Chapter 9
Cytochrome P450-Derived Lipid Mediators and Vascular Responses

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Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
BK_{Ca}	Large conductance Ca^{2+}-activated K^+ channels
COX	Cyclooxygenase
CREB	cAMP-response element-binding protein
CYP	Cytochrome P450
DHA	Docosahexenoic acid
DHET	Dihydroxyeicosatrienoic acid
EDHFs	Endothelium-derived hyperpolarizing factors
EET	Epoxyeicosatrienoic acid
EGF	Epidermal growth factor
EPA	Eicosapentaenoic acid
FABPs	Fatty acid-binding proteins
HETE	Hydroxyeicosatetraenoic acid
K_{Ca}	Ca^{2+}-dependent K^+ channels
MKP-1	MAP kinase phosphatase-1
MMP	Matrix metalloproteinase
NFκB	Nuclear factor κB
NO	Nitric oxide
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
sEH	Soluble epoxide hydrolase;
TRP channels	Transient receptor potential channels

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9.1 The Cytochrome P450 (CYP)/Soluble Epoxide Hydrolase Axis

Cytochrome P450 (CYP) enzymes are membrane-bound, heme-containing terminal oxidases in a multi-enzyme complex that also includes an flavin adenine dinucleotide/flavin mononucleotide (FAD/FMN)-containing nicotinamide adenine dinucleotide phosphate (NADPH)-CYP reductase and cytochrome b_5. CYP enzymes oxidize, peroxidize and/or reduce cholesterol, vitamins, steroids, xenobiotics, and numerous pharmacological substances in an oxygen- and NADPH-dependent manner. Some isoforms are fairly specific in their choice of substrates but many catalyze a large number of chemical reactions and can use an almost unlimited number of biologically occurring and synthetic compounds. Hepatic CYP enzymes are responsible for the metabolism of xenobiotica and many pharmaceuticals, but they also utilize endogenous compounds as substrates, such as cholesterol and fatty acids. Even though many CYP isozymes can oxidize a spectrum of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) such as retinoic acid, linoleic acid, eicosapentaenoic acid (EPA), and docosahexenoic acid (DHA; Fig. 9.1), they are often referred to as the third pathway of arachidonic acid metabolism, mainly because more is known about the biological actions of these products [1].

Interest in the vascular actions of CYP enzymes followed reports that the epoxides of arachidonic acid (the epoxyeicosatrienoic acids [EETs]) were endothelium-derived hyperpolarizing factors (EDHFs) [2, 3], while the 20-hydroxyeicosatetraenoic acid (HETE) generated by ω-hydroxylases belonging to the CYP4A family were potent vasoconstrictors (for review see Harder et al. and Imig et al. [4, 5]). Initial reports focused on the effects of arachidonic acid metabolites on membrane potential, but it is now generally appreciated that these compounds mediate a number of membrane potential-independent effects and regulate angiogenesis [6, 7]. The arachidonic acid-metabolizing CYP enzymes with prominent roles in vascular regulation are the epoxygenases of the CYP2 gene family (e.g. CYP2B, 2C8, 2C9, 2C10, and 2J2 in humans; 2C34 in pigs; 2C11, 2C23, and 2J4 in rats) and the arachidonic acid ω-hydroxylases belonging to the CYP4A family which form subterminal and ω-terminal HETEs [8, 9].

Epoxide generation is thought to be determined by both the level of epoxygenase expression and the availability of the PUFA substrate, which in the case of arachidonic acid is determined by the activity of phospholipases such as phospholipase A_2. Intracellular levels of the epoxides are tightly regulated and metabolism occurs relatively rapidly by hydrolysis, β-oxidation, and chain elongation [10]. The soluble epoxide hydrolase (sEH) is the most important epoxide-metabolizing enzyme that generates dihydroxy fatty acids (or diols). For a long time, the latter were considered to be less active that the parent epoxides but recent evidence has challenged this assumption (see section 9.6). There are, of course, exceptions to every rule and some epoxides are not great sEH substrates—the best-studied exception is probably 5,6-EET which is more rapidly metabolized by cyclooxygenases (COXs) [11, 12].

