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Action of epoxyeicosatrienoic acids (EETs) on cellular function

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Abstract

Epoxyeicosatrienoic acids (EETs), which function primarily as autocrine and paracrine mediators in the cardiovascular and renal systems, are synthesized from arachidonic acid by cytochrome P450 epoxygenases. They activate smooth muscle large conductance Ca^{2+}-activated K^+ channels, producing hyperpolarization and vasorelaxation. EETs also have antiinflammatory effects in the vasculature and kidney, stimulate angiogenesis, and have mitogenic effects in the kidney. Many of the functional effects of EETs occur through activation of signal transduction pathways and modulation of gene expression, events probably initiated by binding to a putative cell surface EET receptor. However, EETs are rapidly taken up by cells and are incorporated into and released from phospholipids, suggesting that some functional effects might occur through a direct interaction between the EET and an intracellular effector system. In this regard, EETs and several of their metabolites activate peroxisome proliferator-activated receptor (PPAR) α- and PPARγ, suggesting that some functional effects might result from PPAR activation. EETs are metabolized primarily by conversion to dihydroxyeicosatrienoic acids (DHETs), a reaction catalyzed by soluble epoxide hydrolase (sEH). Many potentially beneficial actions of EETs are attenuated upon conversion to DHETs, which do not appear to be essential under routine conditions. Therefore, sEH is considered as a potential therapeutic target for enhancing the beneficial functions of EETs.

Key words: soluble epoxide hydrolase, eicosanoids, dihydroxyeicosatrienoic acids, cytochrome P450, peroxisome proliferator-activated receptor

Arachidonic acid is converted to eicosanoid mediators by the cyclooxygenase, lipoxygenase and cytochrome P450 (CYP) monooxygenase pathways (5). The CYP pathway produces two types of eicosanoid products, the epoxyeicosatrienoic acids (EETs) formed by CYP epoxygenases and the hydroxyeicosatetraenoic acids (HETEs) formed by CYP ω-oxidases (123). Early studies indicated that the EETs produce important biological effects, particularly in the vascular and renal systems (52, 68, 103), but there was only limited interest in these compounds until the middle 1990s when they were shown to be synthesized in the endothelium and function as an endothelium-dependent hyperpolarizing factor (EDHF) under certain conditions in the coronary circulation (7, 51). Subsequent studies indicated that deletion of soluble epoxide hydrolase (sEH), the enzyme that converts EETs to dihydroxyeicosatrienoic acids (DHETs), decreased blood pressure in male mice (143), and treatment with a selective sEH inhibitor decreased blood pressure in hypertensive rats (171). These results suggested that inhibition of EET conversion to DHET might be a new therapeutic approach for hypertension. Interest was further heightened by the observations that EETs have antiinflammatory effects in the endothelium (120), stimulate angiogenesis (105,115), and prevent arterial smooth muscle migration (151). These findings are described in detail in a number of recent reviews (77, 131, 172).

Signal transduction pathways and transcriptional mechanisms involved in EET function have been identified (11, 56, 89, 150), and attempts are being made to isolate an EET membrane receptor that mediates these effects (144, 164). However, cells also rapidly take up EETs and incorporate them into phospholipids (4, 10, 149), suggesting the possibility of an intracellular mechanism of action. In this regard, heart fatty acid binding protein (H-FABP) binds EETs with K_d values that are only slightly higher than the K_d for arachidonic acid (161), implying that EETs

might bind to other intracellular proteins including transcription factors like peroxisome proliferator-activated receptors (PPAR) that contain fatty acid binding sites. These recent advances will be summarized in this review, with emphasis on the cellular mechanism and EET action, effects of sEH inhibition on these processes, and the potential role of EETs and their metabolites on PPAR-mediated gene expression.

EET PRODUCTION

The epoxygenases that synthesize EETs are primarily members of the CYP 2C and 2J classes. These enzymes are located in the endoplasmic reticulum, and they utilize arachidonic acid hydrolyzed from phospholipids when the Ca^{2+}-dependent type IV phospholipase A_2 is activated and translocated from the cytosol to intracellular membranes (53, 60). The CYP epoxygenases add an epoxide group across one of the four double bonds of arachidonic acid, forming four EET regioisomers, 5,6-, 8,9-, 11,12- and 14,15-EET, as illustrated in Fig. 1. Studies with purified CYP epoxygenases indicate that while each enzyme converts arachidonic acid to all four EET regioisomers, the main products in many cases are 11,12-EET and 14,15-EET (10). Endothelial cells express CYP2C9 and CYP2J2 and are the main source of EETs in the vascular system (51, 120, 132). Bradykinin or methacholine increase endothelial EET production 2- to 5-fold (7, 119), and shear stress also stimulates EET production by endothelial cells (76).

