The Pharmacology of the Cytochrome P450 Epoxygenase/Soluble Epoxide Hydrolase Axis in the Vasculature and Cardiovascular Disease

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Abstract—Over the last 20 years, it has become clear that cytochrome P450 (P450) enzymes generate a spectrum of bioactive lipid mediators from endogenous substrates. However, studies focused on the determining biologic activity of the P450 system have focused largely on the metabolites generated by one substrate (i.e., arachidonic acid). However, epoxides and diols derived from other endogenous substrates, such as linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid, may be generated in higher concentrations and may potentially be of more physiologic relevance. Recent studies that used a combination of phenotyping and lipid array analyses revealed that rather than being inactive products, fatty acid diols play important roles in a number of biologic processes including inflammation, angiogenesis, and metabolic regulation. Moreover, inhibitors of the soluble epoxide hydrolase that increase epoxide but decrease diol levels have potential for the treatment of the metabolic syndrome.

I. Introduction

Cytochrome P450 (P450) enzymes are membrane-bound, heme-containing terminal oxidases. Although the majority of P450 enzymes are primarily expressed in the liver, several can be detected in the cardiovascular system and in inflammatory cells. Most is known about the cardiovascular actions of proteins belonging to the CYP4A, CYP2C, and CYP2J families. ω-Hydroxylases such as the CYP4A enzymes use arachidonic acid to generate the vasoconstrictor 20-hydroxyicosatetraenoic acid (HETE), which is implicated in the regulation of myogenic tone and inflammation. The CYP2C and CYP2J epoxygenases generate epoxyicosatrienoic acids (EETs), which possess vasodilator and anti-inflammatory properties.

The role of 20-HETE in the regulation of microvascular function and angiogenesis was extensively reviewed by others (Harris and Hammock, 2013; Imig, 2013). Thus, this review focuses on the other side of the pathway; that is, the epoxides and diols generated by the sequential actions of P450 epoxygenases and the soluble epoxide hydrolase (sEH). Emphasis is placed on outlining more recent findings on the physiologic and pathophysiological actions of metabolites derived from different ω-3 and ω-6 substrate fatty acids, as well as the role of these metabolites in the regulation of vascular tone, angiogenesis, inflammation, and cardiovascular disease, and their cross-talk with different signaling pathways.

II. Input and Output: Polyunsaturated Fatty Acid Substrates for the Cytochrome P40 Pathway

P450 enzymes generate EETs by catalyzing the epoxidation of the olefin bonds in arachidonic acid to generate four regioisomers: 5,6-EET, 8,9-EET, 11,12-EET, and 15,16-EET. Each of these EETs can occur in an R,S- or S,R-enantionic configuration, which potentially exert different effects and have been attributed numerous biologic properties including a role as endothelium-derived hyperpolarizing factors (EDHF).

ABBREVIATIONS: 2-EG, 2-epoxyeicosatrienoic acid; 12-HHT, 12-hydroxyheptadeca-5,8,10-trienoic acid; 12(S)-hydroxyheptadeca-5,8,10-trienoic acid; 14,15-EEZE, 14,15-epoxyeicosatrienoic acid; 15d-PGJ2, 15-deoxy-Δ6-prostaglandin J2; 19,20-DHDP, 19,20-dihydroxydocosapentaenoic acid; AMPK, AMP-activated protein kinase; AP-1, activating protein 1; ApoE(−/−), apolipoprotein E knockout; BK, large-conductance calcium-activated potassium channel; COX, cyclooxygenase; Cx, connexin; DHA, docosahexaenoic acid; DHT, dihydroxyicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EDHF, endothelium-derived hyperpolarizing factor; EDP, epoxydicosapentaenoic acid; EET, epoxyicosatrienoic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EnNaC, epithelial sodium channel; EnNaC-L, endothelial epithelial sodium channel; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; EnPOME, epoxyeicosatetraenoic acid; ER, extracellular regulated kinase; FFA, free fatty acid; FGF, fibroblast growth factor; GPR120, G protein-coupled receptor 120; HETE, hydroxyeicosatrienoic acid; HO, heme oxygenase; JNK, c-Jun N-terminal kinase; KATP, ATP-sensitive potassium; KATP, ATP-sensitive potassium; LDL, low-density lipoprotein; LOX, lipoxigenase; MGS86, 1-[4-chlorophenylmethyl]-3-[(1,1-dimethylethylthio)-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid; MMP, metalloprotease; NFκB, nuclear factor κB; NO, nitric oxide; NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; O2-, superoxide anion; P450, cytochrome P450; PGE2, prostaglandin E2; PGJ2, prostacyclin; P38K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PPAR, peroxisome proliferator–activated receptor; PUFAs, polyunsaturated fatty acid; sEH, soluble epoxide hydrolase; STAT3, signal transducer and activator of transcription-3; STZ, streptozotocin; t-AUCB, trans-4-[4-(3-adamantyl-1-yl-ureido)-cyclohexyl]oxy]-benzoic acid; TRP, transient receptor potential; TRPC, transient receptor potential cation; TRPV, transient receptor potential vanilloid; TxαS, thromboxane A2; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.
Arachidonic acid is clearly not the only endogenous P450 substrate and P450 enzymes are also able to generate epoxides from other polyunsaturated fatty acids (PUFAs) such as linoleic acid, linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Fig. 1). The latter can be converted into either five regioisomeric epoxyeicosatetraenoic acids (EEQs) or six regioisomeric epoxydocosapentaenoic acids (EDPs) (VanRollins et al., 1984; Barbosa-Sicard et al., 2005; Fer et al., 2008; Konkel and Schunck, 2011). Biologic activity has been attributed to almost all of these P450 derivatives, which, just like the EETs, are reported to activate large-conductance calcium-activated potassium (BK) channels, leading to the hyperpolarization and relaxation of vascular smooth muscle cells (VSMCs) (Lauterbach et al., 2002; Ye et al., 2002; Morin et al., 2009). Moreover, the EEQs and EDPs demonstrate cardiovascular-protective properties, have been linked with angiogenesis and improved wound healing (Liclican and Gronert, 2010; Tian et al., 2010), and also show antihypertensive, antithrombotic, and antiatherosclerotic effects in several rat models (McLennan et al., 1996; Hashimoto et al., 1999; Frenoux et al., 2001). However, the specific enzymes involved in the conversion of linoleic acid, EPA, and DPA are less well studied than those that metabolize arachidonic acid, especially in relation to their effects on cardiovascular homeostasis (Oliw et al., 1996; Bylund et al., 1998a,b) and EPA and DPA “antagonists” have not been widely tested.

An epoxygenase is not always just an epoxygenase because superoxide anions (\( \text{O}_2^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), and hydroxyl radicals can also be generated during the P450 reaction cycle when the electrons for the reduction of the central heme iron are transferred on the activated bound oxygen molecule (Coon et al., 1992; Bondy and Naderi, 1994; Puntarulo and Cedernaum, 1998). This means that under certain conditions, the P450 enzymes known to be expressed in the cardiovascular system have the potential to contribute to the generation of oxygen-derived free radicals and the actions of the latter can even mask potential beneficial effects of the lipid mediators generated (see section IV).

A. Regulation of Cytochrome P450 Enzyme Expression and Activity

PUFA epoxide production may change as a consequence of altered P450 expression (by induction or repression) or altered activity (Fig. 2). Little is known about the regulation of vascular P450 expression; although P450 protein can be convincingly demonstrated in native endothelial cells, mRNA and protein levels generally rapidly decrease after cell isolation. Such findings may highlight the importance of physiologic stimuli for the control of P450 levels. Indeed, the exposure of cultured endothelial cells to either cyclic stretch or fluid shear stress can restore CYP2C protein expression as well as endothelial EET production (Fisslthaler et al., 2001). Changes in oxygen tension also affect P450 expression and the promoter regions of several CYP2 genes contain hypoxia-responsive elements. For example, hypoxia upregulates CYP2C8/CYP2C9 expression in vitro in cultured human endothelial cells (Michaelis et al., 2005), whereas experimental ischemia increases the expression of the rat homolog CYP2C11 in the brain in vivo (Alkayed et al., 2002). These changes can be functionally relevant (at least in resistance-sized arteries) because the hypoxia-induced induction of CYP2C protein in arteries from animals exposed to hypoxia was proposed to functionally antagonize agonist-induced contraction by increasing hyperpolarization and

Fig. 1. The P450/sEH axis and PUFA metabolism. P450-dependent metabolism of \( \omega-6 \) (AA and LA) and \( \omega-3 \) (EPA) PUFAs and metabolism of the epoxides generated to the corresponding diols by the sEH. The P450 pathway is frequently referred to as the third pathways for AA metabolism (i.e., in addition to COXs and LOXs) because most is known about the biologic actions of the AA epoxides (e.g., 11,12-EET). AA, arachidonic acid; DHEQ, dihydroxyeicosatetraenoic acid; LA, linoleic acid.
Lower O2 tensions (Marden et al., 2003) induce hypoxia because the expression of CYP2J2 decreases at 2003). Clearly not all P450 enzymes are induced by the CYP2C inhibitor sulfaphenazole (Earley et al., 2002). In the latter study, these changes were prevented by the development of myogenic tone (Earley and Walker, 2002). In the latter study, these changes were paralleled by an increase in the expression of a murine Cyp2c protein recognized by an antibody directed against the human CYP2C9 isofrom. In addition, the hypoxia-induced decrease in the myogenic response was normalized by the CYP2C inhibitor sulfaphenazole (Earley et al., 2003). Clearly not all P450 enzymes are induced by hypoxia because the expression of CYP2J2 decreases at lower O2 tensions (Marden et al., 2003).

Given the size of the P450 family of proteins, it is not surprising that there is considerable interisoform variation in the regulation of gene expression and mRNA stability as well as post-translational modification of the P450 protein. Regulation of the CYP2 family involves nuclear receptors related to the steroid hormone receptor superfamily, such as the constitutive androstane receptor and the retinoic acid receptor. CYP2C9, which is highly homologous to CYP2C8, is inducible in primary human hepatocytes by xenobiotics, including dexamethasone and phenobarbital (Gerbal-Chaloin et al., 2001). Similarly, the induction of CYP2C protein occurs in the endothelial cells of porcine coronary arteries exposed to cortisol (Bauersachs et al., 2002). These changes are explained by the fact that the CYP2C8 and CYP2C9 promoters contain glucocorticoid-responsive elements that are recognized and transactivated by the glucocorticoid receptor (Ged and Beaune, 1991; de Morais et al., 1993; Gerbal-Chaloin et al., 2002).

Almost nothing is known about the regulation of the P450 enzymes that are enriched in vascular cells by either microRNAs or long noncoding RNAs. However, the microRNA let-7b is reported to target CYP2J2 (Chen et al., 2012). This is of particular interest because the let-7 family is highly expressed in the cardiovascular system and the altered expression of let-7 members has been implicated in cardiac hypertrophy and fibrosis, as well as cardiomyopathy arrhythmia, hypertension, atherosclerosis, and angiogenesis (for review, see Bao et al., 2013). Although it is not an epoxygenase, the CYP1B1 that has been implicated in angiogenesis and cancer is tightly regulated by the angiogenic microRNA miR-27b (Tsuchiya et al., 2006; Devlin et al., 2010; Chuturgoon et al., 2014). The link to this particular microRNA is interesting because it was previously described as a “regulator hub in lipid metabolism” (Vickers et al., 2013). Indeed, miR-27b levels are significantly upregulated by a high-fat diet and hepatic miR-27b and its target genes are inversely altered in a mouse model of dyslipidemia and atherosclerosis (Vickers et al., 2013). Whether CYP1B1 is involved in the latter is unclear, but the enzyme was recently linked with protection against angiotensin II–induced hypertension in female mice (Jennings et al., 2014).

Once the protein is expressed, P450 activity is thought to be determined mainly by the availability of its substrates (Capdevila et al., 2000). Since phospholipase A2 inhibitors attenuate P450-dependent vasodilator responses, the activation cascade is thought to involve a stimulus-induced increase in intracellular Ca2+, followed by the activation of phospholipase A2 that then liberates the PUFAsubstrate (e.g., arachidonic acid) from membrane phospholipids. The increase in substrate immediately results in the activation of P450 enzymes and the generation of its products. Although this sequence of events is certainly plausible, it is highly likely that additional mechanisms, such as phosphorylation, play a role in regulating P450 activity. Indeed, some P450 enzymes (CYP2B1, CYP2B2, and CYP2E1) are reported to be phosphorylated by protein kinase A (PKA), and the consequences of P450 phosphorylation range from the regulation of activity (Oesch-Bartlomowicz et al., 2001) and subcellular localization (Anandatheerthavarada et al., 1999; Korsmeyer et al., 1999) to proteasome degradation (Eliasson et al., 1992; Korsmeyer et al., 1999). Although there has been little advancement in this area over the last few years, other P450 enzymes such as the hepatic-enriched CYP3A4 and CYP2E1 are phosphorylated by PKA and protein kinase C, which accelerates their ubiquitination and endoplasmic reticulum–associated degradation (Correa et al., 2014).

High concentrations of nitric oxide (NO) can attenuate P450 expression and activity. The effects on mRNA levels are most likely attributable to suppression of the DNA-binding activity of transcription factors, such as hepatocyte nuclear factor 4. The latter regulates levels of several P450 enzymes (Vossen and Erard, 2002) and at the same time can promote the proteasomal degradation of P450 proteins (Lee et al., 2008). NO can also interact with P450 enzymes to affect their activity in different ways: 1) NO can reversibly bind to the heme moiety of P450 enzymes, forming iron-nitrosyl complexes; or 2) it can irreversibly react with cysteine residues. Both modifications result in enzyme inactivation in vitro (Minamiyama et al., 1997). Although high concentrations of NO do inhibit the vascular CYP2C enzymes in...
vitro (Bauersachs et al., 1996), whether these processes are biologically relevant and can occur at physiologically relevant concentrations of NO is not entirely clear. If it were the case, the endothelial P450 enzymes would be expected to be largely inactive in the healthy vasculature, which constantly generates NO. This, in turn, would imply that P450-derived metabolites would play a more dominant role in the homeostasis of diseased vessels in which the production of endothelium-derived NO is markedly decreased. However, there are clear physiologic consequences of P450 activation in vascular cells (e.g., on Akt, PKA, and Ca$^{2+}$ signaling) that can be demonstrated even in the presence of a fully functional endothelial nitric oxide synthase (eNOS).

Although useful information can be obtained from overexpression systems or by studying the cellular response to specific PUFA metabolites, it has proven difficult to determine the physiologic and pathophysiologic roles of P450 enzymes in vivo. Investigation of these issues through generation of tissue-specific P450 knockout mice would be ideal, but are not practicable because the mouse carries 15 CYP2C and 8 CYP2J isoforms with overlapping metabolic profiles and tissue distributions (Nelson et al., 2004). Moreover, the knockdown of some P450 enzymes was previously reported to induce a compensatory upregulation of another isoform (Holla et al., 2001; Nakagawa et al., 2006). To avoid such problems, investigators have generated mice with tissue-specific overexpression of the human CYP2C8 or human CYP2J2 enzymes that have generate higher than normal epoxide levels (Edin et al., 2011). An alternative to modulating epoxide production is to prevent their metabolism by inhibiting the sEH, which is highly conserved between species. Indeed, the deletion of the sEH in mice is an effective way of manipulating fatty acid epoxide and diol levels in vivo (Sinal et al., 2000). To date, global sEH-deficient animals (Sinal et al., 2000) as well as “floxed” and “inducible” tissue-specific sEH mice have been generated (Hu et al., 2014).

**B. Soluble Epoxide Hydrolase**

Intracellular levels of the epoxides are tightly regulated and metabolism occurs relatively rapidly. Although the sEH (EPHX2) is the most important fatty acid epoxide-metabolizing enzyme, the arachidonic acid epoxides can also be subjected to $\beta$-oxidation and chain elongation. There is of course an exception to every rule. In this case, it is 5,6-EET, which is not a substrate for the sEH and is more rapidly metabolized by cyclooxygenase (COX) (Chiamvimonvat et al., 2007). Several of the EET- and sEH-derived dihydroxy-ecosatrienoic acids (DHETs) are also biologically active, although generally less so than the parent epoxides (for review, see Harris and Hammock, 2013). The sEH is not restricted to the arachidonic pathway and is also able to metabolize other PUFA epoxides (e.g., $\omega$-3 DHA and EPA epoxides) to generate mediators that have rarely been studied in the vasculature but may turn out to be more potent than the EETs in some organs.