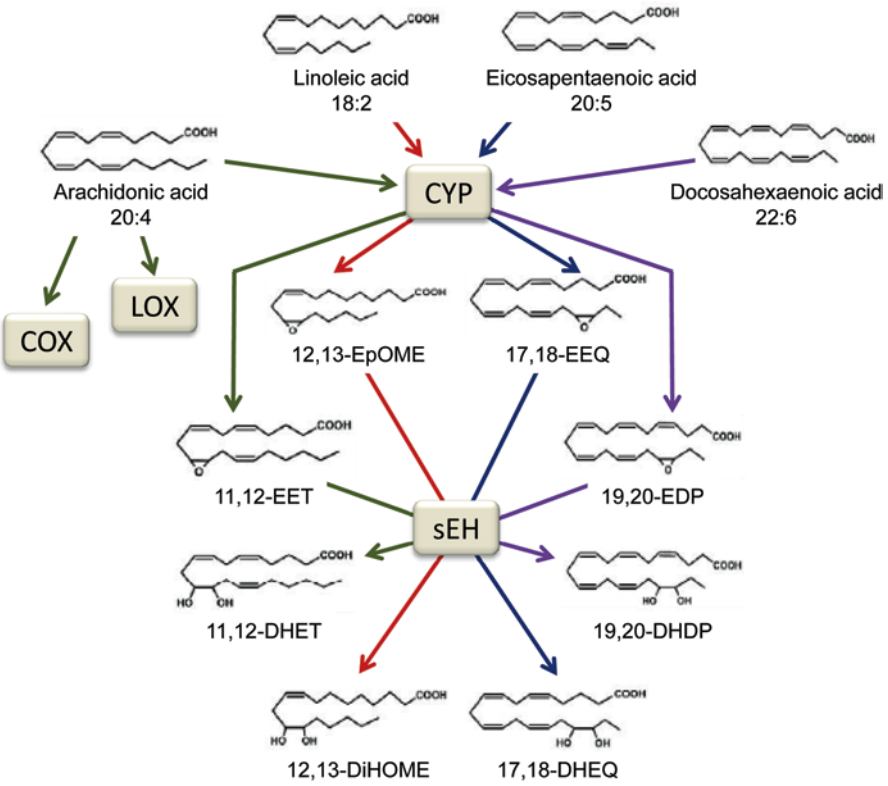

Fig. 9.1 Cytochrome P450 (*CYP*)-dependent metabolism of n-6 (arachidonic and linoleic acid) and n-3 (eicosapentaenoic and docosahexaenoic acid), and metabolism of the epoxides generated to the corresponding diols by the soluble epoxide hydrolase (*sEH*). *DHDP* dihydroxydocosapentaenoic acid, *DHEQ* dihydroxyeicosatetraenoic acid, *DHET* dihydroxyeicosatrienoic acid, *DiHOME* dihydroxyoctadecenoic acid, *EDP* epoxydocosapentaenoic acid, *EEQ* epoxyeicosatetraenoic acid, *EET* epoxyeicosatrienoic acid, *EpOME* epoxyoctadecenoic acid. Reproduced from Fleming [9], with permission

9.2 Regulation of CYP Expression and Activity

EET production may change as a consequence of altered CYP expression (by induction or repression) or altered activity. Little is known about the regulation of vascular CYP expression, and although CYP2C protein has been convincingly demonstrated in native endothelial cells, messenger RNA (mRNA) and protein levels rapidly decrease after cell isolation, so that in passaged cultured endothelial cells, mRNA can only be detected using reverse transcriptase-polymerase chain reaction (RT-PCR) [3, 13]. Such findings indicate that CYP2C proteins are relatively unstable and that transcriptional processes play an important role in determining CYP expression levels, and at the same time highlight the importance of physiological stimuli in the control of CYP 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 [14].

The expression of several CYP enzymes is modulated by changes in oxygen tension; for example, hypoxia downregulates CYP2J2 [15] but upregulates CYP2C8/9 expression in cultured human endothelial cells [16], while transient cerebral ischemia induces CYP2C11 in rats [17]. The promoter regions of several CYP2C genes contain hypoxia-responsive elements, a finding which may explain the observation that the myogenic contraction, as well as the constrictor response to phenylephrine, is attenuated in mesenteric arteries from rats exposed to hypoxia for 48 h [18]. This phenomenon can be attributed to the hypoxia-induced induction of CYP expression as CYP2C protein was elevated above control in arteries from rats exposed to hypoxia, and both the vascular smooth muscle hyperpolarization and the hypoxia-induced decrease in the myogenic response were normalized by the CYP2C inhibitor sulfaphenazole [19].

Given the size of the CYP family of proteins, it is not surprising that there is considerable inter-isoform variation in the regulation of gene expression and mRNA stability, as well as post-translational modification of the CYP 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. Retinoic acid is a CYP2C (CYP2C8) substrate, thus the regulation of CYP2C8 expression by a receptor that is activated by an endogenous substrate such as retinoic acid is not surprising. CYP2C9, which is highly homologous to CYP2C8, is inducible in primary human hepatocytes by xenobiotics, including dexamethasone and phenobarbital [20]. The CYP2C8 and 2C9 promoters contain a glucocorticoid-responsive element that is recognized and transactivated by human glucocorticoid receptor [21–23]. Identification of this functional element provides a rational mechanistic basis for the induction of CYP2C protein, and an increase in EDHF-mediated responses in porcine coronary arteries by cortisol [24]. Retinoic acid is not the only CYP substrate that affects CYP protein levels, and the expression of many of the CYP enzymes can be induced by a substrate excess. Indeed, a number of cardiovascular drugs currently in clinical use are metabolized, at least in part, by CYP2C family members. While this process mainly occurs in the liver and is associated with the induction of the metabolizing enzyme, the expression of CYP2C enzymes in endothelial cells can also be affected. For example, the HMG-CoA reductase inhibitor fluvastatin, which is metabolized by CYP2C9 in the liver [25, 26], also increases CYP2C expression in cultured and native porcine coronary artery endothelial cells [13]. The L-type Ca^{2+} channel blocker nifedipine also elicits a very marked increase in the expression of CYP2C in porcine coronary arteries, and enhances EDHF-mediated responses [13, 27].