EETs are usually considered as a single entity, but in reality, they are eight separate compounds, each with somewhat different properties and functions. As shown in Fig. 1, there are four regioisomers, each stemming from one of the four double bonds of arachidonic acid. Although not shown in Fig. 1, each regioisomer actually represents two EET isomers because the epoxide group can attach at each of the double bonds in two different configurations, producing

R/S and S/R enantiomers of each EET regioisomer. To complicate matters further, the enantiomeric distribution of the same regioisomer produced by two different CYP epoxygenases can differ markedly; for example, 11,12-EET produced by human CYP2C8 is 82 % R/S, whereas the distribution produced by CYP2C10 is 69% S/R (24). Furthermore, two regioisomers produced by the same enzyme can have different stereochemical distributions. For example, CYP2J2 produces 11,12- and 14,15-EET. The 11,12-EET is a racemic mixture, whereas 76 % of the 14,15-EET is the R/S enantiomer (167).

The functional effectiveness of two enantiomers also can differ. As an illustration, 11(R),12(S)-EET relaxes small renal arteries preconstricted with phenylephrine, but 11(S),12(R)-EET is inactive. Likewise, 11(R),12(S)-EET but not the S/R enantiomer increases the activity of the large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in cell-attached patches of renal vascular smooth muscle cells (183). 14(R),15(S)-EET also is a better ligand than 14(S),15(R)-EET for binding to guinea pig mononuclear cells (164), but the other enantiomer, 14(S),15(R)-EET, is more potent in activating smooth muscle BK_{Ca} channels and dilating bovine coronary arteries (9). In contrast, no stereoselectivity was observed for EET-mediated dilation of canine or porcine microvessels (177). Another consideration regarding stereoselectivity is that the two enantiomers might have different functions. CYP2J2 expressed in human kidney forms equal amounts of both 11,12-EET enantiomers (167), but only 11(R),12(S)-EET produces relaxation of small renal arteries (183). However, in addition to vasorelaxation, EETs have anti-inflammatory and naturetic effects in the kidney (77,180), and it is possible that the S/R enantiomer might contribute to these effects. Thus, stereoselectivity is a very complex issue that complicates the investigation of some but not all aspects of EET function.

EET in Phospholipids

Small amounts of 8,9-, 11,12- and 14,15-EET are present in the plasma, liver and kidney, with 14,15-EET being the most abundant regioisomer (83-85). More than 90 % of the EET contained in rat plasma is present in phospholipids, mostly in the low-density lipoproteins. The EETs in human kidney cortex and rat liver are contained almost entirely in the *sn*-2 position of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). The main enantiomers are 8(S),9(R)-EET, 11(S),12(R)-EET and 14(R),15(S)-EET, which is similar to the distribution of R/S enantiomeric forms synthesized in the kidney and liver (83).

The presence of EETs in tissue phospholipids suggests that membrane lipid structural effects may be involved in some EET functions (10). Consistent with this possibility, PC containing 11,12-EET in the *sn*-2 position inhibits the open probability of the cardiac L-type Ca^{2+} channel reconstituted in a planar lipid bilayer (13). However, based on the values reported for rat liver phospholipids (84), EETs comprise only about 0.011% of the total fatty acyl chains in PC, 0.013% in PE, and 0.016 % in PI. While transient increases probably occur when cells are exposed to a bolus of EETs, it seems unlikely that the increase will be sufficient to have a generalized effect on membrane physical properties. On the other hand, perhaps the lipid microenvironment in localized domains might be perturbed sufficiently to produce a functional change, such as observed when the reconstituted L-type Ca^{2+} channel is exposed to PC containing 11,12-EET.

EET METABOLISM

Fig. 2a presents an overview of the EET metabolic pathways. This is a composite of results obtained primarily from incubations of radiolabeled EETs with cultured cells, including murine mastocytoma cells (4), rat astrocytes (142), porcine and human endothelial cells (46,155, 158), porcine arterial smooth muscle cells (44), human skin fibroblasts (40), and COS-7 cells (44). While Fig. 2a provides a general representation of EET metabolism, there are qualitative and quantitative differences among the four EET regioisomers and in the various cell types.