Given that the sEH metabolizes fatty acid epoxides to their corresponding diols, an increase in sEH expression or activity would be expected to decrease EET as well as EPA- and DHA-derived epoxide bioavailability, contribute to the blunting of endothelium-dependent vasodilation, and promote hypertension. In fact, there are several examples of hypertension being associated with elevated sEH expression and/or activity, including the spontaneously hypertensive rat (Yu et al., 2000; Imig, 2005). Moreover, there have been numerous reports showing that specific sEH inhibitors are effective at preventing as well as reversing experimental hypertension (for review, see Imig and Hammock, 2009; Harris and Hammock, 2013).

The sEH protein is a homodimer composed of two 60-kDa monomers joined by a proline-rich bridge (Beetham et al., 1993). Each monomer consists of an N-terminal domain that displays lipid phosphatase activity, and a larger C terminus that processes classic $a/b$-hydrolase activity (Cronin et al., 2003; Newman et al., 2003). The activity of the lipid phosphatase has been associated with the metabolism of lysophosphatidic acid (Morisseau et al., 2012; Oguro and Imaoka, 2012), peroxisome proliferator–activated receptor (PPAR) activity, the isoprenoid/cholesterol biosynthesis pathway, and cholesterol-related disorders (Enayetallah et al., 2008). Indeed, in addition to demonstrating enhanced circulating EET levels (Sinal et al., 2000), male sEH$^{-/-}$ mice exhibit decreased plasma cholesterol and testosterone levels (Luria et al., 2009). Moreover, the isoprenoid pyro- and monophosphates that are reported to be substrates for the N-terminal domain of the enzyme (Tran et al., 2005; Enayetallah and Grant, 2006) are also used for isoprenylation of small G proteins involved in multiple cell signaling pathways, especially those linked to redox stress and inflammation (Kovacs et al., 2002). A tentative link between the serine phosphorylation of the eNOS and the phosphatase activity of sEH was suggested (Hou et al., 2012); however, it has not been possible to detect any such interaction in native or primary cultures of endothelial cells (I. Fleming, unpublished observations).

The exact physiologic relevance of the lipid phosphatase activity is currently unclear because currently available sEH inhibitors act solely on the hydrolase domain (Newman et al., 2003). Although several groups are actively developing specific inhibitors of the lipid phosphatase domain (Tran et al., 2005; Hahn et al., 2011), these have yet to be tested in physiologic models. At the moment, hints on the importance of the lipid phosphatase activity can be gleaned from studies that compare the phenotype of sEH$^{-/-}$ mice with that of wild-type mice receiving classic sEH inhibitors for prolonged periods. The majority of effects seen after
sEH inhibitor therapy are also seen in sEH−/− mice but there are exceptions. One such example is the enhanced pulmonary vascular muscularization and decreased voluntary exercise capacity seen in sEH−/− animals that could not be reproduced by chronic sEH inhibitor treatment (Keserü et al., 2010). Other differences include the fact that sEH deletion, but not inhibition, renders mice more sensitive to anesthetics (unpublished observation) and reduces survival after cardiac arrest (Hutchens et al., 2008). Although they clearly require more detailed investigation, such observations provide an indirect indication of a physiologic role for the phosphatase domain.

Relatively little is known about the mechanisms that control sEH expression and activity. The sEH promoter contains several specificity protein 1 (Tanaka et al., 2008a) and activating protein 1 (AP-1) sites (Ai et al., 2007). Methylation of one of the former has been implicated in gene silencing (Zhang et al., 2010), whereas the activation of AP-1 is implicated in the angiotensin II–induced upregulation of the enzyme (Ai et al., 2007). There may also be a role for activating transcription factor 6 because homocysteine activates this transcription factor and increases sEH RNA levels (Zhang et al., 2012). Whether hypoxia-inducible factors directly regulate gene expression is unknown but hypoxia induces the rapid downregulation of the sEH in vitro and in vivo (Keserü et al., 2010). Metabolic diseases are associated with increased sEH expression in different organs (see below) but it is not known how long the activity of the protein is maintained. This may be a particularly important consideration in diabetes because the sEH can be tyrosine nitrated on two tyrosine residues (Tyr383 and Tyr466) that are thought to be crucial for enzymatic activity (Barbosa-Sicard et al., 2009).

There are other amino acids within the sEH dimer that are putative targets of electrophilic oxidants such as 15-deoxy-Δ-prostaglandin J2 (15d-PGJ2). The latter prostaglandin is generated in inflamed cells and tissues as a consequence of upregulation of COX-2. 15d-PGJ2 is an endogenous PPARγ ligand but it can also directly adduct to redox active protein thiols to modulate protein function. Known targets identified to date include the proinflammatory transcription factors nuclear factor-κB (NFκB), signal transducer and activator of transcription-3 (STAT3), and AP-1, as well as the anti-inflammatory transcription factor nuclear regulatory factor 2 (for review, see Surh et al., 2011). Cysteine 521 adjacent to the catalytic center of the sEH C-terminal (hydrolase) domain was reportedly targeted by 15d-PGJ2 with the consequence that sEH activity was reduced (Charles et al., 2011). Thus, the adduction of 15d-PGJ2 to the sEH would be expected to elicit the same anti-inflammatory effects as pharmacological sEH inhibition. However, the concentrations of 15d-PGJ2 required to induce these effects were high and also target several of the P450 enzymes expressed in the vasculature (I. Fleming, unpublished observation) and have yet to be demonstrated in vivo. It will be interesting to determine whether other electrophilic mediators, such as the nitrofatty acids (Schopfer et al., 2011), are able to target the sEH in the same way they adduct to and influence the 5-lipoxygenase (LOX) (Awwad et al., 2014).

III. Mechanisms of Epoxyeicosatrienoic Acid Action

As outlined above, the P450 enzymes can metabolize a series of substrates to their corresponding epoxides. However, given that most is known about the biologic actions of the epoxides of arachidonic acid, the following sections deal mostly with these metabolites.

Vascular biologists and physiologists became increasingly interested in the epoxides of arachidonic acid as vasodilators underlying some aspects of NO- and prostacyclin (PGI2)–independent vasodilation in resistance-sized arteries and a number of vascular beds, particularly the renal and coronary systems. NO- and PGI2-independent but K+ channel inhibitor–sensitive vasodilation initially tended to be grouped under the umbrella term of EDHF, which is now recognized as an oversimplification. Indeed, several entirely distinct mechanisms are known to contribute to endothelial cell hyperpolarization and vasodilation, and although P450 enzymes are implicated in EDHF-mediated responses, these enzymes are not ubiquitously expressed throughout the vascular tree (Busse et al., 2002; Campbell and Fleming, 2010). However, the vasodilator actions of the EETs are linked to vascular hyperpolarization (Fig. 3).

A. Calcium-Activated Potassium Channels

Calcium-activated potassium (KCa) channels were the first reported targets of PUFAs epoxides in the vascular system with 5,6-EET, 8,9-EET, 11,12–EET, and 14,15-EET initially found to be equally effective when it came to relaxing precontracted bovine coronary arteries. Indeed, both 11,12-EET and 14,15-EET increased VSMC KCa channel opening (Campbell et al., 1996). On the basis of their pharmacological sensitivity to a series of inhibitors, the channels in question were identified as large-conductance calcium-activated potassium (BK or KCa1.1) channels (Campbell et al., 1996). Thereafter followed demonstrations that the inhibition and downregulation of CYP2C epoxygenases attenuated the NO- and PGI2-independent or EDHF-like responses in conductance (Fisslthaler et al., 1999) as well as resistance arteries (Bolz et al., 2000).

As the term EDHF implies, it was initially thought that the P450- and endothelium-dependent hyperpolarization of VSMCs was initiated by the generation of a P450 metabolite in endothelial cells. This supposedly diffusible factor was then transferred to VSMCs, where it activated smooth muscle cell BK channels to elicit hyperpolarization and relaxation. A historical look at
the literature reveals that identifying which KCa channels contributed to the EDHF phenomenon was a matter of prolonged debate. The involvement of iberiotoxin-sensitive BK channels proved to be a major difference between P450-dependent EDHF responses and those studied by other groups in which hyperpolarization was sensitive to the combination of charybdotoxin and apamin, which inhibit small conductance KCa channels and intermediate conductance KCa channels (Zygmont and Högestätt, 1996). However, it is now appreciated that one of the most important events in the generation of EDHF-like responses is the rapid opening of small conductance KCa channels (KCa2.3) and intermediate conductance KCa channels (KCa3.1) that leads to the hyperpolarization of endothelial cells, an event that precedes the hyperpolarization of VSMCs (Busse et al., 2002). It is then the subtle change in K+ that elicits the activation of smooth muscle cell K+ channels and/or the Na+/K+ -ATPase (Edwards et al., 1998). The role of PUFA epoxides in this process is most probably linked to their ability to increase the Ca2+ response to endothelial cell agonists (see section III.D), and to potentiate endothelial cell KCa activation and hyperpolarization (Fleming et al., 2007). However, EET transfer between cells does occur when they are generated in larger amounts (Popp et al., 1996; Gauthier et al., 2005), especially when sEH activity is reduced.

Is the EET-induced activation of BK channels direct or indirect and do all of the stereoisomers and regioisomers elicit the same effects? There are differences in the ability of different stereoisomers to activate BK channels in different tissues because 14(S),15(R)-EET, but not 14(R),15(S)-EET, was found to increase BK channel activity in coronary smooth muscle cells (Campbell et al., 2006), whereas 11(R),12(S)-EET effectively increased BK channel open probability in rat renal smooth muscle cells (Zou et al., 1996). It also appears that the EET-induced activation of BK channels is not simply the result of the direct binding of the epoxide to an extracellular domain of the channel because 11,12-EET activates BK channels in cell-attached patches of smooth muscle cells and endothelial cells, it is without effect in inside-out patches. Such observations imply that some cytosolic component or cellular signaling pathway that is absent in inside-out patches is required for EET-stimulated responses. The missing components may well be G proteins and GTP because the addition of GTP to the cytoplasmic surface of inside-out patches restored the ability of 11,12-EET to open BK channels. Moreover, BK channel activation by EETs can be prevented by the G protein inhibitor GDPβS as well as by an anti-Gαs antibody (Li and Campbell, 1997; Hayabuchi et al., 1998; Fukao et al., 2001). A relatively recent finding is that EETs can also target BK channels present in the mitochondrial membrane to elicit seemingly paradox effects. At least in pulmonary VSMCs treated with NO synthase and COX inhibitors, 11,12-EET was found to induce depolarization, whereas the BK channel opener NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) elicited hyperpolarization. Such results indicated there was no effect of the EET on classic plasma membrane BK channels. Rather, 11,12-EET elicited an iberiotoxin-sensitive loss of mitochondrial membrane potential leading to plasma membrane depolarization—effects that were dependent on the presence of the BKβ1 subunit and were not observed in cells from BKβ1−/− mice. Again these effects of 11,12-EET relied on the activation of PKA. Mechanistically, it seems that 11,12-EET induces the physical association of the BKα and BKβ1 subunits on the mitochondrial membrane (Loot et al., 2012). Whether this is a phenomenon exclusive to pulmonary VSMCs remains to be clarified but is of potential interest given that mitochondrial BK channels in the heart are thought to play a role in protection from ischemic injury (for review, see Singh et al., 2012) and both EETs and sEH inhibition protect mitochondrial function after stress (Katragadda et al., 2009; Batchu et al., 2012b).

B. ATP-Sensitive Potassium Channels

Although inhibitors of ATP-sensitive potassium (KATP) channels have no effect on EDHF-mediated responses,
K<sub>ATP</sub> channels have been implicated in some aspects of EET biology, particularly in the heart. In this case, it seems that the 11,12-EET–induced activation of K<sub>ATP</sub> channels is dependent on the G<sub>s</sub> protein (Ye et al., 2006) and the activation of PKA (Ye et al., 2005) and/or the phosphatidylinositol 3-kinase (PI3-K) (Batchu et al., 2012a), albeit with eventual organ-specific differences in the mechanism of channel activation (Lu et al., 2006; Bodiga et al., 2009). On the whole, EET-induced activation of K<sub>ATP</sub> channels seems to be cardioprotective and EETs can regulate cardiac electrophysiology (Lu et al., 2006) and prevent the increase in intracellular Ca<sup>2+</sup> that is generally associated with ischemia-reperfusion injury (Ye et al., 2006b). More recently, the cardioprotective effect of EETs administered prior to ischemia was attributed to the activation of the eNOS and increased NO production, whereas K<sub>ATP</sub> channel activation and the mitochondrial permeability transition pore were involved in the beneficial effects of the EETs when administered just prior to reperfusion (Gross et al., 2013). The actions of EETs in other organs, such as insulin secretion in pancreatic islets, may also be linked to K<sub>ATP</sub> channel activation (Sharoyko et al., 2007).

C. Epithelial Sodium Channels

In the kidney, particularly in the cortical collecting duct, both arachidonic acid and 11,12-EET inhibit epithelial sodium channel (ENaC) activity (Wei et al., 2004; Wang et al., 2009b). Initially, this effect was thought to be limited to 11,12-EET with 5,6-EET, 8,9-EET, and 14,15-EET being ineffective; however, this no longer seems to be the case (Pavlov et al., 2011; Pidkovka et al., 2013). Endogenously, the EETs that regulate ENaC activity have been found to act as second mediators for the actions of adenosine on the channel (Wei et al., 2006) and are likely to be derived from Cyp2c23 in rats (Wei et al., 2004) or Cyp2e44 in mice (Sun et al., 2012b)—both homologs of the human CYP2C8/9 enzymes. Interestingly, high dietary potassium stimulates the renal expression of Cyp2c23 and enhances the inhibitory effect of arachidonic acid and 11,12-EET on the ENaC (Sun et al., 2010). Given its role in the regulation of sodium transport and body fluid volume, it is clear that changes in EET levels and thus its effects on ENaC can affect blood pressure. It is unclear whether these effects, like other actions of the EETs, are dependent on G<sub>s</sub>, cAMP, or PKA activation. To date, only a nonspecific effect on ENaC activity due to the interaction between the channel and the lipid has been suggested (Wang et al., 2009b).

D. Transient Receptor Potential Channels

The transient receptor potential (TRP) channels are a large (>30 members including splice variants) family of cation channels that can be divided into six subfamilies in mammals. With few exceptions, all of the TRP channels are nonselective cation channels with varying preferences for Ca<sup>2+</sup> over Na<sup>+</sup>. To date, the epoxides of arachidonic acid had been linked to the activation of the transient receptor potential vanilloid (TRPV) channels as well as at least two of the “canonical or classical” transient receptor potential cation (TRPC) channels that contribute to capacitative Ca<sup>2+</sup> entry (Large et al., 2009; Fuchs et al., 2010).

Several TRP channels are activated or modulated by EETs, but there seems to be a cell-dependent specificity in the TRP isoforms that are activated by the specific EET regioisomers. For example, 5,6-EET, but not 11,12-EET, can activate TRPV4 channels in endothelial cells (Watanabe et al., 2003; Vriens et al., 2005) and this mechanism underlies the EDHF-like, flow-induced vasodilation of murine carotid arteries (Loot et al., 2008). Fitting with the fact that 5,6-EET is the regioisomer metabolized by COX rather than the sEH, COX inhibition was able to enhance flow-induced, NO- and PGI2-independent vasodilation, whereas sEH inhibition was without effect. On the other hand, 11,12-EET, but not 14,15-EET or 5,6-EET, can enhance the bradykinin-induced capacitative Ca<sup>2+</sup> influx in endothelial cells and pulmonary VSMCs by a TRPC3 and/or TRPC6-dependent process (Fleming et al., 2007; Keserü et al., 2008).

How can the epoxides of arachidonic acid influence TRP channel activity? In order for TRP channels to facilitate cation influx, they need to be present at the plasma membrane. Interestingly, in the absence of cell stimulation, many TRP channels are physically concentrated around the nucleus. This implies that a further important determinant of TRP channel activation is their rapid intracellular translocation. In vascular endothelial cells and pulmonary VSMCs, 11,12-EET was found to stimulate the rapid (within 10 seconds) translocation of TRPC6 and TRPC3 channels to caveolin-rich areas in the plasma membrane (Fleming et al., 2007). The increase in intracellular Ca<sup>2+</sup> then increased K<sub>Ca</sub> channel activation and endothelial cell hyperpolarization. Thus, it seems clear that the most likely way by which 11,12-EET contributes to NO- and PGI2-independent relaxations, or rather those responses attributed to an EDHF, actually reflects the amplification of K<sub>Ca</sub> channel opening elicited by increasing the number of TRPC channels incorporated into caveolae.