Once the protein is expressed, CYP activity is thought to be determined mainly by the availability of its substrates [28]. Since phospholipase A_2 inhibitors attenuate CYP-dependent EDHF responses, the activation cascade is thought to involve a stimulus-induced increase in intracellular Ca^{2+}, followed by the activation of phospholipase A_2, which then liberates the PUFA substrate (i.e. arachidonic acid) from membrane phospholipids. The increase in substrate immediately results in

the activation of CYP enzymes (when expressed) and the generation of vasoactive products. While this sequence of events is certainly plausible, it is highly likely that additional mechanisms, such as phosphorylation, play a role in regulating CYP activity. Indeed, some CYP enzymes (CYP2B1, 2B2, and 2E1) are reported to be phosphorylated by protein kinase A (PKA), and the consequences of CYP phosphorylation range from the regulation of activity [29] and subcellular localization [30, 31] to proteasome degradation [30, 32].

An additional mechanism thought to modulate CYP activity is nitrosation by nitric oxide (NO), which can interact with CYP enzymes in two ways. NO reversibly binds to the heme moiety of CYP enzymes, forming iron-nitrosyl complexes, and it can irreversibly react with cysteine residues [33]. Both NO–CYP adducts are enzymatically inactive *in vitro*. As endothelial CYP enzymes of the 2C family were found to be inhibited by NO, the role of EETs in the regulation of vascular tone in the healthy vasculature which constantly generates NO was suggested to be of minor importance compared with that in circumstances of an endothelial dysfunction in which the bioavailability of NO is impaired [34]. However, there are clear physiological consequences of EET activation in endothelial cells (e.g. on Akt, PKA, and transient receptor potential [TRP] channels) that can be demonstrated, even in the presence of a fully functional endothelial NO synthase. Thus, whether or not physiologically relevant (low nmol/L) levels of NO really affect CYP epoxygenase activity *in vivo*, remains to be determined.

9.3 The Soluble Epoxide Hydrolase

The sEH protein is a homodimer composed of two 60 kDa monomers joined by a proline-rich bridge [35], with each monomer consisting of an N-terminal domain that displays lipid phosphatase activity and a larger C-terminal that processes classical α/β-hydrolase activity [36, 37]. Surprisingly little is known about the mechanisms that regulate sEH activity. There have been a number of studies linking changes in sEH expression with inflammatory or hormonal stimuli [38, 39]. Two tyrosine residues (Tyr383 and Tyr466) in the active site of the hydrolase are reportedly essential for enzyme activity [40], and these were recently shown to be nitrated by peroxynitrite *in vitro* and *in vivo* in mouse models of type 1 and type 2 diabetes, leading to a decrease in sEH activity [41]. It is currently only possible to speculate about the involvement of sEH tyrosine nitration in the amplification of inflammation associated with diabetes, but at least one sEH polymorphism, which results in decreased enzymatic activity, has previously been associated with human insulin resistance [42]. The sEH was also recently reported to be nitrosated in leptin-stimulated wild-type but not endothelial NO synthase knockout mice, suggesting that the effects of NO on PUFA metabolism may be partly related to the modulation of sEH activity [43].

Inhibition or deletion of the sEH increases tissue and circulating levels of the PUFA epoxides at the same time as decreasing diol production, and has pronounced

effects on blood pressure [44, 45], inflammation [46], progenitor cell proliferation, angiogenesis and vascular repair [47]. The particular effectiveness of sEH inhibitors against hypertension associated with activation of the renin-angiotensin system is most likely related to the fact that angiotensin II markedly increases sEH expression *in vivo* [39]. Interestingly, hypoxia does the opposite and markedly downregulates sEH promoter activity and thus protein expression in the lung [48]. There are other examples of hypertension being associated with elevated sEH expression and/or activity, such as the spontaneously hypertensive rat. In these animals, elevated sEH expression is linked to an increase in the renal metabolism of EETs to dihydroxyeicosatrienoic acids (DHETs), and sEH inhibitors blunt the development of hypertension [44]. Initial reports also documented that $sEH^{-/-}$ mice have lower blood pressure and elevated EET levels than their wild-type littermates [49]. However, the blood pressure phenotype now seems to be controversial as the loss of the hydrolase can be compensated by elevated concentrations of the pressor and vasoconstrictor eicosanoid, 20-HETE, as well as increased lipoxygenase-derived hydroxylation and prostanoid production [50]. Despite the lack of alteration in blood pressure, hearts from these $sEH^{-/-}$ animals show improved recovery of left ventricular contractility and less infarction than hearts from wild-type mice after ischemia [51], and have a survival advantage following acute systemic inflammation [50]. Several of the metabolites generated by the sEH, such as the DHETs generated from the EETs, are also biologically active but generally less so than the parent epoxides. However, the DHETs are not as readily incorporated into membrane lipids as the EETs, and the latter are thought to be the form in which the majority of endothelium-derived EETs leave the cell [52].

