatically in their sEH levels. Although the first demonstration of an sEH to lower blood pressure was in the SHR, subsequent studies have shown variable levels of blood pressure lowering in this model¹¹³⁻¹¹⁵ which could be in part due to polymorphisms in the *Ephx2* gene between SHR strains.^{113,116} There is also conflicting results on sEH-mediated changes in blood pressure in *Ephx2*^{-/-} mice. The initial *Ephx2*^{-/-} male mice had decreased blood pressures that could not be confirmed when these mice were back bred into a C57/BL6 background or in an independently generated *Ephx2*^{-/-} mice colony.^{117,118} More recently studies in a second *Ephx2*^{-/-} C57/BL6 back bred colony did not demonstrate lower blood pressures in males at baseline but DOCA-salt induced hypertension was attenuated.¹¹⁹ Interestingly, each *Ephx2*^{-/-} mouse colony demonstrated high EET levels and high 20-HETE levels that could have offset some of the blood pressure effects.¹¹⁸ Even though the anti-hypertensive actions of sEHs have been variable, the ability of sEHs to protect from end organ damage associated with cardiovascular diseases has been much more consistent.

Kidney Protective Properties

Chronic sEH treatment attenuated renal vascular and glomerular injury in rats with angiotensin-induced hypertension, demonstrating that sEHs provided protection from end organ damage associated with cardiovascular disease.^{67,80} In this model, decreases in collagen expression in glomeruli and tubular cells as well as decreased vascular hypertrophy were observed. Moreover, urinary albumin excretion was decreased and macrophage infiltration was reduced. These studies also determined that sEH treatment starting either at the onset or after the establishment of hypertension provided similar protection to the kidney. Although in these hypertensive animal models, the renal protection afforded by sEHs could have been a result of the decrease in blood pressure, a more recent study in diabetic Goto-Kakizaki rats clearly demonstrated that AUDA provides renal protection independent of lowering blood pressure.¹⁰⁷ Moreover, the elevated plasma cholesterol and triglyceride levels observed in the diabetic Goto-Kakizaki rats were not lowered by AUDA treatment.¹⁰⁷ In addition to the studies animal models of chronic progressive kidney disease, sEHs can provide protection from acute renal injury induced by the chemotherapeutic agent cisplatin in mice.¹²⁰ Inhibition of sEH decreased blood urea nitrogen levels for up to 96 hours and reduced tubular damage associated with cisplatin.¹²⁰ Overall, these studies have consistently found improved renal vascular function, decreased glomerular injury and a decrease in renal inflammation that demonstrate the promise of sEHs as a treatment for acute and chronic kidney disease.

Cardiac Protective Properties

A major therapeutic potential for sEHs is the cardiac protective properties, especially from myocardium ischemic events. *Ephx2* gene deficient mice have improved recovery of left ventricular developed pressure (LVDP) and reduced infarct size following ischemia and reperfusion and are also protected from developing pressure overload induced heart failure and cardiac arrhythmias.¹⁰⁴ The ability of sEHs to improve cardiac function has been established in various experimental models and species.^{19,104,108,121} AUDA reduces the cardiac infarct size in dogs and this protection is similar to that observed with 14,15-EET administration.¹⁹ Similar findings were observed in mice that were administered AUDA-BE and subjected to left coronary artery occlusion followed by reperfusion.¹⁰⁴ Furthermore, in

dogs and mice the EET antagonist, 14,15-EEZE inhibits the protection to the heart provided by sEHs.^{19,104} Acute myocardial infarction hypertension can result in cardiac hypertrophy due to ventricular remodeling.^{101,104,111,121} The first evidence that sEHs could attenuate cardiovascular hypertrophy was the observation that heart weight and collagen were decreased in sEH-treated deoxycorticosterone (DOCA) salt hypertensive rats.¹¹¹ Likewise, cardiac hypertrophy in stroke-prone SHR and angiotensin infused rats was prevented by inhibition of sEH.^{101,122} The cardiac protective actions of sEHs have also been found in mice with pressure overload induced myocardial hypertrophy, where sEHs prevented the development or reversed left ventricular hypertrophy,^{104,121} which was linked to the ability of sEHs to block NF- κ B activation.¹²¹ Although there is overwhelming evidence that *Ephx2* deficiency and sEHs provide cardiac protection, *Ephx2* knockout mice had reduced survival from cardiac arrest and cardiopulmonary resuscitation.¹²³ Future experimental evidence is required to determine the potential for sEHs as therapies for various heart ailments.