Is the effect of PUFA epoxides on TRP channels direct? This was initially proposed to be a possibility because some of the EETs were speculated to act as endogenous ligands for TRP channels (Watanabe et al., 2003), several of which possess an arachidonic acid binding site and can be activated by EETs (Clapham, 2003). Although the latter may account for altered open probability of membrane TRP channels, the translocation process is clearly PKA and Rho dependent (Fleming et al., 2007; Keserü et al., 2008). The EETs may not be the only arachidonic acid metabolites that can affect TRP channels because the arachidonic acid–induced
activation of TRPV4 in human coronary arterioles that is sensitive to P450 inhibition could not be attributed to EETs (Zheng et al., 2013a).

The fact that lipid epoxides can activate TRP channels also implies that PUFA epoxides are likely to be integrated into different pain signaling pathways. This is indeed the case and although the majority of studies imply that sEH inhibition has analgesic properties (Inceoglu et al., 2006, 2007, 2011; Terashvili et al., 2008, 2013), other studies have yielded contradictory results and indicate that at least 8,9-EET can sensitize TRPA1-expressing nociceptors to increase pain (Brenneis et al., 2011). In addition, in a model of mechanical pain, 5,6-EET is synthesized after the acute activation of nociceptors and can produce mechanical hypersensitivity via TRPA1 channel activation at central afferent terminals in the spinal cord (Sisignano et al., 2012). The truth is probably somewhere in the middle because EETs or inhibition of the sEH have also been reported to lead to antihyperalgesia by at least two spinal mechanisms, first by repressing the induction of the COX2 gene and second by rapidly upregulating an acute neurosteroid-producing gene (steroidogenic acute regulatory protein D1) (Inceoglu et al., 2008). Furthermore, any potentiating effect of the EETs (particularly 14,15-EET) on TRP channel activation at central afferent terminals in the spinal cord (Sisignano et al., 2012). The truth is probably somewhere in the middle because EETs or inhibition of the sEH have also been reported to lead to antihyperalgesia by at least two spinal mechanisms, first by repressing the induction of the COX2 gene and second by rapidly upregulating an acute neurosteroid-producing gene (steroidogenic acute regulatory protein D1) (Inceoglu et al., 2008). Furthermore, any potentiating effect of the EETs (particularly 14,15-EET) on TRP channels may be offset by the activation of β-endorphin and Met-enkephalin, which subsequently act on μ- and δ-opioid receptors to produce antinoiception (Terashvili et al., 2008).

E. Gap Junctional Communication

Gap junctions are one important mechanism by which signals, including hyperpolarization and depolarization, can be transferred between endothelial cells as well as between endothelial and VSMCs. The latter are aqueous pores connecting adjacent cells, and are formed by the association of connexin (Cx) proteins in a classic pentalaminar structure. Of the gap junctions in a gap junctional plaque, about 80% are thought to be “open,” thus facilitating the transfer of electrical and chemical signals between adjacent cells (Meens et al., 2013). However, numerous intracellular events, such as changes in intracellular pH, membrane potential, and Cx phosphorylation, can enhance or decrease the passage of ions and small molecules through the connexon pore (Nielsen et al., 2012; Hervé and Derangeon, 2013). Functional gap junctional communication between vascular cells has been implicated in both ascending dilatation and the P450 inhibitor–sensitive, NO- and PGI2-independent dilatation of many vascular beds. Therefore, given the finding that EDHF-mediated responses are attenuated after the inhibition of gap junctional communication (Chaytor et al., 1997, 1998; Taylor et al., 1998; Griffith and Taylor, 1999; Hutcheson et al., 1999), it seemed logical to determine whether P450 metabolites such as 11,12-EET could alter Cx trafficking or gap junctional communication. Certainly, in the porcine coronary artery (which demonstrates a particularly strong 11,12-EET component to the EDHF response), agonist-induced hyperpolarization was attenuated by gap junction blockers (Edwards et al., 2000). More direct assessment of cell–cell communication revealed that P450 activation and 11,12-EET formation elicited a biphasic effect on the electrical coupling and transfer of Lucifer Yellow between endothelial cells, consisting of a transient increase in coupling followed by a sustained uncoupling (Popp et al., 2002). The initial facilitation phase was sensitive to the P450 inhibition as well as cAMP analogs and PKA inhibitors and attributed to the recruitment of connection 43 to gap junctional plaques. The transient nature of the response, on the other hand, was related to the extracellular regulated kinase (ERK) 1/2–dependent phosphorylation of Cx43, which resulted in the inhibition of gap junctional communication (Brandes et al., 2002; Popp et al., 2002). These findings were reported for isolated arteries (Weston et al., 2005; McSherry et al., 2006). Thus, a second mechanism by which EETs can contribute to NO- and PGI2-independent vascular relaxation is via the facilitation of endothelial and possibly also myoendothelial gap junctional communication. Although it is clear that Cx43 is not the most relevant connexin in intact arteries (Wit and Griffith, 2010), studies on the effects of PUFA epoxides on Cx40 or Cx37 are lacking.

There may also be links between the P450/sEH axis and Cx expression because the downregulation of P450 in osteoblasts was found to decrease Cx43 expression as well as gap junctional communication (Polusani et al., 2011). In addition, the increase in P450-dependent, EDHF-like responses in mesenteric arteries from cirrhotic rats was associated with increased Cx40 and Cx43 mRNA and protein expression (Bolognesi et al., 2011).

F. Epoxide Receptors

1. Membrane Epoxyeicosatrienoic Acid Receptors.

One characteristic of many EET-induced cellular responses, such as cell proliferation, gap junctional communication, or TRP channel translocation, is the dependence on PKA activation (Wong et al., 2000; Fukao et al., 2001; Popp et al., 2002; Fleming et al., 2007). Given that high-affinity EET binding sites have been described on the surface of some cells, several groups have speculated that a specific EET membrane receptor may exist (Yang et al., 2008; Chen et al., 2009c, 2011c; Pfister et al., 2010). This concept is supported by the fact that there are also differences in responsiveness to different EET stereoisomers and not just the regioisomers. For example, 11(R),12(S)-EET is a more potent activator of renal artery KCa channels (Zou et al., 1996) and rat airway electrical parameters
(Pascual et al., 1998), as well as endothelial cell TRP channel translocation and angiogenesis (Ding et al., 2014), than 11(S),12(R)-EET. However, this is not a universal observation because 11(S),12(R)-EET is reportedly more effective than 11(R),12(S)-EET in activating cardiac K_{ATP} channels (Lu et al., 2002).

In support of the concept of a membrane-bound EET receptor that recognizes defined structural components within the EETs, is the fact that a series of stable and specific EET agonists and antagonists has been generated (Gauthier et al., 2002; Falck et al., 2003; Yang et al., 2008). The fact that the epoxides of arachidonic acid increase intracellular Ca^{2+} may hint at an effect mediated by G_{q/11} proteins, but the effects are indirect and involve TRP channel translocation. Rather, the existence of a G_{q}-coupled EET receptor fits better with the numerous reports of a reliance on cAMP/PKA for EET-induced signaling (Inceoglu et al., 2007). Certainly, biochemical studies measuring GTP binding to G proteins in endothelial cells confirm the importance of a G protein and indicate that 11,12-EET increases GTPγ35S binding to G_{q}, but not G_{i}, proteins (Node et al., 2001). However, although many of the effects of the EETs rely on PKA activation, the EET-induced changes in cAMP tend to be small and inconsistent, particularly in endothelial cells.

2. G Protein–Coupled Receptor 120. G protein–coupled receptor 120 (GPR120), otherwise known as free fatty acid (FFA) receptor 4 or ω-3 fatty acid receptor 1, functions as a receptor for unsaturated long-chain free fatty (Hara et al., 2011; Oh and Lagakos, 2011; Oh and Olefsky, 2012). FFA4 plays an important role in mediating anti-inflammatory effects (Oh et al., 2010), regulating adipogenesis (Gotoh et al., 2007) as well as lipid and glucose metabolism (Hirasawa et al., 2005; Tanaka et al., 2008b), and functioning as a tumor-promoting receptor in human colorectal carcinoma (Wu et al., 2013). Given that many of these actions parallel those of the EETs and/or sEH inhibition, is there any possibility that GPR120 is an EET receptor? There is little that speaks in favor of this being the case, because GPR120 is known to be both G_{i/0} coupled and G_{q/11} coupled, rather than G_{q}-coupled. However, because GPR120 is a receptor for ω-3 PUFAs, it may well turn out that some of the global effects associated with sEH inhibition in vivo might indeed be linked to the activation of GPR120.

There are additional orphan receptors, such as GPR40 (FFA1), GPR41 (FFA3), GPR43 (FFA2), and GPR84, that can be activated by FFAs (Oh and Lagakos, 2011). However, when an EET photoaffinity label was used to screen binding to HEK293 cells expressing 79 such receptors (including all of those listed here), no apparent binding was detected (Chen et al., 2011c). In the same experiments, U937 cells showed a single 47-kDa radiolabeled band indicating that the photoaffloring process allowed the detection of an endogenous EET receptor, which remains elusive.

3. Thromboxane Receptors. The ability of the EETs to functionally antagonize thromboxane A_{2} (TXA_{2})–mediated effects at the level of vascular tone have long been recognized. A more direct interaction at the molecular levels was recently proposed because 14,15-EET was able to relax murine mesenteric arteries and the rat aorta precontracted with a TXA_{2} analog (which induced a Ca^{2+}-independent, Rho kinase–dependent contraction), but not arteries precontracted with a Ca^{2+}-elevating α_{1} adrenoceptor agonist (Behm et al., 2009). Instead of interpreting the results on the basis of the mechanism by which the different vasoconstrictors used actually induce contraction, it was suggested that 14,15-EET evokes relaxation via a thromboxane receptor–dependent mechanism. This suggestion was supported by the observation that 14,15-EET prevented the binding of a selective thromboxane receptor antagonist without having significant effects on other prostanoid or leukotriene receptors (Behm et al., 2009). It is, however, unlikely that the physiologic actions of the EETs can be attributed to thromboxane receptor antagonism largely because the effect was only observed using supraphysiologic concentrations of EETs (for review, see Ellingsworth et al., 2014).

4. Intracellular Epoxycisatrienoic Acid Receptors. Because PUFA epoxides are generated intracellularly, the highest concentrations of these mediators are expected to be reached in the cytosol, especially in cells that express the P450s. Therefore, it is more than likely that these intracellular lipid mediators interact with intracellular receptor molecules such as the PPAR family of nuclear receptors.

There have been numerous reports comparing the effects of PPARα and PPARγ activation with the effects of the EETs on vascular cell proliferation, migration, and inflammation (Wray and Bishop-Bailey, 2008). Different regioisomers may even target different PPAR isoforms because ω-hydroxylated 14,15-EET and 14,15-DHET (Cowart et al., 2002; Fang et al., 2005) as well as 8,9-EET and 11,12-EET (Ng et al., 2007; Wray et al., 2009) are reported to bind with a high affinity to PPARα, whereas the EETs generated in endothelial cells in response to fluid shear stress target PPARγ (Liu et al., 2005).

Despite the wealth of articles, there has been some confusion regarding the importance of PPAR activation in mediating the effects of the PUFA epoxides—largely because some of the early sEH inhibitors that were initially found to result in PPARα activation did so via off-target effects (Fang et al., 2006). In addition, the expression of the sEH is reportedly regulated by PPARγ (Pang et al., 2011), whereas several vascular relevant P450 enzymes are known to be regulated by PPARα activity (Muller et al., 2004; Pozzi et al., 2007; Wang et al., 2009a). In an effort to optimize the anti-inflammatory potential of the P450-sEH axis and the PPARs, several groups have developed dual sEH inhibitors/PPARγ agonists (Imig
et al., 2012; la Buscató et al., 2012) with potentially promising effects for the treatment of the metabolic syndrome (Imig et al., 2012).

**G. Membrane Integration**

A cell surface receptor may not be essential to initiate the biologic actions of the PUFA-derived epoxides and diols because they are able to incorporate into membrane phospholipids and potentially influence cell signaling and biologic responses by modulating the lipid microenvironment therein (Kitson et al., 2012). Certainly, the fish oils EPA and DHA are readily incorporated into phospholipids and the resulting polyunsaturated phospholipids are able to infiltrate lipid rafts as well as form nonraft domains (Williams et al., 2012). DHA, from which 19,20-dihydroxydocosapentaenoic acid (19,20-DHDP) is derived, is particularly interesting in this respect because it tends to incorporate more readily into lipid rafts than EPA (Williams et al., 2012).

There is also evidence that EETs and DHETs can be incorporated into the sn-2 position of phospholipids, especially phosphatidylcholine and phosphatidylinositol phospholipids (Capdevila et al., 1987; Karara et al., 1991; VanRollins et al., 1996; Nakamura et al., 1997; Fang et al., 2003). In endothelial cells, the incorporation of EETs into a phospholipid pool is reported to be catalyzed by acyl coenzyme synthase (Weintraub et al., 1997), and a similar protein kinase C–modulated phenomenon has been described in astroglial cells (Shivachar et al., 1995). This contrasts with the report that EETs can be generated nonenzymatically from EET-containing phospholipids by free radical oxidation (Nakamura et al., 1997). Although the physiologic relevance of these processes remains to be determined, preloading isolated porcine coronary arteries with EET and DHET has been shown to enhance endothelium-dependent, but not endothelium-independent, relaxation. Such observations suggest that these esterified lipids are an intracellular storage form of EET, from which they can be liberated upon cell activation, independently of P450 activity (Weintraub et al., 1997). Whether the various stereo/regioisomers are incorporated differently into membrane lipids remains to be determined. Perhaps addition to membrane phospholipids is a more likely mechanism of action of the PUFA-derived diols and can account for the exclusion of presenilin-1 and cholesterol from lipid rafts after exposure to 19,20-DHDP, but not 19,20-EDP (Hu et al., 2014).

It is also feasible that PUFA epoxide or diol-containing phospholipids can be metabolized by other enzymes to generate a new class of lipid mediators. There is a precedent for such reactions because both the kidney and spleen can produce 2-epoxyeicosatrienoylglycerols (2-EGs) that contain 11,12-EET or 14,15-EET (Chen et al., 2007). 2-(14,15)-EG is reported to stimulate the proliferation of renal proximal tubule cells by causing the release of ligands that activate the epidermal growth factor receptor (EGFR) (Chen et al., 2007), and this novel group of P450-dependent metabolites of arachidonic acid were reported to activate cannabinoid receptors CB1 and CB2 with high affinity to elicit biologic effects in vitro and in vivo (Chen et al., 2008). Although the exact mechanisms by which the EETs elicit their biologic effects remain unclear, the 2-EGs are the first reported P450-dependent arachidonic acid metabolites that can activate G protein–coupled cell membrane receptors and suggest a functional link between the P450/sEH axis and the endocannabinoid system.

**IV. Physiology and Pathophysiology**

**A. Cytochrome P450–Derived Mediators in the Vasculature**

It is relatively easy to specifically target P450 enzymes in experimental animals or isolated arteries to demonstrate the importance of P450 enzymes and the sEH for the regulation of vascular tone. However, the evidence for a vasodilator role of these epoxides in humans is less direct and relies on the use of P450 inhibitors that cannot be guaranteed to be completely selective. Compounds such as sulfaphenazole, which is one of the most selective inhibitors available for CYP2C9 (Khojasteh et al., 2011), have revealed an important role for P450 metabolites in vascular homeostasis under certain conditions. Although several studies failed to demonstrate any effects of sulfaphenazole on forearm vasodilatation in healthy subjects (Michaelis and Fleming, 2006), components of the flow-induced vasodilatation of skeletal muscle arterioles (Hillig et al., 2003) and the radial artery (Bellien et al., 2006a,b; Fischer et al., 2007) were attenuated by the P450 inhibitor. Is there a way to explain this apparent discrepancy? Certainly, the P450 enzymes are not expressed in all endothelial cells and are highest in coronary arteries and in small arterioles. Thus, the net effect of P450 inhibition depends on its expression profile and the contribution of P450-expressing arteries/arterioles to the overall change in blood flow measured. In this respect, it is important to note that the studies reporting a significant effect of sulfaphenazole in healthy subjects looked at specific vessels and confirmed the expression of CYP2C protein in these vessels (Hillig et al., 2003; Fischer et al., 2007). Clearly, disease can affect responses, because forearm vasodilatation to acetylcholine was blunted by P450 inhibitors in patients with hypercholesterolemia (Ozkor et al., 2011) and primary hyperparathyroidism (Virdis et al., 2010). Assessing the contribution of P450s to cardiovascular disease may, however, be hampered by the fact that several routinely prescribed cardiovascular pharmaceuticals, especially the Ca<sup>2+</sup> antagonist nifedipine, and the 3-hydroxy-3-methylglutaryl–CoA reductase inhibitor fluvastatin (Fisslthaler et al., 2000, 2003), can increase vascular P450 expression.
B. Cytochrome P450–Derived Mediators in the Heart

The heart expresses both P450 epoxygenases and \(\omega\)-hydroxylases, meaning that cardiac myocytes can generate EETs as well as 20-HETE from arachidonic acid—metabolites that have contrasting effects on cardiac myocyte function (Seubert et al., 2007). The actions of the EETs are perhaps the best studied because these epoxides are reported to modulate cardiac function via cardiac L-type \(\text{Ca}^{2+}\) currents (Xiao et al., 2004), \(K_v\text{TTP}\) currents (Lu et al., 2006), and even T-type \(\text{Ca}^{2+}\) currents (Cazade et al., 2013)—observations that fit well with the smaller QT prolongation and the less marked ST elevation in isolated murine hearts after perfusion with 14,15-EET (Batchu et al., 2009). Like many other actions of the EETs, the positive effects on cardiac myocytes reportedly relied on the activation of PKA.