The exact physiological role of the lipid phosphatase activity associated with the N-terminal domain of the sEH is currently unclear as there are currently no selective inhibitors of this domain (sEH inhibitors act on the hydrolase domain and do not affect the phosphatase activity [36]). However, the lipid phosphatase has been associated with cholesterol-related disorders, peroxisome proliferator-activated receptor (PPAR) activity, and the isoprenoid/cholesterol biosynthesis pathway [53]. Indeed, in addition to demonstrating enhanced circulating EET levels [49], male $sEH^{-/-}$ mice exhibit decreased plasma cholesterol and testosterone levels [54]. Moreover, it seems that isoprenoid pyro- and monophosphates are substrates for the N-terminal domain of the enzyme [55, 56], and these lipid phosphates are metabolic precursors of cholesterol biosynthesis and are also utilized for isoprenylation of small G-proteins involved in multiple cell signaling pathways [57]. Lysophosphatidic acids are involved in regulating cell survival, apoptosis, motility, shape, differentiation, gene transcription, and malignant transformation, and are reportedly excellent substrates for the lipid phosphatase [58, 59]. However, to what extent this can affect physiology/pathophysiology needs to be determined.

It is interesting to note that even though most current sEH literature attributes the hydrolase domain to the cardiovascular effects seen in humans, the human sEH single nucleotide polymorphism most often associated with cardiovascular disease (R287Q) encodes a protein with significantly lower rather than elevated hydrolase activity [60]. Thus, solely incriminating the hydrolase domain for adverse

cardio- and pulmonary-vascular effects seems premature and highlights the importance of further investigating the independent roles of the hydrolase and phosphatase domains. Indeed, some aspects of the phenotype of $sEH^{-/-}$ mice (e.g. pulmonary vascular muscularization) cannot be reproduced by chronic sEH inhibitor treatment, which may be an indirect indication of a physiological role for the phosphatase domain [61].

9.4 How do Lipid Epoxides Initiate Cellular Signaling?

Most is known about the actions of the epoxides or arachidonic acid or EETs for which several modes of signal initiation have been proposed. One of them involves the transactivation of the epidermal growth factor (EGF) receptor in endothelial cells, and the activation of this particular signaling pathway has been linked to cell proliferation and angiogenesis [62–64]. For actions other than angiogenesis, a separate mechanism has been proposed as a high-affinity EET binding site was reported to exist on monocytes and U937 cells [65–67]. Competition studies showed a specific high-affinity binding of 14,15- and 11,12-EET to a receptor that seems to be protein in nature [66, 68]. In addition, in isolated membranes, [^{3}H]-14,15-EET binding was found to be specific, reversible, and saturable, and the ligand was not displaced by antagonists of the thromboxane, platelet-activating factor, or leukotriene receptors. However, binding was inhibited by 14,15- and 11,12-EETs, but not by inactive analogs of 14,15-EET or 15-HETE. Importantly, ligand binding was inhibited by GTPγS, indicating that the binding site or receptor is coupled to a G protein. Such findings are in agreement with other reports indicating the involvement of a G protein in the actions of the EETs [69, 70]. One characteristic of many EET-induced cellular responses such as gap junctional communication [71] and TRP channel translocation [72] is their ability to increase intracellular cyclic adenosine monophosphate (cAMP) levels and activate PKA [66, 73]. Moreover, an EET analog that is able to induce the complete relaxation of bovine coronary arteries also does so by increasing cAMP levels [74, 75]. Putting the evidence of a protein receptor on cell membranes together with that indicating a reliance on cAMP/PKA for EET-induced signaling, the existence of a Gαs-coupled EET receptor has been postulated [76]. However, to-date no specific EET receptor has been identified.

Many lipids also interact with intracellular fatty acid receptors such as the PPARs, and the EETs are no different. For example, ω-hydroxylated 14,15-EET and 14,15-DHET [77] are reported to bind with a high affinity to PPAR-α, while the EETs generated in endothelial cells in response to fluid shear stress increase PPAR-γ transcriptional activity [78]. Additional intracellular receptors for CYP products have not yet been identified, but one possibility is that these oxidized fatty acids bind to fatty acid binding proteins (FABPs) such as heart type (H) FABP [79], which in turn mediate some of the physiologically relevant actions of these intermediates, possibly including the activation of PPARs [80].