Ischemic stroke protection & Vascular Disease

Another potential therapeutic use for sEHs is protection from ischemic brain damage that accompanies stroke. Chronic treatment with AUDA to stroke-prone SHR decreases cerebral infarct size after middle cerebral artery occlusion.^{103,114,115,124} Interestingly, blood pressure was not lowered in these hypertensive rats supporting the notion that the cerebral protective effects were independent of blood pressure.¹¹⁴ *Ephx2* deficient mice have decreased infarct size following a cerebral ischemia.^{103,124} Ischemic stroke protection has also been determined in a mouse model of focal ischemia reperfusion injury, where administration of AUDA-BE or exogenous EETs resulted in at least a 50% reduction in infarct volume.^{124,125} Moreover, administration of sEHs one hour prior to the onset or at the start of reperfusion provided cerebral protection.^{124,125}

The mechanisms by which sEHs protect the brain from ischemic damage appear to be multi-modal and involve the cerebral vasculature and neurons.^{124,125} EETs and sEHs can protect the neurons through anti-apoptotic and anti-inflammatory actions and vasodilator EETs mediate cerebral blood flow regulation and could contribute to brain protection.^{124,125} Angiogenic and attenuated vascular remodeling that allow for enhanced perfusion of the ischemic area have been observed in the stroke-prone SHR treated with sEHs.¹¹⁵ However, these vascular changes do not occur in normotensive animals that also demonstrate decreased infarct volume when treated with sEHs.¹¹⁵ Taken together, these findings indicate that sEHs have broad pharmacological potential for treating ischemic stroke.

Other areas that are beginning to be explored include effects of sEHs on vascular remodeling, angiogenesis, diabetes, and atherosclerosis. Inhibition of sEH decreased vascular hypertrophy in hypertension and decreases vascular smooth muscle cell proliferation.^{51,52,67} Angiogenic actions of EETs have been demonstrated in mice that were enhanced in the presence of a sEH.⁴⁸ Increased microvascular densities and increased middle cerebral artery compliance were associated with AUDA treatment in the stroke-prone SHR.^{114,115} More recent studies demonstrate that sEHs or *Ephx2* deletion antagonizes neointimal formation *in vivo* by mechanisms that are endothelium dependent.^{105,126} Along these lines, atherosclerosis in apolipoprotein e knockout mice was reduced by sEH treatment.¹⁰⁵ Thus, sEHs may have therapeutic potential for specific types of vascular remodeling and atherosclerosis.

Anti-inflammatory Properties

The anti-inflammatory actions of sEHs have been demonstrated to be a key component to end organ protection in cardiovascular disease models.^{18,65} There is also strong evidence

that sEHs have efficacy for the treatment of inflammatory diseases.^{64,66,109,127} AUDA-BE reduced the production of cytokines and pro-inflammatory lipid mediators and diminished lipopolysaccharide-induced mortality in mice.¹²⁷ Furthermore, topical application of sEHs reduced lipopolysaccharide-induced thermal hyperalgesia and mechanical allodynia inflammatory pain in rats.⁶⁶ Although there is ample evidence that sEH inhibition is anti-inflammatory, *Ephx2* deficient or sEH administration to wild-type mice did not reduce lipopolysaccharide-induced inflammatory gene expression or neutrophil accumulation in the liver.¹⁰⁹

Lung inflammation is another area where sEHs could have therapeutic value. Mice exposed to tobacco smoke had only three aortic endothelial cell genes that demonstrated a 3-fold or greater increase in expression, one of which was *Ephx2*.⁶⁸ Inhibition of sEH reduces macrophage infiltration into the rat lung exposed to tobacco smoke and was further reduced by the combination of AUDA-BE and EET treatment.⁶⁸ On the whole, these experimental findings reveal that sEHs provide beneficial anti-inflammatory and analgesic actions.