CYP2J2, CYP2C8, and CYP2C9 are all highly expressed in the heart and vasculature (Wu et al., 1996; Delozier et al., 2007), but the overall consequence of P450 activation on cardiac function depends of the relative expression of the different isoforms. Indeed, whereas the overexpression of CYP2J2 is generally protective [versus streptozotocin (STZ)–induced diabetic cardiomyopathy (Ma et al., 2013) or doxorubicin-induced cardiotoxicity (Zhang et al., 2009b)], the overexpression of CYP2C8 is deleterious, most probably because of its ability to synthesize reactive oxygen species (Edin et al., 2011). Certainly, the CYP2C inhibitor sulphasphenazole reduces infarct sizes in isolated rat hearts by inhibiting CYP2C-induced oxygen-derived free radical production during reperfusion (Granville et al., 2004). The accumulation of cardiotoxic PUFA metabolites may also affect cardiac function, because this was impaired after CYP2C8 overexpression and linked to increased levels of the linoleic acid diol 9,10-dihydroxyoctadecenoic acid (9,10-DiHOME) (Edin et al., 2011).

Although the classification of increased CYP2J2 expression as protective and increased CYP2C8/CYP2C9 expression as potentially deleterious fits with several in vitro studies, the situation is not simple because although cardioprotection against ischemia-induced dysfunction was observed in young mice overexpressing CYP2J2, the effect was lost in older (11–13 months) animals. Because protection was recovered after the administration of an sEH inhibitor, the loss of protection may have been related to an age-related increase in the expression of the sEH or the accumulation of a cardiotoxic metabolite. The latter seemed to be the case because the older transgenic animals demonstrated increased levels of 9,10-DiHOME, which was accompanied by increased oxidative stress (Chaudhary et al., 2013b). Whether levels of CYP2C epoxygenases were increased in the affected animals was not determined.

Early stages of diabetic cardiomyopathy are associated with increased sEH expression and activity (Dewey et al., 2013), and there are a wealth of reports demonstrating the beneficial effects of sEH inhibitors in different models of cardiac hypertrophy and failure (for review, see Alsaaad et al., 2013). sEH inhibition not only affects cardiac myocyte function, but also prevents the adverse cardiac remodeling that occurs after myocardial infarction that can eventually lead to heart failure (Sirish et al., 2013). Although these effects were linked to the attenuated proliferation of cardiac fibroblasts and reduced fibroblast infiltration into the heart, which are partly attributable to decreased production of proinflammatory cytokines and chemokines (Sirish et al., 2013) and the downregulation of fibroblast growth factor (FGF)-2 (Zhang et al., 2014), more exact molecular mechanisms are unknown. Such observations may imply a role for PUFA epoxides or diols in the process of endothelial–mesenchymal transition but this has not yet been investigated directly. A further interesting effect of sEH deletion on cardiac cells relates to the expression of caveolar structural proteins or caveolins. There is one report of caveolin-1 and caveolin-3 levels being decreased after ischemia in wild-type mice, a phenomenon that was accompanied by the loss of caveolae and damage to mitochondrial cristae. These effects were attenuated in sEH–/– mice as well as in wild-type mice given a sEH inhibitor (Chaudhary et al., 2013a). Again, the molecular mechanisms responsible for the maintenance of caveolar structure and caveolin expression are not known.

Although more information is available on the molecular actions of the arachidonic acid epoxides, it may well turn out that the epoxides of EPA and DHA are more important for cardioprotection. There are numerous indications that the fish oils have beneficial effects on health and insulin sensitivity. For example, the EPA-derived metabolite 17,18-EEQ and the DHA-derived 19,20-EDP are highly active antiarrhythmic agents in neonatal rat cardiomyocytes (Arnold et al., 2010; Falck et al., 2011; Westphal et al., 2011). Although a similar effect of preoperative supplementation of \(\omega-3\) PUFAs was reported on postoperative atrial fibrillation after heart surgery in humans (Haberka et al., 2011; Moertl et al., 2011; Costanzo et al., 2013), these findings were recently questioned (Mozaffarian et al., 2012; Mariani et al., 2013; Fares et al., 2014).

C. Regulation of Blood Pressure

There are a number of mechanisms by which EETs can potentially affect blood pressure. These include direct effects on the vasculature and the regulation of renal sodium excretion by affecting the expression and/or activity of ion channels such as the BK channels, inwardly rectifying K channels (Wang et al., 2008), or \(\text{ENaC}\). Working out which P450 does what is not easy because there are major differences in the P450 isoforms expressed by mice and humans. In animals, the genetic deletion of one P450 isoform frequently leads to the
up- or downregulation of a second P450 enzyme that may play a more significant role in the phenotype observed than the enzyme originally targeted. For example, the disruption of Cyp4a14 led to male-specific hypertension that was linked to androgen-mediated increases in renal CYP4A12 protein levels, as well as the increased generation of prohypertensive 20-HETE biosynthesis and increased renal vascular resistance (Holla et al., 2001). On the other hand, targeting Cyp4a10 resulted in dietary salt-sensitive hypertension, which was unrelated to differences in 20-HETE formation between wild-type and knockout mice but was related to decreased expression of the Cyp2c44 epoxygenase (Nakagawa et al., 2006).

There is a phenomenal amount of data demonstrating the importance of the P450/sEH axis in renal physiology and the ability of EETs to increase renal blood flow and the glomerular filtration rate as well as to decrease renal vascular resistance, as reviewed extensively elsewhere (Imig and Hammock, 2009; Elmarakby, 2012; Capdevila and Wang, 2013). In the kidney, EETs and 20-HETE are also produced in the proximal tubule, the thick ascending loop of Henle, and the cortical collecting duct, where they modulate ion transport. Thus, in addition to their actions on the kidney vasculature, arachidonic acid metabolites are also effective regulators of sodium excretion.

Cyp2c44 is of particular interest because it is thought to be the predominant epoxygenase in the mouse kidney and is closely related to the human CYP2C8 and CYP2C9 isoforms (Capdevila and Wang, 2013). It is expressed in the cortical collecting duct and plays a role in the regulation of blood pressure by inhibiting the ENaC (Sun et al., 2012b). The genetic deletion of Cyp2c44 blunts the inhibition of the ENaC to cause salt-sensitive hypertension and although arachidonic acid is a poor Cyp2c44 substrate, 11,12-EET was reported to inhibit the ENaC in wild-type and Cyp2c44−/− mice (Sun et al., 2012b). At the molecular level, it seems that the inhibition of the ENaC by 11,12-EET is linked to protein phosphatase 2A (Capdevila and Wang, 2013), cAMP, the activation of ERK1/2, and the subsequent threonine phosphorylation of the ENaCα subunit (Pútkovka et al., 2013; Capdevila et al., 2014). It is not known whether other Cyp2c44-derived epoxides of linoleic acid or DHA (which are better substrates for the enzyme) can exhibit similar effects. However, a diet rich in EPA/DHA was reported to downregulate the angiotensin II–induced increase in the mRNA encoding the ENaCα subunit (Ulu et al., 2013). The latter effects were even more pronounced when the diet was combined with sEH inhibitor treatment and linked to lower blood pressure and the attenuated expression of other inflammatory genes such as the 5-LOX and the increased expression of the anti-inflammatory angiotensin-converting enzyme-2 (Ulu et al., 2013).

Endothelial cells also express ENaC and endothelial epithelial sodium channel (ENaC) have been classed as a major regulator of mechanical stiffness and the production of the endothelium-derived NO (Kusche-Vihrog et al., 2014). To our knowledge, nothing is known about the ability of 11,12-EET to affect ENaC channels or endothelial cell stiffness, but the fact that EETs have been attributed to vasodilator and antihypertensive properties would fit with their ability to attenuate ENaC activation and thus increase NO production. Little is known about the mechanisms and pathways that regulate Cyp2c44 protein expression in disease, but the enzyme is downregulated in inflammatory conditions (Theken et al., 2011) and upregulated by hypoxia (unpublished observation). Whether cardiovascular diseases are associated with altered Cyp2c44 expression is unknown.

There are as yet unexplored potential links between P450 metabolites and hormones that regulate obesity-associated hypertension because the ENaC is also targeted by the potent orexigenic peptide ghrelin in the cortical collecting duct through cAMP-dependent trafficking of the channel (Kemp et al., 2013). In obesity, ghrelin levels are increased and as the peptide stimulates appetite and food intake together with renal sodium absorption, it may contribute to the increase in blood pressure that is frequently associated with obesity. It would be interesting to determine whether the antihypertensive actions of sEH inhibition in diet-induced obesity (Iyer et al., 2012) are also related to effects on ENaC or ENaC activity.

Perhaps the most convincing evidence for a role of the P450/sEH axis in the regulation of blood pressure was the report that male animals lacking the sEH demonstrate an elevated circulating level of EETs and a significantly lower blood pressure (Sinal et al., 2000). It should be pointed out, however, that sEH inhibitors rarely affect basal blood pressure in healthy male or female animals (Jung et al., 2005), and the hypotensive phenotype initially observed in sEH−/− mice slowly disappeared with continual backcrossing into the C57Black/6 genetic background (Luria et al., 2007). The latter phenomenon may be linked to a shift in renal metabolism toward 20-HETE and LOX-derived hydroxylation and prostanooid production as metabolic compensation for the loss of the Ephx2 gene. Experiments planned to study the consequences of the inducible knockdown of the sEH in adult mice should help to clarify the situation and the importance of sEH substrates and metabolites in the regulation of basal blood pressure.

Effects on basal blood pressure aside, sEH inhibitors are very effective in the treatment of hypertension associated with activation of the renin-angiotensin system (for review, see Imig, 2010). This fact is explained by the finding that angiotensin II is a potent inducer of sEH expression the rat vasculature (Ai et al., 2007) as well as the rat heart (Ai et al., 2009).
D. Inflammation and Resolution of Inflammation

Interest in the role of the PUFA epoxides as anti-inflammatory mediators increased after 11,12-EET or the overexpression of CYP2J2 was reported to attenuate the upregulation of vascular cell adhesion molecule-1 induced by a series of inflammatory mediators in vitro and in vivo (Node et al., 1999). At the molecular level, these effects were attributed to the inhibition of the IkB kinase and the subsequent degradation of IkBa, meaning that the NFkB subunit Rel A remained bound to IkBa and was thus unable to translocate to the nucleus. Interestingly, these effects were selectively induced by 11,12-EET, whereas 14,15-EET was ineffective—potentially indicating that the effects may be EET-receptor mediated. Since the initial report, the overexpression of CYP2J2 has been linked with an impressive list of anti-inflammatory activities that includes the activation of PPARα (Wray et al., 2009), inhibition of cardiac apoptosis and cardiac dysfunction (Zhao et al., 2012), the inhibition of endoplasmic reticulum stress in heart failure (Wang et al., 2014), and the initiation of a protective autophagic response limiting mitochondrial dysfunction and reducing cellular death (Samokhvalov et al., 2013). However, not all P450 enzymes were created equal, because although the overexpression of CYP2J2 (for which arachidonic acid is a relatively poor substrate) attenuates adhesion molecule expression, the overexpression of CYP2C8 or CYP2C9 (which more readily metabolize arachidonic acid) in endothelial cells results in exactly the opposite (i.e., increased adhesion molecule expression) (Fleming et al., 2001). This may imply that PUFA mediators derived from lipids other than arachidonic acid may account for some of the anti-inflammatory effects observed, especially in in vivo models, or that there are other major differences in enzyme activity that are of physiologic consequence. There is evidence to support both of these possibilities. Certainly, the already mentioned generation of reactive oxygen species by CYP2C8 and CYP2C9 is able to override any direct anti-inflammatory action of the EETs generated at the same time, at least as far as NFkB activation and adhesion molecule expression are concerned (Fleming et al., 2001).

The anti-inflammatory actions of sEH inhibitors have been largely attributed to increased tissue and circulating levels of PUFA epoxides, such as the EETs. It is also important to note, however, that there are some situations in which sEH inhibition cannot be expected to have much of a beneficial effect. These include conditions associated with chronically reduced tissue oxygen levels, which in turn decrease sEH promoter activity and gene expression (Keserü et al., 2010), as well as poorly controlled diabetes, in which peroxynitrite production results in the tyrosine nitration of the active site of the sEH and thereby results in its inhibition (Barbosa-Sicard et al., 2009).

Although research focused for a considerable time on the anti-inflammatory actions of the P450-derived metabolites of arachidonic acid, it has now become clear that bioactive lipids generated from other PUFAs also possess anti-inflammatory activity (for recent comprehensive reviews, see Konkel and Schunck, 2011; Westphal et al., 2011). Some of these effects are likely related to direct intracellular actions and some to actions at the cellular level e.g., on the mobilization of the bone marrow–derived Lin− Sca-1+cKit+ cells that give rise to neutrophils and monocytes (Frömel et al., 2012). Certainly, the inflammation-induced tissue infiltration of lymphocytes, neutrophils, and macrophages can be reduced by sEH inhibition (Smith et al., 2005; Manhiani et al., 2009; Zhang et al., 2009a; Revermann et al., 2010; Deng et al., 2011). However, elucidating which lipid mediators regulate which process in vivo is complicated and the evidence available to date points to a potential role of arachidonic acid and linoleic acid diols, rather than PUFA epoxides (Frömel et al., 2012).

DHA- and EPA-derived P450 metabolites play a role in cardiac protection (Westphal et al., 2011) but what about inflammation? The answer is currently unclear because when talking about fish oils and the resolution of inflammation, it is not epoxides or diols but so-called resolvins, protectins, and maresins that immediately come to mind (Serhan, 2011; Serhan and Petasis, 2011). No single enzyme can generate the resolvins whose synthesis has been attributed to two sequential oxygenation reactions. The first reaction is oxygenation by enzymes such as COX2 or 15-LOX, whose expression is induced during inflammation. The second reaction is thought to be catalyzed by 5-LOX, which is abundant in infiltrated cells in inflamed tissues. This complicated synthetic relationship accounts for the global inability to detect resolvins under normal conditions.

Is there any potential link between the resolvins/protectins and the P450/sEH axis? Not a lot of work has been done in this area to our knowledge, but it is striking that hypoxia is generally required for resolvin generation—a situation that results in the upregulation of P450 enzymes and the simultaneous down-regulation of the sEH. Moreover, three murine P450 mono-oxygenases (Cyp1a1, Cyp1a2, and Cyp1b1) have been implicated in the generation of protectin D1 at least during peritonitis (Divanovic et al., 2013). There is additional indirect evidence implicating P450 oxygenases in the first reaction step because a nonselective P450 inhibitor was found to reduce the synthesis of the lipoxin precursor 15-HETE in hepatocytes, whereas the P450 inducer phenobarbital increased its production (Titos et al., 1999). In addition, the genetic deletion or the long-term inhibition of the sEH results in a marked increase in plasma levels of 5-LOX and 15-LOX–derived metabolites that are required for resolvin...
production (Jung et al., 2010). However, the latter effects were not linked to the resolution of inflammation; however, an increase in its severity and the coadministration of a sEH inhibitor together with COX or LOX inhibitors enhanced their anti-inflammatory effects (Liu et al., 2010b). Although the topic is of interest, the elucidation of possible biosynthetic pathways and an estimation of the anti-inflammatory potential of these novel lipid mediators is hampered by widespread difficulties in detecting their existence in vitro as well as in vivo (Balas et al., 2014).