A further proposed mechanism involves the incorporation of EETs into the plasma membrane, where they associate with effector molecules such as small G proteins [69] or change the lipid bilayer order, fluidity, and volume, and thereby regulate the flux of ions (e.g. Ca^{2+}) across the membrane [81]. Certainly, the EETs can be esterified to phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositols [28]. Moreover, at least in the pancreas, it has been suggested that the long-chain acyl-coenzyme A (acyl-CoA) synthetase 4 activates EETs to form EET-CoAs that are incorporated into glycerophospholipids [82].

Given that enhanced EET production has frequently been correlated with an increase in intracellular cAMP levels, it is not entirely surprising that increased CYP expression and EET production are associated with activation of the cAMP-response element-binding protein (CREB) which underlies the EET-induced expression of COX-2 [83]. However, the first transcription factor reported to be regulated by CYP-derived EETs was nuclear factor κB (NF-κB) [84]. Indeed, the EET-dependent inhibition of the I-κB kinase led to classification of the EETs as anti-inflammatory mediators. However, this classification is complicated by the fact that some CYP epoxygenases can generate physiologically relevant levels of superoxide anions which tend to activate NF-κB, and thus functionally antagonize the inhibitory effects of EETs and promote the expression of adhesion molecules on endothelial cells [85]. The reason why CYP epoxygenases of the 2C family generate superoxide anions [85, 86] while the 2J enzymes do not [87, 88] is currently unclear but is probably related to substrate binding and metabolism. However, the differential ability to generate free radicals accounts for the disparate effects of these isozymes on vascular protection.

Other transcription factors that are reported to be modulated by EETs/DHETs are PPARα [77, 89, 90] and FOXO3a [91]. While the nuclear localization of FOXO3a is regulated by the EET-dependent activation of Akt [91], much less is known about the mechanisms involved in the EET-dependent activation of PPARα, or indeed the consequences of this effect.

9.5 CYP and Cardiovascular Function

9.5.1 Vascular Reactivity

The realization that EETs, especially 11,12- and 14,15-EET, can activate large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) on vascular smooth muscle cells to elicit hyperpolarization and relaxation led to their identification as a class of EDHF [2, 3]. The latter term is now recognized as an oversimplification as there are three principal mechanisms linked to the EDHF phenomenon: (i) an increase in endothelial $[Ca^{2+}]_i$ following cell stimulation triggers the synthesis of a metabolite which is essential for the subsequent EDHF-mediated responses; (ii) K^+, released from endothelial cells via Ca^{2+}-dependent K^+ (K_{Ca}) channels, induces smooth muscle hyperpolarization by activating inwardly rectifying K^+ channels and/or the Na^+/K^+-

ATPase on vascular smooth muscle cells; and (iii) endothelial cell hyperpolarization is transmitted to the vascular smooth muscle via gap junctions. The strengths and weaknesses of the arguments for each of these specific types of EDHF has been discussed at length [92] but each of them appears to be valid in certain vascular beds. Interestingly, all of these mechanisms can be modulated by EETs.

In endothelial cells, the activation of K_{Ca} channels by EETs is preceded by an increase in intracellular Ca^{2+} levels that can be accounted for by an increased open probability of nonselective cation channels of the TRP family. How this happens was initially attributed to the presence of an arachidonic acid-binding site in some of the TRP channels that can be activated by the parent lipid [93, 94] as well as the EETs [93, 95]. However, while relatively high concentrations of the EETs may affect TRP channels directly, more physiological concentrations activate TRP channels in a PKA-dependent manner that involves their translocation to caveolin-rich areas in the plasma membrane [72, 96]. There appear to be regioisomer-specific differences in EET-induced TRP channel translocation and activation as 5,6-EET, but not 11,12-EET, can activate TRPV4 in endothelial cells [93, 95], a phenomenon that underlies the EDHF-dependent, flow-induced vasodilatation [96]. On the other hand, 11,12-EET, but not 14,15-EET or 5,6-EET, enhance the bradykinin-induced capacitive Ca^{2+} influx in endothelial cells by stimulating the translocation of TRPC6 and TRPC3 to caveolin-rich areas in the plasma membrane [72].

9.5.2 Pulmonary Circulation

While increasing intracellular Ca^{2+} in endothelial cells elicits vasodilatation, the same process in vascular smooth muscle cells does exactly the opposite. This means that when EETs activate TRPC6 channels in pulmonary smooth muscle cells, an increase in pulmonary vascular tone would be expected. The fact that activation of TRPC6 channels plays a role in regulating hypoxic pulmonary vasoconstriction (a physiological mechanism by which pulmonary arteries constrict in hypoxic lung areas in order to redirect blood flow to areas with greater oxygen supply) was demonstrated using mice lacking the channel. Indeed, in pulmonary vascular smooth muscle cells from these animals, hypoxia completely failed to cause Ca^{2+} entry. It should be noted here that the TRPC6 is reported to primarily conduct Na^{+}, and Ca^{2+} follows secondarily through voltage-gated Ca^{2+} channels or by the Na^{+}/Ca^{2+} exchanger. In line with the disturbed Ca^{2+} entry, these animals completely lacked the initial, acute phase of hypoxia-induced pulmonary constriction [97]. Moreover, $TRPC6^{-/-}$ mice did not respond to 11,12-EET, although the eicosanoid induced a pronounced increase in pulmonary pressure in $TRPC6^{+/-}$ littermates. Furthermore, inhibition of the sEH potentiated the hypoxic pulmonary vasoconstriction in the heterozygous mice, but had no effect in the $TRPC6^{-/-}$ mice [48]. In line with the functional data, hypoxia and 11,12-EET caused the translocation of TRPC6 to caveolae in isolated pulmonary vascular smooth muscle cells. In addition, hypoxia-induced translocation of the channel could be prevented by pretreating the cells with an EET antagonist [48]. More recently [98], the site for pulmonary oxygen sensing