Therapeutic Potential and Challenges

Based on studies in a wide variety of experimental animal models of cardiovascular disease, significant interest has been generated in the therapeutic potential that can be afforded by sEHs. Human studies are also providing evidence for a contribution of sEH to cardiovascular as well as other disease states. One consideration is that the long-term treatment of many cardiovascular diseases requires a very high safety profile. To date the very large therapeutic index makes sEHs very attractive in this respect. However, the requirement to perform clinical trials in addition to currently accepted treatments and the high bar for safety of new therapies makes the route to the clinic very expensive. It could be that other disease indications will be more attractive routes for the first sEH phase 3 trials. The findings from animal studies and the initial clinical trials will undoubtedly be expanded on in the future and clinical translational studies will ultimately determine the best therapeutic uses and limitations for sEHs. What are these therapeutic potentials and what challenges lie ahead?

Future for Novel Therapeutic Applications

The therapeutic potential for sEHs for treating cardiovascular diseases appears to be exceptionally promising. Patients with cardiovascular diseases are treated many times with multiple medications for conditions such as high blood pressure, hypercholesterolemia, high blood glucose and hyperlipidemia to name just a few. There is ever increasing evidence that sEHs can synergize with existing medications and could be designed as combinational drugs.^{27,64,128} COX-2 protein is decreased by sEHs resulting in decreased PGE2 levels while maintaining the PGI2 to TXA2 ratio, suggesting that low dose COX-2 inhibition in combination with sEHs have additive to synergistic anti-hyperalgesic and anti-inflammatory effects without a decrease in the cardiotoxic PGI2 to TX ratio.⁶⁴ The complexity of the arachidonate cascade suggests that sEHs will have interactions with other NSAIDs, 5-LOX and LT receptor antagonists currently on the market, which could be beneficial or detrimental as therapies for cardiovascular diseases. Further evaluation of sEHs and interactions with other eicosanoid pathways will undoubtedly provide some surprises with the potential for improving cardiovascular therapeutics.

Another interesting finding has been that AUDA has weak PPAR α agonistic activity, suggesting that one could design combinational drugs from this sEH,¹²⁸ that could be beneficial for patients with hyperlipidemia and hypertension. Although AUDA failed to lower blood pressure, cholesterol or triglyceride levels in hypertensive and diabetic Goto-Kakizaki rats,¹⁰⁷ urea based alkanolic acid sEHs can transactivate PPAR α and this PPAR α

activity attenuates vascular smooth muscle cell proliferation.²⁷ One could also envision designing a sEHI with PPAR γ agonistic activity for the treatment of cardiometabolic syndrome. This is an interesting prospect since PPAR γ agonists have the unwanted effect of causing fluid retention that could be detrimental to patients with heart failure and other cardiovascular diseases.¹²⁹ Interestingly, sEHIs and EETs are natriuretic, i.e. they increase sodium and water excretion, and could lessen the fluid retaining state during PPAR γ agonist treatment.^{20,65,79,102} Overall there is great potential for sEHIs as anti-hypertensive treatment that could be used in combination with other medications for patients with poor cardiovascular health, and they may be particularly valuable in patients with co-morbidities.

In addition, sEHIs could have broad neural protective actions. There is now mounting evidence that sEHIs provide protection from brain damage following cerebral ischemia by means that are independent of vascular actions, possibly due to sEHI-induced increase in the neuronal expression of pro-survival anti-apoptotic genes.¹¹⁵ This is further support by data demonstrating that ischemic preconditioning in the brain involves a hypoxic inducible factor- α mediated increase in the CYP2c11 epoxigenase enzyme in astrocytes.¹³⁰

Recent studies also indicate that 14,15-EET activates opioid receptors in the ventrolateral periaqueductal gray area of the brain to produce antinociception.¹³¹ Interestingly, topical application of sEHIs can reduce inflammatory-induced pain and demonstrates the promise for sEHIs as analgesics.^{66,132} On the other hand, the potential for sEHIs to treat neurological disorders such as Alzheimer's or multiple sclerosis has yet to be explored.