**E. Angiogenesis, Tumors, and Atherosclerosis**

1. **Angiogenesis.** Angiogenesis is a tightly regulated and organized process and although numerous studies have addressed the role of specific proteins at the different stages of vascular development (Potente et al., 2011), the role of lipids is less clear. Although COXs and prostaglandins have been the focus of some studies (Salvado et al., 2012), little is known about the role of P450-derived lipid mediators.

   a. **Cytochrome P450.** The first link between P450 enzymes and angiogenesis was obtained in cocultures of astrocytes and endothelial cells in which EETs released from astrocytes increased endothelial cell proliferation and elicited the formation of capillary-like structures (Munzenmaier and Harder, 2000; Zhang and Harder, 2002). In addition, the overexpression of the CYP2C9 epoxygenase in and/or the application of 11,12-EET or 14,15-EET to monocultures of endothelial cells was associated with angiogenesis (Medhora et al., 2003; Michaelis et al., 2003). In vivo data rapidly followed to support these in vitro findings, with EETs reported to induce angiogenesis in the chick chorioallantoic membrane (Michaelis et al., 2003) and the vascularization of Matrigel plugs implanted into wild-type mice (Medhora et al., 2003; Webler et al., 2008a). Moreover, the overexpression of the human CYP2C11 and CYP2J2 enzymes in the ischemic rat hindlimb model was found to increase muscle capillary density (Wang et al., 2005). Such studies could not address the importance of endogenously generated P450 metabolites and were difficult to back up in knockout models because there are major differences in P450 isoform expression between species. This is particularly true for the CYP2C family of proteins, which have been most frequently linked to angiogenesis.

   More detailed analysis of the mechanisms involved in P450-induced angiogenesis therefore focused on the EETs and their ability to transactivate the EGFR (Chen et al., 2002; Michaelis et al., 2003). 14,15-EET was initially suggested to act as a second messenger after activation of the EGFR, however it appears that 14,15-EET can also elicit the release of heparin-binding EGF-like growth factor from a renal epithelial cell line via a process involving the activation of matrix metalloproteases (MMPs) (Chen et al., 2002). Although the MMP involved was not identified, a similar mechanism was responsible for the transactivation of the EGFR in endothelial cells that linked 11,12-EET with the EGFR followed by the activation of the kinase Akt and an enhanced expression of cyclin D1 (Michaelis et al., 2003). Since the early reports, all four EET regioisomers have now been reported to elicit an increase in Akt phosphorylation and cell proliferation in murine endothelial cells but only the proliferative effects of 5,6-EET and 14,15-EET are reportedly sensitive to a PI3-K inhibitor, whereas the 8,9-EET– and 11,12-EET–induced increase in \(^{3}H\)thymidine incorporation seems to be dependent on the activation of the p38 mitogen-activated protein kinase (Pozzi et al., 2005). By contrast, in bovine aortic endothelial cells, 8,9-EET–, 11,12-EET–, and 14,15-EET–induced cell proliferation can be attenuated by MEK, ERK and PI3-K inhibition (Wang et al., 2005). Other signaling pathways also contribute to the increase in cyclin D1 expression including the mitogen-activated protein kinase phosphatase-1, which decreases c-Jun N-terminal kinase (JNK) activity (Potente et al., 2002). The activation of Akt by EETs also induces the phosphorylation and therefore inhibition of the forkhead factors FOXO1 and FOXO3a and subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor p27\(^{kip1}\) (Potente et al., 2003). The involvement of this mechanism into the CYP2C9-induced endothelial cell proliferation could be demonstrated by the transfection of CYP2C9-overexpressing cells with either a dominant negative Akt or a constitutively active FoxO3a, which both inhibit CYP2C9-induced endothelial cell proliferation (Potente et al., 2003). Although there is a precedent for the negative regulation of JNK after activation of Akt, inasmuch as Akt has been reported to phosphorylate and inactivate the kinase SEK1 and thus inactivate its substrate JNK (Park et al., 2002), it remains unclear whether these pathways are linked to each other or are simply activated in parallel.

   The majority of the latter studies were performed using cultured cells and either the exogenous application of EETs, P450 inhibitors, or in cells overexpressing the epoxygenase of interest. As a consequence, there was relatively little evidence linking endogenous EET production with proliferation and angiogenesis. However, it is now clear that hypoxia-induced angiogenesis, which is largely attributed to the activation of hypoxia-inducible factor 1\(\alpha\) and increased production of vascular endothelial cell growth factor (VEGF) (Levy, 1995), also involves P450 activation and increased EET production. Indeed, both hypoxia and VEGF are effective inducers of P450 enzyme expression in human endothelial cells (Michaelis et al., 2005; Webler et al., 2008b) and the “EET antagonist” 14,15-EEZE [14,15-epoxyeicosa-5(Z)-enoic acid] almost abolished VEGF-induced endothelial tube formation in vitro and in vivo.
The latter effect was specific for VEGF-induced vascularization of Matrigel plugs and angiogenesis induced by basic FGF was unaffected. Similarly targeting the Cyp2c44 in murine endothelial cells blocks cellular responses to VEGF (Yang et al., 2009). P450 activity may in turn also affect VEGF expression as at least in human dermal microvascular endothelial cells, 14,15-EET was is reported to stimulate the tyrosine phosphorylation of STAT3 and its translocation from the cytoplasm to the nucleus as well as the subsequent increase in VEGF levels (Cheranov et al., 2008).

Is there any differentiation between arterial and venous sprouting regarding the effects of the epoxides? There is circumstantial evidence that the EETs tend to promote venous differentiation inasmuch as 1) EphB4, a classic venous marker (Gerety et al., 1999; Shin et al., 2001), is upregulated in native and cultured endothelial cells exposed to 11,12-EET, and 2) EET-induced angiogenesis in vivo can be attenuated by the small interfering RNA–mediated downregulation of EphB4 (Webler et al., 2008a). In addition, the arterial vasculature is almost normal in zebrafish embryos that lack the sEH, whereas major defects in the zebrafish trunk venous network are evident. The most prominent alteration being the development of an enlarged single channeled caudal vein and lack of the caudal venous plexus (Frömel et al., 2012).

Is there a negative side to the enhancement of angiogenesis by P450 epoxides? This may well be the case, especially when considering the reports that link increased EET production or reduced EET metabolism with cancer metastasis (see below). A recent report also links the Tie2-driven overexpression of the human CYP2C8 isoform with retinal pathologic neovascularization in mice (Shao et al., 2014). Although not definitively shown, it was suggested that the CYP2C8 expressed in monocytes/macrophages was upregulated during oxygen-induced retinopathy, whereas the sEH was suppressed to result in an increased retinal epidioxide/diol ratio. Interestingly, retinal neovascularization increased when animals were given a ω-3 PUFA–rich diet, whereas there was no effect of the diet in animals maintained under normoxic conditions (Shao et al., 2014). Apart from emphasizing the importance that diet has on the balance of lipid mediators generated by the sEH axis, this report underscores the need to carefully screen lipid profiles in different tissues before jumping to conclusions about which of the P450-derived lipid mediators are the most bioactive.

b. CYP1B1. CYP1B1 is worth mentioning at this point even though the enzyme is an estrogen-metabolizing P450 hydroxylase. CYP1B1 induction is an important factor in determining risk associated with hormone-mediated cancers, particularly because CYP1B1 is induced by hypoxia (Kirwan et al., 2012); this is probably because its expression is regulated by the AMP-activated protein kinase (AMPK) (Dallaglio et al., 2014) and is involved in the metabolism of some clinically relevant anticancer agents (Sissung et al., 2006).

What makes CYP1B1 of interest in angiogenesis is that its deletion impaired revascularization in a model of oxygen-induced retinopathy in mice (Tang et al., 2009). This effect was linked with a decrease in the expression of eNOS (Tang et al., 2010), as well as a corresponding increase in intracellular oxidative stress and increased production of thrombospondin-2, an endogenous inhibitor of angiogenesis (Tang et al., 2009; Palenski et al., 2013). Interestingly, estrogen-induced angiogenesis has also been attributed to changes in eNOS, thrombospondin, and free radical generation, making it tempting to speculate that CYP1B1 may actually mediate the effects of the hormone. Certainly, the CYP1B1-derived metabolites of β-estradiol promote angiogenesis in uterine artery endothelial cells (Jobe et al., 2010). Rather intriguingly, residues 41–48 of human CYP1B1 are part of a mitochondrial import signal and the cleavage of CYP1B1 by serine proteases results in its targeting to mitochondria, which is associated with oxidative stress and mitochondrial dysfunction (Bansal et al., 2014). Given that angiogenic endothelial cells undergo changes in metabolism (the so-called Warburg effect) (Polet and Feron, 2013), it will be interesting to determine whether CYP1B1 can also alter endothelial cell metabolism and mitochondrial function. Effects on CYP1B1 may also explain the antiangiogenic actions of the antidiabetic drug metformin, which prevents the tumor cell supernatant–induced upregulation of CYP1B1 in endothelial cells (Dallaglio et al., 2014).

c. Soluble epoxide hydrolase. The majority of studies addressing P450 enzymes and angiogenesis have concentrated on the molecular actions of the EETs, and largely ignored other ω-6 and ω-3 PUFA epoxides. One way around these problems is to address the consequences of inhibiting the activity or decreasing the expression of the sEH on a biologic response and elucidating the lipid mediator responsible with the help of liquid chromatography coupled to tandem mass spectrometry–based lipid profiling. Exactly such an approach was used to study the role of PUFA epoxides and diols in postnatal retinal angiogenesis and revealed that the genetic deletion and inhibition of the sEH activates Notch signaling and attenuates sprouting angiogenesis in the murine retina. Mechanistically these effects could be attributed to the lack of the sEH product and DHA diol 19,20-DHDP (Hu et al., 2014). Moreover, 19,20-DHDP was able to rescue the defective angiogenesis in sEH−/− mice as well as in animals lacking the Fbxw7 ubiquitin ligase, which demonstrate strong basal activity of the Notch signaling cascade. In these animals, the parent epoxide (19,20-EDP) was without any apparent effect but may even contribute to the phenotype observed because DHA-derived EDPs...
have been reported to attenuate VEGF-induced angiogenesis by interfering with the agonist-induced phosphorylation of VEGF receptor 2 (Zhang et al., 2013a).

Interestingly, in the murine retina, the major site of sEH expression was not the endothelium but Müller glia cells. These cells develop and maintain close contact with both superficial vessels and deeper capillaries via their multiple end feet (Newman and Reichenbach, 1996) and have been implicated in angiogenesis by virtue of their ability to produce angiogenic substances in response to hypoxia (Pierce et al., 1995; Stone et al., 1995; Robbins et al., 1997). Given that the Müller cell-specific deletion of VEGF-A inhibited neovascularization in a mouse model of oxygen-induced retinopathy without affecting physiologic vascularization or retinal morphology (Bai et al., 2009), it was assumed that Müller cells play a greater role in proliferative retinopathy than physiologic angiogenesis. However, the deletion of the sEH in Müller cells clearly affected endothelial cell proliferation as well as Notch signaling and tip cell filopodia formation in mice, indicating that Müller cells make a more important contribution to retinal angiogenesis than generally appreciated. Mechanistically, 19,20-DHDP was found to promote angiogenesis by inducing the redistribution of presenilin 1 from lipid rafts, thus inhibiting the γ-secretase and Notch signaling (Hu et al., 2014).

Attenuated PUFA diol levels, rather than elevated epoxide levels, have also been linked to defects in the formation of the caudal vein plexus in zebrafish, a complex vessel network that originates from the single channeled caudal vein 24–48 hours postfertilization and serves as a transient hematopoietic tissue in which hematopoiesis and angiogenesis occur in a close physical proximity (Murayama et al., 2006). In zebrafish, the consequences of sEH inhibition or deletion could be rescued by the application of 12,13-DiHOME or to a lesser extent by 11,12-DHET, whereas the corresponding epoxides were without effect (Frömel et al., 2012). A major consequence of the lack of diol production was a decrease in the generation of a subpopulation of hematopoietic cells resulting in fewer cmyb/lmo2 double-positive cells being detected within the caudal vein plexus. The latter findings could also be transferred to the mouse, in which the sEH was detected in bone marrow–derived hematopoietic progenitor cells (i.e., Lin−cKit−) cells, which were able to generate 9,10-epoxyoctadecanoenoic acid (EpOME) and 11,12-EpOME/DiHOME as well as 11,12-EET/DHET and 14,15-EET/DHET. To parallel the studies in zebrafish, the ability of the sEH to influence the formation of short-term repopulating hematopoietic progenitor cells colonies was assessed in spleens from irradiated wild-type animals. Again, solvent-treated bone marrow cells from sEH−/− mice formed significantly fewer colonies than cells from wild-type mice, whereas incubation of cells from sEH−/− mice with 11,12-DHET or 12,13-DiHOME effectively restored the colony-forming ability of the cells. Given that genetic deletion as well as inhibition of the sEH attenuated progenitor cell proliferation (a process known to be linked to canonical Wnt signaling; Cerdan and Bhatia, 2010), a potential link between the epoxides and diols and β-catenin activity was studied. It turned out that 12,13-DiHOME and 11,12-DHET, but not 11,12-EET or 12,13-EpOME, were able to induce the nuclear translocation of β-catenin in Lin− bone marrow cells. Lin−Sca-1+cKit+ cells give rise to neutrophils and monocytes, which contribute to angiogenesis. Thus when the vascularization of an acellular matrix was assessed 4 days after implantation into mice treated with the bone marrow cell mobilizing agent granulocyte colony stimulating factor, significantly more endothelial cells (CD31+) and pericytes/smooth muscle cells (α-actin+) were recovered from the wild-type than sEH−/− mice. These observations have clear implications for vascular repair because the recovery of blood flow and vascularization after hindlimb ischemia was attenuated in sEH−/− mice. The latter phenotype was found to be bone marrow cell–dependent because transplantation with wild-type bone marrow normalized vascular repair in sEH−/− animals, as did the infusion of 12,13-DiHOME (Frömel et al., 2012). Such studies revealed a previously uncharacterized interaction between the P450/sEH pathway and hematopoietic progenitor cell proliferation, mobilization, and their subsequent role in vascularization. Thus, it appears that the fatty acid diols affect hematopoietic stem cells by a mechanism similar to that of eicosanoids such as prostaglandin E2 (PGE2) and 15-HETE (i.e., via the stabilization of β-catenin) (North et al., 2007; Chen et al., 2009b; Goessling et al., 2009; Kinder et al., 2010).

2. Tumors. Many of the compounds that promote angiogenesis have also been implicated in tumor development; the same is true for the PUFA epoxides. In fact, several different tumor cell types have been shown to express P450 proteins (e.g., CYP2J2 and CYP2C) and to generate EETs, which in turn accelerate tumor cell proliferation in vitro and protect against tumor necrosis factor-α–induced apoptosis (Yokose et al., 1999; Jiang et al., 2005; Chen et al., 2009a, 2011a). Indeed, terfenadine-related inhibitors of CYP2J2 exhibit strong antitumor activity in vitro and in vivo (Chen et al., 2009a; Xu et al., 2011). The expression of different P450 isoforms is highly variable between different individuals and different tumors, with CYP2C9 being the most abundantly expressed epoxygenase in several human malignant neoplasms (Enayetallah et al., 2006) and esophageal adenocarcinoma (Schmelzle et al., 2011). Making conclusions about the role of P450s in human cancer development is hampered by the fact that P450 expression can be affected by exogenous factors including the drugs used.
to treat the disease. Many of these compounds (e.g., paclitaxel and tamoxifen) are metabolized by CYP2C enzymes and thus may induce enzyme expression the phenomenon of “substrate induction” at the same time these compounds could potentially decrease epoxide-producing activity by competing with endogenous P450 substrates (for review, see Xu et al., 2011). What about the sEH? Because the enzyme is downregulated when the pO2 drops (Keserü et al., 2008), the sEH is likely to be downregulated in tumors, especially those with a hypoxic core. Indeed, sEH expression was reported to decrease in renal and hepatic tumors (Enayetallah et al., 2006), thus essentially increasing PUFAs epoxide levels and angiogenic and eventually metastatic potential.