All EET regioisomers are incorporated into cell phospholipids, mostly into the *sn*-2 position, and are hydrolyzed from phospholipids by phospholipase A_2 (4, 47, 149). The main EET catabolic pathway is conversion to the corresponding DHET by sEH. This enzyme effectively utilizes 8,9-, 11,12- and 14,15-EET, whereas 5,6-EET is a poor substrate. A 16-carbon epoxy-fatty acid accumulates when either 11,12-EET or 14,15-EET undergoes partial β-oxidation. A 22-carbon product is formed from 11,12-EET and 14,15-EET by chain-elongation. However, β-oxidation and chain-elongation are prominent metabolic pathways only in cells with low inherent sEH activity or when a sEH inhibitor is added (39, 46). A methyl-terminal hydroxyl group can be inserted to 8,9-, 11,12- and 14,15-EET by CYP ω-oxidases (22). Fig. 2b shows the structures of these four classes of EET metabolites, DHET, the 16- and 22-carbon epoxides, and the ω-hydroxy-derivative, using 14,15-EET and its products as the example.

EETs can form glutathione conjugates (146). However, the functional significance of this reaction is questionable because the K_m for 14,15-EET, the best substrate for glutathione-S-transferase, is 10 μM.

Only 5,6- and 8,9-EET are substrates for cyclooxygenase. 5,6-EET is converted to a prostaglandin analog, 5,6-epoxy-prostaglandin (PG) E_1, which functions as a renal vasodilator

(12). 8(S),9(R)-EET can undergo only a partial cyclooxygenase reaction and is converted to 11-hydroxy-8,9-EET, a renal vasoconstrictor and mitogen for glomerular mesangial cells (73,178).

EET Incorporation into Cell Lipids

Incorporation of EETs into phospholipids occurs through a coenzyme A-dependent process (84, 158). The largest amount of EET is incorporated into PC, but in most cases PI contains a higher percentage of the 14,15-EET uptake than any of the other regioisomers (142, 155). Most of the radiolabeled EET is incorporated without chemical modification. However, small amounts of 11,12-DHET, 14,15-DHET, and a 22-carbon chain-elongation product of 14,15-EET, 16,17-epoxy-$\Delta^{6,9,12}$-docosatrienoic acid (16,17-EDT), have been detected in the phospholipids (39, 155, 158). A small amount 14,15-EET also is incorporated into endothelial and astrocyte triglycerides, and some unesterified 14,15-EET is present in astrocytes and endothelial cells (142, 155, 158). Likewise, a small amount of the 8,9-EET that is incorporated into arterial smooth muscle cells remains in unesterified form (44). The presence of intracellular unesterified EET suggests that EET binding to cytosolic FABP, which has been observed in vitro (161), might occur in the intact cell. Modeling of in vitro data suggests that binding to FABP may modulate the intracellular metabolism of EETs (162), as illustrated in Fig. 2a. The presence of unesterified EET also suggests the possibility that binding might occur to other intracellular proteins that contain fatty acid binding sites.

Incubations of endothelial cells with 14,15-EET and smooth muscle cells with 11,12-EET indicate that after these EETs initially accumulate in the cell lipids, they are continuously and progressively hydrolyzed and released into the extracellular fluid as DHETs (42, 155). This occurs under basal conditions, suggesting that any perturbation of membrane structural domains

or signaling properties that might occur as a result of EET incorporation into phospholipids is only transient. A much larger and faster EET efflux occurs when calcium ionophore A23187 is added to endothelial cells containing 14,15-EET (158, 159). The material released into the extracellular fluid remains largely as 14,15-EET if a sEH inhibitor is present (39). These results suggest that EETs might be temporarily stored in endothelial phospholipids and rapidly released as a bolus when the endothelium is exposed to an agonist (149). Such a mechanism could explain the potentiation of bradykinin-stimulated vasorelaxation produced by EETs (158).