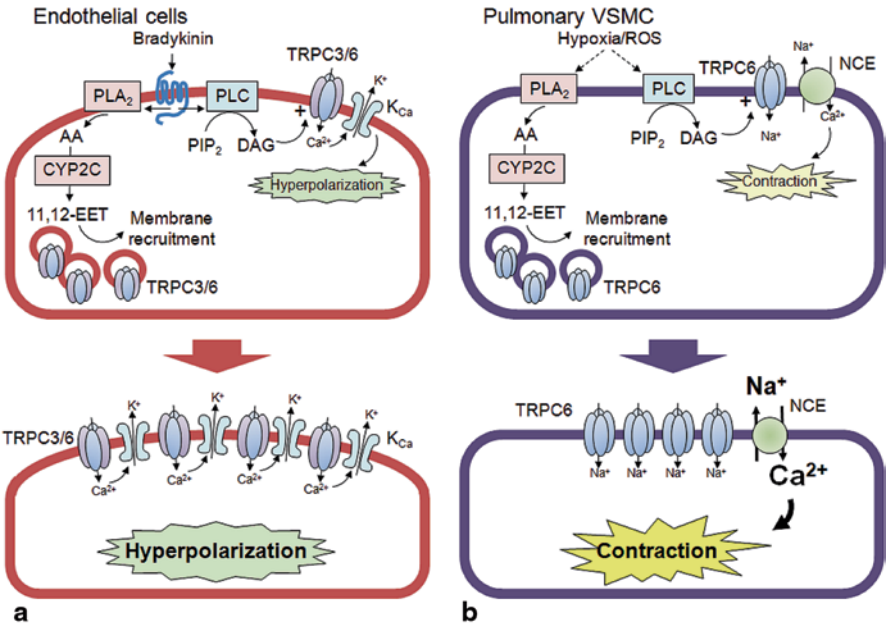

Fig. 9.2 Proposed mechanism for the differential consequences of epoxyeicosatrienoic acid (*EET*)-induced transient receptor potential (*TRP*) channel activation in the systemic and pulmonary circulations. **a** In the systemic circulation, EETs are generated in endothelial cells in response to stimulation (e.g. by bradykinin) following the activation of phospholipase A_2 and cytochrome P450 (*CYP*)2C epoxygenases. *EET*-induced activation of protein kinase A (*PKA*) results in the translocation of *TRP* channels to the plasma membrane to potentiate the activation on K_{Ca} channels initiated by the phospholipase C (*PLC*)-induced conversion of phosphatidylinositol 4,5-bisphosphate (*PIP_2*) to diacylglycerol (*DAG*). The overall consequence is hyperpolarization and vasodilatation. **b** While activating *TRPC6* in endothelial cells elicits vasodilatation, the same process in vascular smooth muscle cells does exactly the opposite as the TRPC6 channels in vascular smooth muscle cells (*VSMC*) primarily conduct Na^+, Ca^{2+} follows secondarily through voltage-gated Ca^{2+} channels or by the Na^+, Ca^{2+} exchanger (*NCE*). Reproduced from Loot and Fleming [99], with permission

was identified at alveolocapillary level, from which the hypoxic signal is propagated as endothelial membrane depolarization to upstream arterioles in an EET- and Cx40-dependent manner (Fig. 9.2; [99]).

The evidence for a vasodilator role of the arachidonic acid epoxides in humans is, needless to say, indirect and relies on the use of CYP inhibitors that cannot be guaranteed to be completely selective. That said, sulfaphenazole is one of the most selective inhibitors available for CYP2C9 [100], and while several studies failed to demonstrate any effects of sulfaphenazole on forearm vasodilatation in healthy subjects [86, 101, 102], a component of the flow-induced vasodilatation of skeletal muscle arterioles [103] and the radial artery [104–106], both of which have been shown to express CYP2C protein, is attenuated by the CYP inhibitor. Clearly, however, disease can affect responses as forearm vasodilator responses to acetylcholine could be blunted by CYP inhibitors in patients with hypercholesterolemia and reduced NO-dependent vasodilatation [107].