Although many actions of the EETs on angiogenesis can contribute to tumor growth, it seems that additional mechanisms and indeed additional enzymes can be expected to be implicated in cancer cell proliferation. For example, CYP3A4 and CYP2C8 (both of which generate EETs) expression is increased in breast cancer and correlates with decreased overall survival. Both proteins are also reported to be specifically required for the growth of a series of breast cancer lines. EETs have been attributed to effects in solid tumors as well as in malignant hematologic diseases, and CYP2J2 was recently detected in five human-derived malignant hematologic cell lines and in leukemia cells from peripheral blood and bone marrow in 86% of patients with malignant hematologic diseases. Increased EET generation was linked, as in endothelial cells, to activation of the AMPK, JNK, and PI3-K/Akt signaling pathways, as well as increased EGFR phosphorylation levels (Chen et al., 2011a). However, whereas 11,12-EET is the isoform most frequently linked to endothelial cell angiogenesis, 14,15-EET was required for tumor cell proliferation and mechanistically attributed to Stat3 phosphorylation (Mitra et al., 2011).

In addition to potentially promoting tumor growth and tumor angiogenesis, EETs (generated as a consequence of the overexpression of P450 enzymes) have also been linked to increased tumor metastasis (Jiang et al., 2007; Panigrahy et al., 2012). The increased rate of metastasis has been linked to a number of different processes including the upregulation of VEGF, CD44, and prometastatic MMPs and downregulation of the antiangiogenic factor thrombospondin-1 and the antimetastatic genes CD82 and nm-23 (Jiang et al., 2007; Panigrahy et al., 2012). It therefore follows that P450 inhibitors may be potential antitumor agents. Certainly, CYP2J2 inhibitors have been shown to repress tumor growth in vitro and in vivo as well as to decrease lung metastasis (Chen et al., 2009a; Panigrahy et al., 2012). Perhaps a more selective approach is to target the actions of specific EET regioisomers, and at least three EET antagonists have been reported to inhibit EET-induced cell invasion and migration as well as increased EGFR phosphorylation and Akt activation (Nithipatikom et al., 2010). Dietary intervention to shift the ω-6/ω-3 PUFA balance may also be a useful approach as DHA-derived epoxides (i.e., the EDPs inhibit angiogenesis, primary tumor growth, and metastasis) (Zhang et al., 2013a).

3. Atherosclerosis. It may seem strange to talk about atherosclerosis in a section immediately after angiogenesis but there are strong links between the two processes. Generally, signaling pathways that promote angiogenesis also affect atherosclerosis. There are also hints that disturbed levels of EETs, DHETs, and 20-HETE are associated with the severity of vascular dysfunction in humans with coronary artery disease (Theken et al., 2012b; Schuck et al., 2013). Added to this is the interest in the potential benefits of supplemental and/or dietary ω3 PUFAs in disease prevention, particularly in the prevention of atherosclerotic plaque formation and the stabilization of the rupture-prone “vulnerable” plaque (for review, see Fares et al., 2014).

a. CYP2C. P450 enzymes and cardiovascular disease are potentially linked at two levels. The first relates to the role of EETs in the regulation of vascular homeostasis (described above) and indicates that P450 enzymes may influence the pathogenesis or progression of disease. Although there is currently little information available regarding alterations in P450 levels in human disease, epoxygenase levels are linked to hypertension in the spontaneously hypertensive rat (Spector and Norris, 2007), and there have been some links (however tentative) between P450 polymorphisms and cardiovascular disease (Zordoky and El-Kadi, 2010). At least one study indicates that individuals carrying the CYP2C9*3 polymorphism, which is linked with loss of function, are at a lower risk for the development of hypertension (Yu et al., 2004). How does this fit with the concept that P450-derived epoxides are vasodilators? The most likely explanation is that decreased CYP2C activity also results in the decreased generation of reaction by-products such as reactive oxygen species.

Certainly, all P450 enzymes are not created equal. Indeed, although the overexpression of CYP2J2 in coronary arteries generates EETs that attenuate NFκB activation and adhesion molecule expression (Node et al., 1999), the overexpression of CYP2C8/CYP2C9 results in the generation of enough oxygen-derived free radicals to increase NFκB activation and adhesion molecule expression (Fleming et al., 2001) to mask the beneficial effects of the epoxide. This is also linked to attenuated NO-mediated vasodilatation, the development of endothelial dysfunction in animal models as well as humans (Fleming et al., 2001; Fichtlscherer et al., 2004; Sun et al., 2012a; Zhou et al., 2012), as well as myocardial cell apoptosis (Ishihara and Shimamoto, 2012). It is currently unclear why CYP2C8/CYP2C9 generates physiologically relevant amounts of reactive oxygen species that can increase susceptibility to
the characteristics of the two enzymes are such that the biologic consequences of P450 activation. Certainly, the most relevant enzymes remains unclear, as does et al., 2008; Bystrom et al., 2011), but whether these are P450 enzymes in monocytes/macrophages (Nakayama CYP2J2 and CYP2C8 were identified as EET-generating these cells, little is known about the ability of monocyte- and LOX pathways have been extensively studied in integral part of the inflammatory response and the COX

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rate effects of the overexpression of these isozymes on vascular protection (Fleming et al., 2001; Yang et al., 2001). Whether this can account for the reports from blinded clinical trials that high linoleic acid intakes indicate potential deleterious effects on long-term health (Cunnane, 2003) remains to be demonstrated.

As always, with polymorphism studies, conflicting associations have also been reported, for example, because polymorphisms in the CYP2C19 gene (which also generates EETs) have been associated with hypertension (Ma et al., 2011) and a higher possibility of coronary artery disease, especially in combination with smoking (Yang et al., 2010). High-performance liquid chromatography was also recently used to identify six variants of CYP2C8 and detect differences in variant frequencies between controls and hypertensive patients. Nonhypertensives had a significantly higher prevalence of C35322C compared with the hypertensive patients, but the functional significance of the single nucleotide polymorphism requires further study (Teh et al., 2010).

The second aspect that should not be overlooked is that most patients receive one or more pharmacological agents that can in turn affect P450 activity and/or expression. Indeed, the sulfaphenazole-sensitive CYP2C9 that modulates vasodilatation in some situations is second only to CYP3A in the metabolism of marketed drugs (Khojasteh et al., 2005), although the latter reports were poorly quantified and apparently controversial (Wu et al., 2006). However, it now seems that the enzyme can metabolize arachidonic acid to generate EETs, with a slight preference for the 11,12-EET and 14,15-EET regioisomers, as well as linoleic acid and EPA (Frömel et al., 2013). At the functional level, macrophage CYP2S1 expression was increased by lipopolysaccharide and interferon-γ (to generate classically activated or M1 polarized macrophages) and oxidized low-density lipoprotein (LDL) but not by interleukin-4 and interleukin-13 (to generate alternatively activated or M2 macrophages). In agreement with these findings, inflamed human tonsils (which should contain predominantly M1 polarized macrophages) showed a high level of CYP2S1 and CD68 colocalization, whereas alternatively activated macrophages in breast cancer metastases in the human lung expressed no detectable CYP2S1. Oxidized LDL also significantly increased CYP2S1 levels in vitro and the enzyme was present in macrophage foam cells recovered from apolipoprotein E knockout (ApoE−/−) mice fed a high-fat diet as well in aortae from similarly treated animals but not wild-type animals. The latter findings fit well with the previous report that treating monocytes with minimally modified LDL elicited a significant increase in 11,12-EET formation (Honda et al., 1999). In human atherosclerotic plaques, CYP2S1 was colocalized with the monocyte marker CD68 and could be detected in intimal xanthoma, in fibrous cap atheroma, as well as in fibrous cap atheroma with hemorrhage (Frömel et al., 2013).

b. CYP2S1. Despite the fact that monocytes are an integral part of the inflammatory response and the COX and LOX pathways have been extensively studied in these cells, little is known about the ability of monocyte-derived macrophages to generate fatty acid epoxides. CYP2J2 and CYP2C8 were identified as EET-generating P450 enzymes in monocytes/macrophages (Nakayama et al., 2008; Bystrom et al., 2011), but whether these are the most relevant enzymes remains unclear, as does the biologic consequences of P450 activation. Certainly, the characteristics of the two enzymes are such that different biologic reactions would be expected after activation.

A more recent study used a proteomic approach to identify CYP2S1 in microsomes prepared from freshly isolated and cultured CD14+ human blood-derived monocytes (Frömel et al., 2013). The human and mouse isoforms are 78% identical at the amino acid level (Rivera et al., 2002) and in both species, CYP2S1 seems to be the only member of the new 2S family (Saarikoski et al., 2005). Interestingly, available CYP2C antibodies crossreacted with CYP2S1, initially leading to the suggestion that a CYP2C was present in monocytes.

The major substrates for human CYP2S1 have not been fully established but preliminary reports suggested that the recombinant CYP2S1 is involved in the metabolism of retinoic acids (Marill et al., 2003; Smith et al., 2003; Saarikoski et al., 2005) and naphthalene (Karlgren et al., 2005), although the latter reports were poorly quantified and apparently controversial (Wu et al., 2006). However, it now seems that the enzyme can metabolize arachidonic acid to generate EETs, with a slight preference for the 11,12-EET and 14,15-EET regioisomers, as well as linoleic acid and EPA (Frömel et al., 2013). At the functional level, macrophage CYP2S1 expression was increased by lipopolysaccharide and interferon-γ (to generate classically activated or M1 polarized macrophages) and oxidized low-density lipoprotein (LDL) but not by interleukin-4 and interleukin-13 (to generate alternatively activated or M2 macrophages). In agreement with these findings, inflamed human tonsils (which should contain predominantly M1 polarized macrophages) showed a high level of CYP2S1 and CD68 colocalization, whereas alternatively activated macrophages in breast cancer metastases in the human lung expressed no detectable CYP2S1. Oxidized LDL also significantly increased CYP2S1 levels in vitro and the enzyme was present in macrophage foam cells recovered from apolipoprotein E knockout (ApoE−/−) mice fed a high-fat diet as well in aortae from similarly treated animals but not wild-type animals. The latter findings fit well with the previous report that treating monocytes with minimally modified LDL elicited a significant increase in 11,12-EET formation (Honda et al., 1999). In human atherosclerotic plaques, CYP2S1 was colocalized with the monocyte marker CD68 and could be detected in intimal xanthoma, in fibrous cap atheroma, as well as in fibrous cap atheroma with hemorrhage (Frömel et al., 2013).

How does the expression of CYP2S1 alter macrophage function? Although the inhibition and down-regulation of CYP2S1 led to an increased phagocytic uptake of bioparticles, inhibition of the sEH tended to decrease phagocytosis. The anti-inflammatory effects associated with CYP2S1 expression and activity could have been due to the ability of the enzyme to generate an anti-inflammatory factor (e.g., EETs) or the
metabolism/downregulation of an immune modulator factor. One such factor is PGE2 and CYP2S1 was reported to metabolize the PGE2 precursors PGG2 and PGH2 to 12-HHT (12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid) (Bui et al., 2012; Madanayake et al., 2012). Thus, perhaps from the macrophage polarization point of view, the most relevant CYP2S1 substrates may well be PGG2 and PGH2 because the subsequent decrease in the immunomodulator PGE2 would certainly be expected to result in a macrophage subtype with attenuated angiogenic potential (Fig. 4).

c. CYP1B1. This enzyme is again worth mentioning briefly because the sex difference in the hypertensive response to angiotensin II in mice was recently attributed to the differential expression of CYP1B1 (Jennings et al., 2014). Indeed, CYP1B1 as an estrogen-metabolizing enzyme is more highly expressed in female than male mice and angiotensin II caused cardiovascular remodeling and endothelial dysfunction accompanied by oxidative stress in Cyp1b1−/− and ovariectomized Cyp1b1+/− mice but not in Cyp1b1+/+ mice. These protective actions were attributed to the generation of 2-methoxyestradiol and its ability to inhibiting oxidative stress. Whether other cardiovascular disorders, especially those linked with cardiovascular disease, are also associated with altered CYP1B1 activity is not known.

d. Soluble epoxide hydrolase. The sEH has a complicated relationship with cholesterol and sEH−/− mice demonstrate lower levels of plasma cholesterol than wild-type mice (Enayetallah et al., 2008; Luria et al., 2009), a finding apparently explained by reduced hepatic expression of the 3-hydroxy-3-methylglutaryl-CoA, which is rate limiting for cholesterol synthesis (Enayetallah et al., 2008). However, a group of human subjects suffering from familial hypercholesterolemia that was linked to a polymorphism of the Ephx2 gene (Arg287Glu substitution) demonstrated increased, rather than decreased, plasma cholesterol levels (Sato et al., 2004). The apparent contradiction may be explained by the fact that the Arg287Glu polymorphism was linked with a higher isoprenoid phosphatase activity than the wild-type sEH, which may imply that altered N-terminal lipid phosphatase activity rather epoxide hydrolase activity may underlie the phenomenon (Enayetallah and Grant, 2006). The situation is still far from clear but work in cell lines hints that the two catalytic domains of the sEH protein do have opposing effects on cholesterol, with the activity of the phosphatase domain being linked with elevated cholesterol levels and the hydrolase domain being linked to lower cholesterol levels (Enayetallah et al., 2007, 2008). This being the case, it makes sense to expect that sEH inhibitors (which only affect the activity of the C-terminal hydrolase activity) would target the cholesterol-lowering part of this pathway and elicit an increase in plasma cholesterol. The latter is indeed what is observed in vitro using HepG2 cells as well as in vivo in mice (Enayetallah et al., 2008). Whether beneficial effects of C-terminal domain activity are related to the ability of some sEH products (e.g., 19,20-DHDP) to displace cholesterol from lipid rafts (Hu et al., 2014) remains to be determined.

What does all of this mean for the role of the sEH in the development of atherosclerosis and the potential use of sEH inhibitors for the treatment of cardiovascular disease? After all, the population studies have identified links between Ephx2 polymorphism and coronary atherosclerosis (Lee et al., 2006; Wei et al., 2007) as well as stroke (Fornage et al., 2005). In addition, because the sEH is required for the efficient mobilization of bone marrow–derived Lin−Sca-1+cKit+ cells that give rise to neutrophils and monocytes (Frömel et al., 2012), it would make sense to speculate that sEH inhibition may exert an antiatherosclerotic effect. First-generation sEH inhibitors were initially reported to attenuate VSMC proliferation, which led to the suggestion that they may be used to prevent intima proliferation (Davis et al., 2002). However, these findings could not be reproduced using more recently developed sEH inhibitors, or EETs (Davis et al., 2006). In fact, it seems that the VSMCs studied in the first report actually did not actually express the sEH, implying that off-target effects of the early inhibitors were responsible for the decreased VSMC proliferation. More recently, a water-soluble sEH inhibitor was reported to reduce the development of atherosclerosis in ApoE−/− mice fed an atherogenic diet while being simultaneously infused with angiotensin II (Ulu et al.,

Fig. 4. CYP2S1 as a determinant of macrophage polarization. Although CYP2S1 can generate PUFA epoxides, the enzyme is expressed exclusively in classically activated or M1 polarized macrophages in inflamed and atherosclerotic tissues. Alternatively activated or M2 polarized macrophages do not express the enzyme. CYP2S1 may determine polarization by metabolizing the PGE2 precursors PGG2 and PGH2 to 12-HHT, which largely prevents the generation of the important immune modulator factor PGE2. The insert shows immunostaining of human blood-derived monocytes (CYP2S1 in red; actin in green) after 5 days in culture.
Similarly, the angiotensin II–induced development of abdominal aortic aneurysms in ApoE–/– mice was attenuated with a chemically distinct inhibitor (Zhang et al., 2009a). However, other groups have had much more difficulty in demonstrating clear effects; even within one study, differential effects on neointima formation in the femoral artery cuff model versus carotid artery ligation were reported (Revermann et al., 2010). The apparent contradiction in the latter study was attributed to the contrasting roles played by inflammatory cells because the expression of proinflammatory genes was reduced in femoral arteries from sEH–/– mice. It is possible that the two “positive” reports of an antiatherosclerotic effect of sEH inhibition may have been biased by the fact that both relied on the perfusion of angiotensin II, which induces strong vascular inflammation (Marchesi et al., 2008) as well as increases the expression of the sEH in the vascular wall (Ai et al., 2007). Thus, the increase in sEH expression and activity may have exaggerated the importance of the sEH in the development of atherosclerosis seen under “normal” conditions (i.e., without massive activation of the renin-angiotensin system).