β-Oxidation of EETs

As opposed to porcine cells that convert EETs almost entirely to DHETs (44, 155), cultured human endothelial cells, human vascular smooth muscle cells and human skin fibroblasts convert EETs mostly to chain-shortened β-oxidation products (40, 45, 46). This is consistent with the finding that cultured human coronary endothelial cells contain only $1/30^{th}$ the sEH activity of porcine coronary endothelium (46). Studies with mutant fibroblasts indicate that EET β-oxidation occurs in the peroxisomes (40). Although 18- and 14-carbon epoxy-fatty acids are formed, the most abundant β-oxidation product contains 16-carbons (46). As illustrated in Fig. 2b, 14,15-EET is converted primarily to 10,11-epoxy-$\Delta^{4,7}$-hexadecadienoic acid (10,11-EHD) by β-oxidation. Similarly, 11,12-EET is converted to 7,8-epoxy-$\Delta^{4,10}$-hexadecadienoic acid (40). The chain-shortened EET metabolites were not detected in earlier studies done with [1-^{14}C]EETs because the radiolabeled carboxyl carbon is removed in the first β-oxidation cycle. Detection of these metabolites required incubations with [^{3}H]EETs synthesized from [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, so that radioactivity remains in the products even though several carbons are removed from the carboxyl end of the EET. The 16-carbon

intermediates probably accumulate because they contain a Δ^4-*cis*-double bond (see Fig.2b for 10,11-EHD structure). Two additional enzymes, 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase, are necessary for β-oxidation to proceed through an intermediate that contains a Δ^4-*cis*-double bond (91), and it appears that these enzymes are rate-limiting for continued β-oxidation of 11,12-EET and 14,15-EET in the cells that so far have been studied (40, 46).

As opposed to cultured human endothelial and vascular smooth muscle cells where EET β-oxidation is a prominent process, the physiological role of β-oxidation in human vascular tissue is highly questionable in view of recent findings with surgical specimens (45). Human coronary artery and aortic segments converted 14,15-EET entirely to 14,15-DHET. Likewise, human saphenous vein segments converted 14,15-EET entirely to 14,15-DHET, whereas 10,11-EHD was the main product formed by endothelial and smooth muscle cells cultured from the saphenous vein. Western blots showed that freshly isolated human saphenous vein segments contain substantial amounts of sEH protein, whereas detectable amounts were not detected in cultured saphenous vein endothelial and smooth muscle cells. These data indicate that sEH expression decreases markedly when these human cells are grown in culture. Therefore, the high level of EET β-oxidation observed in human endothelial and vascular smooth muscle cells probably is an artifact of the cell culture conditions.

Porcine coronary artery endothelial cells, which contain high levels of sEH and ordinarily form DHETs, convert 11,12- and 14,15-EET to β-oxidation products when the cells are incubated with a selective sEH inhibitor (39). This provides additional evidence that β-oxidation becomes prominent only when the sEH activity is deficient. While β-oxidation appears to be an alternate pathway, there is some evidence that the 16-carbon products that accumulate have bioactivity. For example, 10,11-EHD is almost as potent as 14,15-EET in relaxing isolated

constricted porcine coronary arterioles and inhibiting cytokine-stimulated interleukin-8 production in human coronary endothelial cultures (46). However, 10,11-EHD does not retain the biological activity of EETs in all systems. For example, it is much less potent than 14,15-EET in dilating bovine coronary artery rings (35).

11,12-DHET also is catabolized by β-oxidation when it accumulates in smooth muscle cells. As in the case of the EETs, the main β-oxidation product formed is 7,8-DHHD, the corresponding 16-carbon dihydroxy metabolite that contains a Δ^4-*cis* double bond (42). Although 7,8-DHHD can relax porcine coronary artery rings, it is less potent than 11,12-EET. Therefore, the main function of this β-oxidation pathway appears to be removal of any residual DHET that is retained in the smooth muscle cells.

MECHANISM OF EET ACTION

EETs are autocrine and paracrine mediators that function primarily in the cardiovascular and renal systems. The generally accepted paradigm is that EETs are synthesized from arachidonic acid when cells that express a CYP epoxygenase, such as endothelial cells, are activated. The stimulus activates a cellular phospholipase A_2 that hydrolyzes arachidonic acid from the *sn*-2 position of phospholipids, and the released arachidonic acid is converted to EETs by the CYP epoxygenase. Support for this mechanism is provided by studies with blood vessel preparations showing that CYP epoxygenase inhibitors block EET-mediated vasodilation (131), implying that the EET is formed subsequent to activation of the cell. An alternative possibility that may operate in some circumstances is that the activated phospholipase releases preformed EETs stored in the phospholipids (149). This is consistent with the presence of EETs in hepatic and renal phospholipids (83,84), and the finding that radiolabeled EETs present in endothelial

phospholipids are rapidly released when the cells are exposed to calcium ionophore A23187 (39, 158, 159).