9.5.3 Hypertension and Atherosclerosis

Inhibition of the sEH increases intracellular levels of EETs, and thus prolongs their vasodilator and anti-inflammatory actions. Indeed, pharmacological inhibition of the sEH prevents angiotensin II-induced hypertension in rats and mice, and protects the kidney from hypertension-induced damage [44, 45, 52]. Furthermore, in humans increased sEH activity was associated with more advanced endothelial dysfunction and vascular inflammation [108].

Although the link between the sEH and cholesterol metabolism would make it logical to look at atherosclerosis, the situation is somehow less clear. Certainly, polymorphisms of the sEH have been linked with the risk of atherosclerosis and coronary heart disease [109–111]. Why this is the case is not known, but the initial report that sEH inhibitors can attenuate smooth muscle cell proliferation [112] most probably represented an off-target effect of the substance used [113]. In addition, some of the animal studies failed to deliver consistent results, and although inhibition of the sEH was reported to attenuate atherosclerosis, abdominal aortic aneurysm formation, and dyslipidemia by some researchers [114, 115], our group has been unable to detect clear effects. Furthermore, the effects on vascular remodeling are inconsistent with inhibition of the sEH preventing vascular remodeling in an inflammatory model but not in a blood flow-dependent model of neointima formation [116].

Most of the studies performed to date have focused on vascular smooth muscle cells, and the fact that monocytes express the sEH and a number of CYP enzymes has been largely overlooked. However, this area deserves much more attention as human and murine macrophages within atherosclerotic plaques express CYP2S1, a largely extrahepatic epoxygenase [117]. Interestingly, enzyme expression increased during monocyte differentiation to macrophages, and could be detected in classically activated or M1 macrophages and macrophages present in atherosclerotic plaques and inflamed tonsils, but not in macrophages polarized towards the M2 or alternatively-activated phenotype. Although the enzyme was able to accept several substrates and to generate bioactive epoxides from arachidonic acid, linoleic acid and EPA in an NADPH-dependent manner, perhaps from the macrophage polarization point of view the most relevant substrates seem to be prostaglandins G_2 and H_2 [117]. The resulting decrease in the immunomodulator prostaglandin E2 (PGE_2) would certainly be expected to result in a macrophage subtype with attenuated angiogenic potential, but whether or not the CYP2S1 product 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid actively contributes to inflammation remains to be determined.

9.6 Angiogenesis and Cancer

Given the fact that the activation of K_{Ca} channels has been linked to endothelial cell proliferation [118–120], and EETs activate K_{Ca} channels, it would seem logical to assume that K_{Ca} activation would play a role in EET-induced proliferation. However, although the activation of K_{Ca} channels has been linked to endothelial

cell proliferation induced by basic fibroblast growth factor [118], this mechanism appears to not be involved in the EET-induced proliferation of endothelial cells. The first hint that EETs may affect cell signaling and proliferation was obtained in renal epithelial cells [62, 121] and, soon afterwards, 'authentic EDHF' recovered from the luminal incubate of rhythmically stretched coronary arteries was found to activate a number of kinases, whose function was closely linked with endothelial cell proliferation [122]. Activation of these MAP kinases could be inhibited by treatment with CYP inhibitors, as well as by antisense oligonucleotides directed against CYP2C, and could be mimicked by the treatment of endothelial cells with 11,12-EET or by overexpression of CYP2C8 [122]. More detailed analysis of the mechanisms involved revealed that CYP epoxygenase-derived metabolites of arachidonic acid are able to transactivate the EGF receptor [123, 124]. 14,15-EET was initially suggested to act as a second messenger following activation of the EGF receptor; 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 metalloproteinases (MMP) [123]. Although the MMP involved has not yet been identified, a very similar mechanism seems to be responsible for the transactivation of the EGF receptor in endothelial cells [124]. The EET-mediated activation of the EGF receptor leads, in turn, to the activation of the kinase Akt and an enhanced expression of cyclin D1. All four EET regioisomers have been reported to elicit an increase in Akt phosphorylation and cell proliferation in murine endothelial cells, but only the proliferative effects of 5,6- and 14,15-EET are reportedly sensitive to a phosphatidylinositol 3-kinase (PI3-K) inhibitor, whereas the 8,9- and 11,12-EET-induced increase in [^{3}H] thymidine incorporation seems to be dependent on the activation of the p38 MAP kinase [125]. In contrast, in bovine aortic endothelial cells 8,9-, 11,12-, and 14,15-EET-induced cell proliferation can be attenuated by MEK, ERK, and PI3-K inhibition [126]. Other signaling pathways also contribute to the increase in cyclin D1 expression, including the MAP kinase phosphatase-1 (MKP-1), which decreases c-Jun N-terminal kinase (JNK) activity [127]. Activation of Akt by EETs also induces phosphorylation and therefore inhibition of the forkhead factors FOXO1 and FOXO 3a, and subsequently a decrease in the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [91]. The involvement of this mechanism in 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, both of which inhibit CYP2C9-induced endothelial cell proliferation [91]. 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 [128], it remains unclear whether these pathways are linked to each other or are simply activated in parallel.