*ω*-3 versus *ω*-6 polyunsaturated fatty acids. To date, studies have focused mostly on linking EETs, 20-HETE, and sEH inhibition with inflammation and vascular pathology. Relatively little attention has been paid to the possibility that other PUFA-derived epoxides and diols (which can be regulated by diet) may also play an important role. It is even possible that PUFA substrate availability determines whether the outcome of the P450/sEH axis is pro- or anti-inflammatory. Thus, although increased sEH activity would accelerate the biologic inactivation of the EETs (potentially proinflammatory effect), the increased generation of the DHA-derived diol 19,20-DHDP may be classed as anti-inflammatory. One potential reason is that 19,20-DHDP effectively inhibits the γ-secretase activity and subsequent Notch signaling is reported to prevent angiotensin II–induced abdominal aortic aneurysm formation in vivo in ApoE–/– mice (Zheng et al., 2013b). There is additional evidence hinting that a shift in the *ω*-3/*ω*-6 balance may facilitate cardiovascular disease (if not promote it) because carotid plaques from symptomatic patients were found to be more inflammatory and to contain less EPA and DHA than plaques from asymptomatic patients (Bazan et al., 2009).

Beneficial effects of *ω*-3 supplementation on cardiovascular disease development have yet to be clearly documented in large populations. A recent review of 33 intervention trials investigating the effects of EPA/DPA on fasting and/or postprandial endothelial function found inconsistent effects in healthy subjects, improved endothelial function in overweight dyslipidemic patients and patients with type 2 diabetes, and conflicting results in patients with cardiovascular disease (Egert and Stehle, 2011). Genetic variation may underlie some of the inconsistent results because the available literature hints at gene variants that influence tissue *n*-3 PUFA status and response to fish oil supplementation (Madden et al., 2011). Clearly, more detailed and exhaustive studies are needed to determine the real role of the different PUFA epoxides and diols in the pathophysiology of cardiovascular disease and the therapeutic potential of sEH inhibition either alone or in combination with dietary PUFA substitution/supplementation. Certainly, serum levels of several hydroxy, epoxy, and dihydroxy PUFAs are dependent on the individual status of the parent PUFAs, and dietary supplementation of EPA and DHA directly modulates serum levels of EPA- and DHA-derived metabolites with no apparent differences in the responses between normo- and hyperlipidemic subjects (Schuchardt et al., 2013; Fischer et al., 2014). It also seems that the increase of EPA metabolites along the P450/sEH pathway was highly correlated with the increase of relative EPA levels in erythrocyte membranes, making it tempting to suggest that potential beneficial effects of the fish oils on the cardiovascular system could be attributed to *ω*-3 epoxides (Schuchardt et al., 2014). Certainly, in healthy volunteers, EPA/DHA supplementation primarily resulted in a large increase of EPA-derived P450-dependent epoxy-metabolites followed by increases of EPA- and DHA-derived lipoxygenase-dependent monohydroxy-metabolites including the precursors of resolvin E and D families (Fischer et al., 2014). A similar approach was also found to improve small peripheral artery function in a patient collective with intermediate to high cardiovascular risk (Merino et al., 2014).

### F. Cytochrome P50–Derived Mediators in the Lung

P450 enzymes have long been known to be expressed in the lung but the biologic consequences of their activation was unclear, as contradictory findings were published for different-sized arteries isolated from canine and rabbit lungs (Tan et al., 1997; Zhu et al., 2000a,b; Stephenson et al., 2003; Alvarez et al., 2004). Looking at the pulmonary circulation in vivo in mice or in isolated perfused lungs, rather than isolated vessels, has shed some light on the situation because it appears that P450-derived metabolites such as 11,12-EET increase pulmonary perfusion pressure. Thus, although elevated EET levels act as vasodilators in the systemic circulation, they do exactly the opposite in the pulmonary circulation.

Acute hypoxia increases P450 activity, which means that one of the best ways to increase pulmonary EET levels is to ventilate lungs with low oxygen levels—a procedure that increases pulmonary perfusion pressures (Pokreisz et al., 2006). Inhibiting P450 enzyme activity attenuates the magnitude of the response,
whereas sEH inhibition potentiates it. The EET antagonist 14,15-EEZE decreased the response to hypoxia, whereas 11,12-EET also elicited pulmonary vasoconstriction and 14,15-EET was without effect (Keserü et al., 2008). At the molecular level, it seems that the EET-induced pulmonary vasoconstriction, like the activation of K\(_{Ca}\) channels in endothelial cells is linked to the activation of PKA and the membrane translocation of TRPC6 channels (which is required for effective cation transport). However, because the sEH seems to be largely expressed in pulmonary smooth muscle rather than endothelial cells, TRPC6 channel opening results in depolarization and contraction rather than relaxation. Certainly, a short exposure to hypoxia was sufficient to elicit the rapid translocation of TRPC6 channels from the Golgi apparatus to caveolae in pulmonary smooth muscle cells and the response was sensitive to the EET antagonist 14,15-EEZE. In addition, neither hypoxia nor 11,12-EET was able to elicit pulmonary vasoconstriction in TRPC6\(^{-/-}\) mice (Keserü et al., 2008).

How does the P450/sEH axis fit with the other mechanisms proposed to sense and regulate hypoxic pulmonary vasoconstriction? In a recent elegant study, it was possible to demonstrate that P450 activation and 11,12-EET generation are part of a string of events that involve oxygen sensing at the alveolocapillary level, with subsequent retrograde propagation to upstream arterioles via Cx40 endothelial gap junctions. The transformation of endothelial depolarization into vasoconstriction then involved endothelial \(\alpha_{1G}\) subtype T-type voltage-dependent Ca\(^{2+}\) channels, cytosolic phospholipase A\(_2\), and finally EETs (Wang et al., 2012). The role of Cx40 in the process was demonstrated by the fact that hypoxic pulmonary vasoconstriction was abrogated in lungs from wild-type mice in the presence of Cx40-specific and nonspecific gap junction uncouplers and in lungs from Cx40\(^{-/-}\) mice. The physiologic importance of this cascade of events was highlighted by the fact that hypoxemia in vivo was more severe in Cx40\(^{-/-}\) mice than in wild-type mice.

In the systemic circulation, the generation of P450-derived metabolites of arachidonic acid as well as other events that elicit endothelial cell hyperpolarization are thought to act as a back-up vasodilator system that only plays a significant role in vasoregulation when NO and PGI\(_2\) production are attenuated (Bauersachs et al., 1996). However, all of the effects of P450 activation, 11,12-EET, and sEH inhibition outlined above were observed in the absence of NOS and COX inhibitors. There are however some actions of the 11,12-EET in the lung that can be masked or antagonized by NO, including the activation of BK channels. As outlined above, BK channels on VSMCs were reported to be the initial targets of 11,12-EET in the systemic circulation, a response generally only observed when NO synthesis was inhibited. Also in the lung, hypoxia-induced pulmonary vasoconstriction was identical in wild-type mice and mice lacking the \(\beta1\) subunit of the BK channel. However, a BK\(\beta1\)-dependent component to the EET-induced pulmonary vasoconstriction could be demonstrated after NO synthase inhibition. Rather than targeting BK channel activity on the plasma membrane, it seems that 11,12-EET contributes to pulmonary vasoconstriction by stimulating the association of the \(\alpha\) and \(\beta1\) subunits of mitochondrial BK channels to disrupt the mitochondrial membrane potential and depolarize pulmonary artery smooth muscle cells (Loot et al., 2012). Certainly, a substantial subpopulation of BK channels in pulmonary artery smooth muscle is localized to mitochondria (Roth et al., 2009).

Hypoxia regulates the expression of numerous P450 isoforms so it would seem logical to expect an increase in P450 activity in lungs from animals exposed to chronic hypoxia. Certainly, the exposure of mice to hypoxia for 3 weeks increased the expression of CYP2C29 and elicited pulmonary vascular remodeling (Pokreisz et al., 2006). Interestingly, the same procedure markedly attenuated the expression of the sEH in vivo and in vitro, most probably via altered binding of specificity protein 1 (Tanaka et al., 2008a) and c-Jun (Ai et al., 2007) to the sEH promoter. It was possible to make a causative link between remodeling and P450 activity because P450 inhibition attenuated the increase in pulmonary vascular muscularization observed over 1 week. In addition, small artery muscularization was greater in lungs from sEH\(^{-/-}\) mice than from wild-type mice even under normoxic conditions and enhanced muscularization was accompanied with decreased voluntary exercise capacity (Keserü et al., 2010). Could altered P450 and sEH expression contribute to human lung diseases? Only limited data are available but whereas the sEH was expressed in the wall of pulmonary arteries in lungs from human donors, the enzyme was not detectable in samples from patients with pulmonary hypertension. Such data suggest that a decrease in sEH expression is intimately linked to pathophysiology of hypoxia-induced pulmonary remodeling and hypertension. However, the long-term treatment of wild-type mice (4 months) with sEH inhibitors failed to promote the vascular remodeling and pulmonary hypertension seen after the deletion of the enzyme, indicating that the N-terminal lipid phosphatase of the sEH protein may play a role in the development of pulmonary disease (Keserü et al., 2010).

G. Metabolism and Diabetes

The bioavailability of the P450 metabolizable PUFAs is clearly dependent on diet, with the metabolites of the fish oils associated with protective or anti-inflammatory properties but the metabolites of other PUFAs (e.g., linoleic acid) being linked with enhanced inflammation. The \(\omega-6\) PUFA linoleic acid is the biologic precursor of arachidonic acid and can be metabolized by P450
enlarges and better controlled clinical trials for the sEH that catalyzes the generation of 9,10-DiHOME and 12,13-DiHOME. Linoleic acid levels are high in the Western diet and recent studies indicate that dietary imbalance between ω-6 and ω-3 PUFAs leads to an adverse cardiovascular and metabolic profile, thereby contributing to the pathogenesis of nonalcoholic fatty liver disease, diabetes, and cardiovascular disease (Fig. 5). For example, young obese subjects with pediatric nonalcoholic fatty liver disease and type 2 diabetes have higher levels of oxidized metabolites derived from linoleic acid and lower insulin secretion than subjects with normal glucose tolerance (Santoro et al., 2014). Moreover, at least in rats, linoleic and α-linolenic acid consumption over three generations exerted cumulative effects on the regulation of hepatic expression of genes related to lipid metabolism (Jacometo et al., 2014). Although such studies can only provide indirect evidence for a detrimental consequence of increase linoleic acid metabolism, there have been clear demonstrations of the toxicity of derivatives of linoleic acid. The most impressive link was made by studying patients with acute respiratory distress syndrome who generated high levels of 9,10-DiHOME (Quinlan et al., 1996; Moghaddam et al., 1997; Kumar et al., 2000). Given that 9,10-DiHOME production is catalyzed by the sEH, it is not surprising that sEH inhibitors were able to provide protection in such a situation. However, a simpler approach was simply to reduce the concentration of linoleic acid in the parenteral nutrition given to patients, an intervention that resulted in marked benefits in gas exchange, ventilation requirement, and mortality (Calder, 2010).

Although very high concentrations of linoleic acid–derived diols in critically ill patients are clearly detrimental, more recent studies indicate that low concentrations of 12,13-DiHOME inhibit, rather than potentiate, the respiratory burst in neutrophils (Thompson and Hammock, 2007). In addition, 12,13-DiHOME was shown to activate Wnt signaling and to be required for the regulation of stem and progenitor cell proliferation and mobilization, as well as vascular repair after ischemia (Frömel et al., 2012). All in all, it seems that it is a case of the concentration being important with high levels of linoleic acid linked to inflammation but low levels required for the correct functioning of the immune response.

In contrast with the ω-6 PUFAs, the ω-3 PUFAs are usually attributed anti-inflammatory effects, particularly when considering the metabolic syndrome, type 2 diabetes, and cardiovascular disease (Calviello et al., 2013; Gray et al., 2013; Liu et al., 2013; Robinson and Mazurak, 2013; Pinel et al., 2014; Yates et al., 2014). Despite early positive reports of the benefits of supplementing diets with fish oils on cardiovascular outcomes, larger and better controlled clinical trials have failed to reveal any clear clinical benefit. Unfortunately, it appears that there is a surprisingly high degree of interindividual variability in certain aspects of lipid metabolism (Nording et al., 2013) that renders responsiveness to ω-3 fatty acid supplementation probably too variable to see clear effects in large population studies. Indeed, variables such as age, underlying health status of individuals, dose, duration of exposure, and relative proportion of EPA and DHA in the supplements given (Calviello et al., 2013; Lee and An, 2013; Lovegrove and Griffin, 2013; Fares et al., 2014) can complicate the situation. However, assessing individual responsiveness to ω-3 fatty acids may be a way to increase the effectiveness to PUFA dietary supplementation (Nording et al., 2013).

How could fish oils exert an anti-inflammatory effect? This is difficult to answer because much less is known about the molecular mechanism(s) of action of the fish oils than the actions of the ω-6 PUFAs. However, ω-3 PUFAs can modify tissue and blood lipid metabolism, blood lipid concentrations, blood coagulation, immune function, inflammation, and endothelial function (Wang et al., 2006). EPA and DHA are readily incorporated into cells and tissues to modify membrane properties in lipid rafts as well as nonraft domains (Williams et al., 2012), including the cholesterol content, eicosanoid profiles, signal transduction processes, and gene expression (Lee and An, 2013). To what extent the P450- and sEH-derived metabolites of EPA and DHA actually underlie the actions of the fish oils remains to be determined, but it is interesting to note that the DHA-induced redistribution of cholesterol out of lipid rafts (Wassall and Stillwell, 2008, 2009; Lee et al., 2014) decreases γ-secretase activity in
a neuroblastoma cell line (Grimm et al., 2011), which may in fact be attributable to the actions of the sEH-derived 19,20-DHDP rather than the parent PUFA (Hu et al., 2014). It will be interesting to determine whether there are also differential effects of the DHA-derived epoxides and diols on the association of other signaling molecules with lipid rafts (e.g., EGFR, Hsp90, Akt, and Src) or the fusion of rafts with lysosomes (Lee et al., 2014). Akt, and Src) or the fusion of rafts with lysosomes (Lee et al., 2014).

1. Cytochrome P450. Overnutrition in the form of a high-fat diet affects the P450/sEH axis in experimental animals. In ApoE<sup>−/−</sup> mice, feeding results in a suppression of P450 activity in the liver but in activation of P450 ω-hydroxylase activity in the kidney, thus decreasing EET and increasing 20-HETE levels (Theken et al., 2012a). Interestingly, the angiotensin-converting enzyme inhibitor enalapril reversed the changes in P450 activity in both organs, indicating that there may be a link between angiotensin II generation or bradykinin breakdown (both of which are catalyzed by the angiotensin-converting enzyme) and the P450/sEH axis in situations associated with metabolic dysfunction.

In humans, recent clinical studies have linked obesity with low plasma EET levels and 14,15-EET/14,15-DHET ratios, which would fit with reports of elevated sEH levels. Age, diabetes, and cigarette smoking also were significantly associated with P450 and sEH metabolic activity, whereas only renin-angiotensin system inhibitor use was associated with P450 ω-hydroxylase metabolic activity (Theken et al., 2012b).

Taking a short diversion from the vascular system, it should be mentioned that P450 enzymes are expressed in pancreatic islets. To date, the enzymes have been detected in α, β, δ, and pancreatic polypeptide cells, with CYP2C enzymes probably being the most highly expressed (Chen et al., 2013). Does this mean that the activity of the P450/sEH pathway can alter insulin sensitivity? The evidence is still largely circumstantial but the answer may well be yes.

A recent study reported an interesting potential link between P450 metabolite generation and insulin resistance that involves long-chain acyl-CoA synthetases. It appears that while unesterified EETs inhibit glucose-stimulated insulin secretion, the generation of EET-acyl-CoAs by long-chain fatty acyl-CoA synthetase 4 results in EET esterification into glycerolipids, thus alleviating the intrinsic inhibitory mechanism and promoting glucose-stimulated insulin secretion (Klett et al., 2013). The prolonged exposure (72 hours) of INS 832/13 cells to the ω-6 PUFAs arachidonic acid or linoleic acid resulted in decreased Acsl4 expression and a parallel decrease in glucose-stimulated insulin secretion (Klett et al., 2013). However, the physiologic relevance of the latter observations remains to be demonstrated. Moreover, the observation that 11,12-EET attenuated insulin secretion contradicts an earlier report showing that EETs (5,6-EET) stimulate insulin secretion (Falck et al., 1983). Despite the focus on EETs, it is unclear which of the PUFA, P450/sEH-derived metabolites may play a role in regulating pancreatic homeostasis. Although sEH inhibition protected pancreatic function, this intervention failed to affect the pancreatic and plasma ratios of EETs to DHETs (Chen et al., 2013), indicating that the actions of other PUFA mediators may dominate.