Potential for Unwanted Effects

The potential of unwanted effects must also be considered when developing sEHIs for the treatment of cardiovascular diseases. sEHIs can promote angiogenesis and this could result in acceleration of tumorigenesis in patients with some types of cancer.^{48,115} EETs are potent angiogenic lipids that promote vascularization of tumors *in vivo*.^{48,133,134} Epoxigenase metabolites have been demonstrated to be a component of the VEGF-induced angiogenic ERK1/2, Akt and STAT-3 endothelial cell signaling pathways.^{134,135} Though angiogenesis is a potential unwanted effect with sEHI treatment, there are cardiovascular diseases where angiogenesis would be beneficial. Additionally, these findings have led to the postulate that enhancing sEH activity or inhibiting EET production and/or actions could be a therapeutic target for various cancers.

Another concern is that sEHIs exacerbate hypoxic pulmonary vasoconstriction and hypoxia-induced pulmonary vascular remodeling.^{35,36} Chronic hypoxia elicits pulmonary hypertension and vascular remodeling that is associated with increased EET generation and epoxigenase inhibition reduced the hypoxic pulmonary vasoconstriction.^{35,36} Ephx2 $^{-/-}$ mice also demonstrate an increased pulmonary vasoconstriction in response to hypoxia.³⁵ The concern of pulmonary hypertension may be limited to that induced by hypoxia since in monocrotaline-induced pulmonary hypertension, sEHI reduced vascular remodeling and the development of pulmonary hypertension.¹³⁵ Another possible concern in the lungs is that EETs can increase endothelial cell permeability that could result in an unwanted increase in alveolar fluid volume.^{136,137} On the other hand, 14,15-EET combats TNF- α induced hyperreactivity in human airway smooth muscle cells.¹³⁸ These findings suggest that sEHIs have the potential unwanted effect of pulmonary vasoconstriction but could be beneficial to combat bronchial inflammation.

There are also potential unwanted cardiovascular effects that have the potential to limit the therapeutic utility for sEHIs. Although sEHIs can improve cardiac function following ischemia, Ephx2 deletion or sEHIs delayed blood pressure recovery and resulted in higher mortality after cardiopulmonary resuscitation in mice.¹²³ The effect of sEHI on blood

clotting is also complex. Platelet aggregation could be slowed or inhibited, resulting in enhanced bleeding and hemorrhaging in patients taking sEHIs.^{59,61,139} However, PGI₂ to TXA₂ ratios and other data provide evidence that sEHIs would speed clotting in animals treated with aspirin but delay clotting in animals treated with rofecoxib.^{64,80} This observation seems to be in agreement with the trend that EETs appear to move a variety of biological functions back toward steady state.

Epoxyeicosanoids as a Therapeutic Target

The fact that sEHIs and EETs are angiogenic and have the potential for increasing tumor growth means that inhibiting epoxygenase enzymes or EETs could be a treatment for tumor growth. Interestingly, the CYP2J₂ epoxygenase enzyme has been found to be up-regulated in many tumors.¹⁴⁰ A recent study that explored the possibility that selective inhibition of the CYP2J₂ epoxygenase enzyme would repress tumor growth showed that selective CYP2J₂ inhibitors that decreased EET production had marked antitumor properties in *in vitro* and *in vivo* settings including various human cancer cells.¹⁴¹ One could also imagine that EET antagonists such as 14,15-EEZE or even the use of sEH protein as potential cancer therapeutics.