The first link between EETs and angiogenesis was obtained in co-cultures of astrocytes and endothelial cells. EETs released from astrocytes increased thymidine incorporation into endothelial cells and elicited the formation of capillary-like

structures [129, 130]. Moreover, overexpression of CYP2C9 in, and/or the application of, 11,12- or 14,15-EET to monocultures of endothelial cells was associated with angiogenesis [124, 131]. *In vivo* data rapidly followed to support these *in vitro* findings, and EETs induced angiogenesis in the chick chorioallantoic membrane [124], as well as in EET-impregnated Matrigel plugs in adult rats [131] and an ischemic rat hindlimb model in which the overexpression of different CYP isozymes, including CYP2C11 and 2J2, was found to increase muscle capillary density [126]. Furthermore, tumor growth and metastasis can be increased by sEH inhibition in transgenic mice with high vascular EET levels, i.e. animals that overexpress either the human CYP2C8 or human CYP2J2 specifically in Tie-2-expressing cells, or that were treated with high concentrations of 14,15-EET [132]. All in all, such evidence indicated that activation of the CYP/sEH axis is linked with the promotion of angiogenesis; however, the latter models were somewhat artificial and focused on the products of arachidonic acid metabolism, largely ignoring the biological actions of other lipids that feed into the same CYP/sEH axis.

It was partly to assess the role of the sEH in angiogenesis under more physiological conditions that we determined the effects of the global and induced deletion of the sEH, as well as its pharmacological inhibition in vascular repair after ischemia and in the postnatal murine retina. We found that sEH deletion and inhibition resulted in markedly decreased angiogenesis [133] and vascular repair [47], and provided some of the first experimental data that linked the defect not to the accumulation of a PUFA epoxide but to the lack of diol production. To identify such lipids, liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based lipid profiling approaches are used to screen for the PUFA epoxides or diols most affected by the deletion of the sEH, and, to date, biological activities have been attached to the DHA-derived diol 19,20-dihydroxydocosapentaenoic acid [133] and the linoleic acid-derived diol 12,13-dihydroxyoctadecenoic acid [47]. Interestingly, the signaling pathways targeted by the diols are distinct, as, while the defective vascular repair in $sEH^{-/-}$ mice could be attributed to altered Wnt signaling followed by attenuated progenitor cell proliferation and mobilization [47], defects in the retina could be linked to the translocation of presenilin 1 out of lipid rafts and the subsequent inhibition of the γ-secretase [133]. This means that the take-home message with respect to angiogenesis is that the ω-3/ω-6 profile of a particular tissue is likely to determine the overall effects on angiogenesis. Certainly, while EETs have been well-defined as angiogenic mediators [132, 134], a DHA-derived epoxide was recently reported to inhibit angiogenesis by preventing phosphorylation of the vascular endothelial growth factor receptor-2 (VEGFR2) [135]. This is of relevance since the lipids that feed into the CYP/sEH axis are largely provided by the diet, and regulating dietary intake of specific lipids, e.g. the fish oils EPA and DPA, has been linked with altered epoxide and diol profiles, as well as protection against vascular inflammation and cancer. On the other hand, increased dietary intake of linoleic acid is generally associated with inflammation and increased risk. It will therefore be interesting to determine to what extent diet can alter the influence

of the CYP/sEH axis on angiogenesis and tumor growth [136, 137], as well as the development of the cardiovascular complications associated with the metabolic syndrome [136, 138].

To date, the CYP enzymes linked to angiogenesis have included the human 2C8/2C9 and 2J to enzymes, as well as the rat 2C11 and mouse 2c44 isoforms, all of which are epoxygenases. CYP1B1 is worth mentioning at this point, even though the enzyme is an estrogen-metabolizing CYP hydroxylase. CYP1B1 induction is an important factor in determining risk associated with hormone-mediated cancers, in particular as CYP1B1 is induced by hypoxia [139], probably because its expression is regulated by the AMP-activated protein kinase (AMPK) [140], and is involved in the metabolism of some clinically relevant anticancer agents [141]. In addition, CYP1B1 is tightly regulated by the angiogenic microRNA miR-27b [142–144]. The link to this particular microRNA is interesting as it has previously been described as a "regulator hub in lipid metabolism" [145]. 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 [145]. Whether or not CYP1B1 is involved in the latter is unclear but the enzyme has recently been linked with protection against angiotensin II-induced hypertension in female mice [146].

What makes CYP1B1 of interest in angiogenesis is that its deletion impaired revascularization in a model of oxygen-induced retinopathy in mice [147]. This effect was linked with a decrease in the expression of the endothelial NO synthase [148], as well as a corresponding increase in intracellular oxidative stress and increased production of thrombospondin-2, an endogenous inhibitor of angiogenesis [147, 149]. Interestingly, estrogen-induced angiogenesis has also been attributed to changes in endothelial NO synthase, 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 [150]. 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 [151]. Given that angiogenic endothelial cells undergo changes in metabolism, the so-called Warburg effect [152], it will be interesting to determine whether or not 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 [140].

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