In other organs, P450-derived epoxides are generally thought to be protective and overexpression of the human CYP2J2 in mice attenuates diabetic nephropathy (Chen et al., 2011b) and cardiomyopathy (Ma et al., 2013) in mice made diabetic with STZ. Moreover, a polymorphism in the CYP2J2 gene that results in a functional mutation and reduced gene expression and activity (Spiecker et al., 2004; Liu et al., 2007) has been linked with the risk of a younger onset of type II diabetes, at least in a Chinese population (Wang et al., 2010). The same polymorphism has also been associated with an increased risk of coronary artery disease in Caucasian (Spiecker et al., 2004) and Chinese (Liu et al., 2007) subjects. The impression that P450 products are beneficial as far as insulin sensitivity is concerned is certainly strengthened by the fact that the overexpression of CYP2J3 increased EET generation and reversed insulin resistance and hypertension in vivo in diabetic rats and mice (Xu et al., 2010). CYP2J3 overexpression also enhanced the expression and secretion of the protective adipokine adiponectin. At the molecular level, the CYP2J3 products were shown to prevent the endoplasmic reticulum stress induced in adipose tissue by a high-fat diet (Xu et al., 2013). Effects on adipokine production would be suggestive of effects on adipocyte development or homoeostasis and there is also evidence suggesting that a decrease in EET production is linked with an increase in adipocyte stem cell differentiation and increased levels of inflammatory cytokines, at least in animals with defects in heme oxygenase (HO)–dependent signaling that develop several features of the metabolic syndrome (Sodhi et al., 2009; Burgess et al., 2010; Kim et al., 2010).

2. Soluble Epoxide Hydrolase. The sEH is a PUFA-metabolizing enzyme; therefore, it comes as no surprise that the expression of the enzyme is altered in conditions associated with metabolic stress. Although no data are available regarding caloric restriction, overnutrition in the form of a high-fat diet has been linked with elevated sEH expression (Luria et al., 2011) and/or activity (De Taeye et al., 2010) in adipose tissue and in mesenteric arteries from obese Zucker rats (Zhao et al., 2005). Moreover, sEH inhibition can ameliorate the diet-induced metabolic syndrome in animal models (Luria et al., 2011; Iyer et al., 2012). In humans, the Arg287Gln polymorphism of the Ephx2 gene that results in increased plasma cholesterol levels (Sato et al., 2004) has also been linked with the incidence of
type 2 diabetes (Ohtoshi et al., 2005). However, the sEH has also been linked with type 2 diabetes because sEH expression is reportedly elevated in the hearts from Akita type 1 diabetic mice (Dewey et al., 2013), in macrophages from nonobese diabetic mice (Rodriguez and Clare-Salzler, 2006), as well as in livers (Thomas et al., 1989) and cerebral vessels (Jouhan et al., 2013) from animals made diabetic with STZ. sEH inhibition may therefore be an attractive strategy to address some of the complications of the metabolic syndrome. Indeed, the sEH inhibitor t-AUCh (trans-4-[(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid) was reported to enhance the CD36-mediated recognition and degradation of oxidized LDL and improve cholesterol efflux via the upregulation of ABCAI expression. Furthermore, sEH inhibition attenuated the endoplasmic reticulum stress induced by a high-fat diet (Bettaieb et al., 2013) and blocked tumor necrosis factor-α and adiponectin secretion by cultured adipocytes (Shena et al., 2014). However, longer-term in vivo studies are required because although sEH expression increases, it is not known how long the activity of the protein is maintained. This may be a particularly important consideration in view of the fact that the sEH can be tyrosine nitrad in vivo in diabetic mice (Barbosa-Sicard et al., 2009).

The molecular events underlying the change in sEH expression are currently unclear. However, there is a potential link to HO-dependent signaling. For example, EETs increase the expression of the inducible HO-1 (Sacerdotei et al., 2007) and at least partially account for the positive effects of sEH inhibition on renal function (Botros et al., 2002). EETs have also been suggested to inhibit Bach-1, which is a negative regulator of HO-1 expression. HO-2 is generally considered to be constitutively expressed mice lacking the enzyme are obese and demonstrate insulin resistance as well as high blood pressure. Interestingly, HO-1 and Cyp2c expression were also attenuated in HO-2−/− mice, and sEH inhibition effectively increased EET levels as well as the expression of HO-1, lowered blood pressure, and increased insulin sensitivity at the same time as increasing the generation of the protective adipokine adiponectin and restoring endothelial function (Sodhi et al., 2009). Although HO induction attenuated the development of the metabolic syndrome in obese mice (Burgess et al., 2010), pharmacological HO inhibition negated the protective effects of sEH inhibition in diabetic rats (Elmarakby et al., 2013). Thus, there seems to be an intricate interplay between the P450/sEH axis and the HO pathway. Other molecular targets that may link epoxide levels with cellular energy metabolism include Wnt signaling, PPARγ, CCAAT/enhancer binding protein-α, and AMPK (Vanella et al., 2011). These pathways link EETs not only with diabetes but also with adipogenesis because EETs attenuate the differentiation of human mesenchymal stem cell–derived adipocytes (Kim et al., 2010; Vanella et al., 2011). The sEH is also expressed in pancreatic islets, most probably in β cells (Luo et al., 2010; Chen et al., 2013), and islets from sEH−/− mice secreted more insulin than wild-type littermates when stimulated with glucose (Luo et al., 2010). A similar increase in insulin stimulation was observed when islets from wild-type mice were treated with sEH inhibitors.

Pancreatic β-cell loss and dysfunction are central factors in the pathogenesis of diabetes, and preventing β-cell loss and the loss of β-cell function is an important goal to maintain insulin secretion and glucose homeostasis in diabetes. sEH inhibition has also demonstrated positive effects on β-cell viability by reducing islet cell apoptosis in STZ-treated mice, possibly by attenuating the accumulation of inflammatory factors in the cells and the subsequent generation of proapoptotic cytokines (Luo et al., 2010; Chen et al., 2013). sEH inhibition also increases insulin secretion by mechanisms that are not entirely clear but may be related to Ca2+ signaling because the islet Ca2+ response was enhanced in cells from sEH−/− mice compared with wild-type mice (Luo et al., 2010). TRP channels have recently been identified in β cells, where they are thought to be involved in the regulation of β-cell function and insulin secretion (Colsoul et al., 2011; Islam, 2011). However, whether the increase in insulin secretion in cells treated with an sEH inhibitor can be attributed to the enhanced epoxide-dependent translocation and activation of TRP channels has yet to be studied.

3. EETs and Mitochondria. Reports that sEH deletion or inhibition can protect against the development of diabetes make it tempting to speculate that either the products that accumulate after sEH inhibition improve metabolism or that the generation of “deleterious” metabolites is prevented. Too little has been done in this field to speculate much, but some studies have reported a beneficial effect of the EETs on mitochondrial function. For example, although the induction of cardiac ischemia-reperfusion injury in wild-type mice is associated with mitochondrial fragmentation, this phenomenon was attenuated in transgenic CYP2J2 animals that overexpressed the enzyme selectively in cardiac myocytes (Katragadda et al., 2009). Moreover, 14,15-EET was reported to attenuate the laser-induced loss of the mitochondrial potential in isolated cardiac myocytes (Katragadda et al., 2009). Similarly, EETs have been linked to a decrease in the Ca2+ accumulation associated with anoxia–reoxygenation as well as the maintenance of the cardiac myocyte mitochondrial potential (Batchu et al., 2012a,b). In cancer cells and astrocytes, 11,12-EET attenuated reactive oxygen species generation, loss of mitochondrial function, and caspase activation induced by toxic stimuli the antileukemia drug arsenic trioxide (Liu et al., 2011; Sarkar et al., 2014). On the other hand, 11,12-EET was found to acutely reduce the mitochondrial potential in pulmonary smooth muscle.
cells by promoting the association of the BKα and BKβ1 subunits on the mitochondrial membrane, ultimately leading to the depolarization and contraction of pulmonary artery smooth muscle cells (Loot et al., 2012).

**H. Stem Cells**

Not much is known about the role of the P450/sEH axis in the regulation of stem and progenitor cells, but sEH knockdown in the zebrafish embryo decreases numbers of CD41+ stem and progenitor cells. In adult mice, the sEH is expressed in bone marrow cells, more specifically in Lin−Sca-1−cKit+ cells, and the genetic deletion of the sEH as well as its pharmacological inhibition attenuated the granulocyte colony stimulating factor and hypoxia-induced mobilization of mononuclear cells from the bone marrow. This finding may account for some of the anti-inflammatory effects observed in those models (Frömel et al., 2012). The lack of the sEH also attenuated the ability of bone marrow cells to form colonies in the murine spleen, hinting at a defect in repopulation efficiency. Interestingly, rather than the effects being attributed to the accumulation of an EET or other PUFA epoxide, it turned out that 12,13-DiHOME, and to a lesser extent 14,15-DHET, regulated progenitor cell homeostasis. Although the latter study did not focus on P450 enzymes, others have demonstrated that human mesenchymal stem cells express CYP2J2. It seems that in the latter cells, EETs decrease mesenchymal stem cell differentiation (Kim et al., 2010).

A different approach that also indicates the importance of the P450/sEH axis in the bone marrow involved studying the consequences of downregulation of the P450 reductase, which is an essential electron donor for all microsomal P450 enzymes. The latter studies revealed that attenuated P450 reductase expression enhanced the repopulation efficiency of hematopoietic stem cells (Zhang et al., 2013b). However, elucidating the molecular mechanisms behind this effect would be difficult because the loss of the P450 reductase would be expected to dramatically reduce all P450 activity.

There may also be a role for the other arm of the P450 pathway in the regulation of progenitor cells because 20-HETE derived from CYP4A/F ω-hydroxylases is thought to regulate the angiogenic potential of the circulating progenitor cells previously referred to as endothelial progenitor cells (Guo et al., 2011). The mechanisms are currently not clear but may involve a positive feedback regulation between 20-HETE and the VEGF pathway (Chen et al., 2014).

**V. Pharmacology**

**A. Are Cytochrome P450 Enzymes Druggable Targets?**

On the basis of the evidence outlined, it seems safe enough to conclude that P450 activation and sEH inhibition are linked with vasodilator and anti-inflammatory effects. Exploiting the role of P450 enzymes in cellular functions and a multitude of disease states was considered to make them potential molecular candidates to target pharmacologically. Given the angiogenic actions of some of the P450-derived epoxides, it is tempting to speculate that P450 enzymes might represent a new target for the treatment of tumor angiogenesis and therefore cancer therapy. Pharmacological inhibitors of some P450 isoforms (e.g., CYP1B1) have been identified as promising anticancer agents (Chun and Kim, 2003). However, the majority of work published to date has focused on the consequences of P450 inhibition on the bioavailability of anticancer agents, such as tamoxifen (Coller, 2003; Stearns et al., 2003), rather than determining the consequences of P450 inhibition per se. Certainly, a large number of P450 inhibitors already exist or are being developed (Francis and Delgoda, 2014). However, given that P450 enzymes metabolize xenobiotics (e.g., nifedipine, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, and warfarin), targeting the proximal part of the pathway may lead to unwanted complications and side effects in patients receiving other medications.

**B. What About Targeting the Soluble Epoxide Hydrolase?**

Certainly, a series of pharmacological inhibitors has been developed that demonstrate clear effects in animals models of disease (Imig and Hammock, 2009; Imig, 2012) and excellent availability and pharmacokinetic behavior in rodents, canines (Tsai et al., 2010), and nonhuman primates (Ulu et al., 2012) to yield materials that can be given orally (as well as other routes) with good efficacy. The problem is that although the anti-inflammatory and antihypertensive actions of the sEH inhibitors are well documented (see above), sEH inhibition has also been linked with attenuated progenitor cell proliferation and mobilization (Frömel et al., 2012), attenuated vascular responsiveness in the lung (Keserü et al., 2010), attenuated developmental angiogenesis (Hu et al., 2014), and the promotion of cancer metastasis (Panigrahy et al., 2012). This mixture of positive and negative effects is probably not surprising given the wide array of PUFAs that can enter the P450/sEH pathway as substrates. Indeed, depending on the enrichment in different tissues of the parent PUFA, the epoxides and diols can exhibit widely different and even antagonistic effects. Such findings mean that it is tempting to speculate that P450 enzymes might represent a new target for the treatment of tumor angiogenesis and therefore cancer therapy. Pharmacological inhibitors of some P450 isoforms (e.g., CYP1B1) have been identified as promising anticancer agents (Chun and Kim, 2003). However, the majority of work published to date has focused on the consequences of P450 inhibition on the bioavailability of anticancer agents, such as tamoxifen (Coller, 2003; Stearns et al., 2003), rather than determining the consequences of P450 inhibition per se. Certainly, a large number of P450 inhibitors already exist or are being developed (Francis and Delgoda, 2014). However, given that P450 enzymes metabolize xenobiotics (e.g., nifedipine, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, and warfarin), targeting the proximal part of the pathway may lead to unwanted complications and side effects in patients receiving other medications.
Falck et al., 2009) and show effectiveness in different models (Bukhari et al., 2012; Hye Khan et al., 2013, 2014; Khan et al., 2013). Another approach would be to target specific PUFA epoxide receptors, such as the EET receptor; however, this approach can only begin once the putative EET receptors have been identified.

C. Cross-Talk between the Polyunsaturated Fatty Acid Pathways

At this point, it is important to consider that interfering with the activity of the P450 or sEH enzymes is likely to have consequences on the activity of other fatty acid–utilizing enzymes and blocking the metabolism of P450-derived epoxides could shunt substrate into another pathway. This would be expected to affect biologic function. Taking the use of P450 inhibitors to attenuate tumor cell proliferation as an example, the increased COX or LOX activity associated with P450 inhibition would be expected to result in the generation of metabolites with angiogenic and tumorigenic potential. Indeed, many lipid autoxidants stimulate both epithelial cells and stromal cells to produce VEGF and FGF-2, which in turn induce COX2 and amplify the induction process and levels of PGE2, resulting in the generation of metabolites with angiogenic properties.

The sEH plays a central role in the regulation of other PUFA epoxides in the plasma. This enzyme catalyzes the conversion of 4-hydroxy-2-nonenal to 4-hydroxy-2,3-epoxy-5-octenoic acid. The sEH plays a major role in the regulation of arachidonic acid metabolites in the pathogenesis of cardiac hypertrophy. The sEH inhibitor MK886 (1-[(4-chlorophenyl)methyl]-3-{[(1, 1-dimethylthyl)thio]-α,α-dimethyl-5-(1-methylthyl)-1H-indole-2-propanoic acid) enhances the anti-inflammatory effect and resulted in better control of lipopolysaccharide-induced hypotension as well as hepatic protein expression of COX2 and 5-LOX (Liu et al., 2010a; Hwang et al., 2011). However, metabolomic profiling to monitor representative metabolites and cross-talk among branches of the arachidonic acid cascade revealed that rather than redirecting substrate to a parallel pathway, inhibition of one arm of arachidonic acid metabolism tended to decrease eicosanoid production via a parallel arm, largely by decreasing generation enzyme expression (Hwang et al., 2011). Interestingly, although inhibition of COX or LOX pathways had minimal effect on the levels of P450-derived EETs, a combined approach reduced the levels of sEH-generated DHET and the LOX products (Liu et al., 2010b).

VI. Outlook

Interfering with the generation and hydration of PUFA epoxides has beneficial effects on vascular function and gene expression as well as tissue inflammation and metabolic dysfunction. Thus, drugs that can potentiate the production or prolong the tissue/plasma half-life of these lipid mediators have enormous therapeutic potential, which is largely ignored. Why? Specificity is a problem because although inhibiting the hydration of the EETs may be beneficial, the hydration of other PUFA epoxides may be required for their biologic action (e.g., the conversion of 12,13-EpOME to 12,13-DiHOME in the bone marrow or 19,20-EDP to 19,20-DHDP in the retina). Given the large number of PUFA that can feed into the P450/sEH axis, it is unlikely that global P450 or sEH inhibition will be without side effects. Another reason why research into the cardiovascular actions of the P450-derived epoxides and diols of the bioavailable PUFA has not exploded, despite the evidence accumulated over the last 20 years to demonstrate their importance, is perhaps related to the lack of a receptor. However, the work of John R. Falck and others who have generated a series of compounds whose effects can only be explained by their ability to act as agonists or antagonists of an EET receptor provides strong evidence that a membrane EET receptor exists. The exploitation of such knowledge would be expected to lead the way to the selective therapeutic exploitation of the anti-inflammatory effects of the EETs.

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