On the other hand, epoxyeicosanoids and EET analogs are being pursued as a potential therapeutic target for cardiovascular diseases. Increasing EET levels or overexpressing epoxygenase enzymes are cardioprotective,^{21,65,104,142} first demonstrated when 11,12-EET was added to transplant preservation solutions resulting in improved coronary artery endothelial function.¹⁴³ Sulfonamide analogs of EET were developed 15 years ago and other EET analogs and antagonists have been designed and utilized in *in vitro* perfused vascular and organ experimental conditions.^{31,58,144-146} These EET analogs and antagonists have determined structure activity requirements for the biological EET actions.^{58,145,146} Ultimately determination of binding sites or receptors for EETs could provide new targets for the treatment of cardiovascular diseases. Recent evidence suggests that EET analogs can be effectively designed to administer chronically to SHR and have anti-hypertensive actions.¹⁴⁷ A combinational drug that has EET mimetic actions and sEH activity is another possibility. Since the finding that certain sEHIs can vasodilate mesenteric resistance arteries there has been progress in attempts to design EET analogs that can also inhibit the sEH enzyme.¹⁴⁸ One could envision the possible utility for EET analogs being used for acute myocardial infarction and drug eluting stents. On the whole, there is great potential with pursuing epoxyeicosanoids as a cardiovascular therapeutic target.

Conclusion

There has been striking rapid progress made with the evaluation of sEHIs as a therapy for cardiovascular diseases since the first description of their anti-hypertensive actions in 2000. Future research will be to further explore other non-cardiovascular diseases that could potentially be treated with sEHIs. There is strong evidence that inflammatory diseases, neurological diseases such as Alzheimer's disease and diseases associated with pain may benefit from sEH treatment. Therefore, the potential for sEHIs for the treatment of cardiovascular diseases is great and other potential therapeutic possibilities appear to be on the horizon.

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Figure 1. Therapeutic Targets of the Arachidonate Cascade

Three major pathways the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways can metabolize arachidonic acid. Inhibitors of the COX-1 and COX-2 enzymes are used for the treatment of pain, inflammation and blood clotting and prostacyclin analogs are used to treat pulmonary hypertension. Leukotriene receptor antagonists that inhibit the cysteinyl leukotriene CysLT1 receptor are used to treat asthma and allergies. Soluble epoxide hydrolase inhibitors that increase epoxyeicosatrienoic acid levels are being developed for the treatment of cardiovascular diseases and inflammation.

Figure 2. Soluble Epoxide Hydrolase Inhibitor (sEHI) Structures and Binding to the Enzymatic Pocket

Left Panel: A.) The early compound AUDA contains the central pharmacophore **C** that forms multiple hydrogen bonds in the enzyme catalytic site. The **R₁** or left side of the molecule rests in a hydrophobic pocket of the sEH catalytic tunnel. The hydrophobic right side of AUDA was designed to mimic 14,15-EET. AUDA is a highly potent sEHI but must be formulated carefully for use *in vivo*. **B.)** TPAU is a potent sEHI illustrating that a polar secondary pharmacophore **P** 7–8 Å from from **C** increases solubility while maintaining potency. It uses a piperidine as a **L** or linker group between **C** and **P**. **C.)** AUCB (both *trans* and *cis* isomers are active) has an ether as **P** with a **R₂** on the right side reaching toward the enzyme surface and mimicking the carboxylate of EETs. Both TPAU and AUCB are highly potent and have good oral availability and pharmacokinetic characteristics. AUCB is more generally potent across multiple species. **Right Panel:** Structure of sEH enzymatic pocket with bound sEHI.

Figure 3. Soluble Epoxide Hydrolase Inhibitor (sEHI) Anti-hypertensive and End Organ Protective Actions

A.) sEHI treatment given orally at the onset of hypertension induced by angiotensin infusion lowers blood pressure. **B.)** sEHI treatment when given after the development of hypertension induced by angiotensin infusion lowers blood pressure. **C.)** sEHI decreases renal injury in diabetic hypertensive Goto-Kakizaki rats independent of lowering blood pressure. **D.)** sEHI decreases brain injury associated with cerebral ischemia in stroke-prone spontaneously hypertensive rats (SHRSP) independent of lowering blood pressure. Figure adapted from data in references 79,80,109,117.