Regardless of the details of EET formation, the initial mechanistic steps that mediate the autocrine and paracrine effects of EETs remain uncertain. One possibility is that EETs bind to a membrane receptor linked to an intracellular signal transduction pathway that initiates the functional response. The other is an intracellular mechanism in which EETs or phospholipids containing newly incorporated EETs directly interact with and activate ion channels, signal transduction components or transcription factors that produce the functional response. It is likely that the actions of EETs are mediated by both mechanisms, thus accounting for their diverse effects.

Membrane Receptor Mechanism

Fig. 3 is a schematic illustration of the postulated membrane receptor mechanism. The key element is that the functional response is initiated by EET binding to a plasma membrane EET receptor. This activates signal transduction pathways that regulate ion channels or gene expression, producing a change in cell properties and function. Evidence supporting this mechanism was obtained from studies with human U937 cells, which contain a cell surface protein that functions as a high-affinity stereoselective binding site for 14(R),15(S)-EET (165). Guinea pig mononuclear cells have a similar high-affinity 14,15-EET binding protein which sediments with the particulate material of the cell homogenate (164, 166). 14,15-EET binding increases the intracellular cyclic adenosine monophosphate (cAMP) content and activates protein kinase A (PKA), resulting in down-regulation of the putative receptor (164, 165). Additional evidence for a mechanism involving a G-protein coupled receptor (i.e., a seven transmembrane

receptor) is provided by the observation that 11,12-EET induced activation of the BK_{Ca} channel and tissue-plasminogen activator (t-PA) expression is mediated by the $G\alpha_s$ component of a heterotrimeric GTP-binding protein (59, 95, 96, 121). Angiogenesis initiated by 11,12-EET also involves a cAMP-PKA signaling pathway that induces COX-2 expression (107).

Results obtained with a 14,15-EET-sulfonimide derivative covalently attached in amide linkage to a silica bead provide additional support for a membrane receptor mechanism. Previous work indicated that the 14,15-EET-sulfonimide derivative retains the biological activity of 14,15-EET (17). Further studies with rat aortic smooth muscle cells revealed that although the EET-bead complex was stable and the EET remained in the extracellular fluid during incubation, cAMP-induced aromatase activity was inhibited by the bead complex to the same extent as by 14,15-EET-sulfonamide in solution (144). The interpretation is that 14,15-EET inhibits cAMP-induced aromatase activity by acting at the cell surface.

In addition to the $G\alpha_s$-cAMP-PKA pathway, a number of other signal transduction mechanisms, shown in Fig. 3, have been found to be active in EET functional responses under various conditions. Activation of tyrosine kinase cascade, src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI-3K) /Akt pathways mediate actions of EETs in endothelial cells, arterial smooth muscle cells, glomerular mesangial cells, renal tubular epithelial cells, and myocardium (14, 15, 54, 72, 127, 140, 157). In addition, the antiinflammatory effect produced by 11,12-EET in the endothelium is due to inhibition of cytokine-activated nuclear factor-κB (NF-κB)-mediated transcription. This occurs by inhibition of IKK phosphorylation of IκBα (120, 150). The fact that other agonists typically activate these pathways through membrane receptor mechanisms provides indirect support for an EET receptor

mechanism similar to the illustration in Fig. 3, but so far, the putative EET receptor has not been conclusively identified.

Intracellular Mechanism

An alternative possibility is that EET enters the cell and produces functional effects by directly interacting with intracellular effectors as shown in Fig. 4. According to this proposed mechanism, the EET is present intracellularly as a result of uptake, hydrolysis of phospholipids that contain EET, or synthesis from arachidonic acid by a CYP epoxygenase. The intracellular EET directly interacts with FABP, ion channels or transcription factors that produce functional responses, or it is present in phospholipids that interact with membrane proteins or phospholipid-mediated signal transduction pathways.

This hypothesis is supported by biochemical and cell culture data, but the evidence is largely circumstantial. Although EETs are recovered in the extracellular fluid when they are either synthesized from arachidonic acid or released from phospholipids (132, 158, 159), they probably remain in the cells long enough to initiate an autocrine response. This is suggested by the observation that radiolabeled EET initially present in endothelial phospholipids continued to accumulate in the extracellular fluid for up 20 min after calcium ionophore A23187 was added (39), indicating that some of the EET hydrolyzed from the phospholipids probably remained in the cytosol for several minutes before being released to the medium. Likewise, cellular EET uptake from the extracellular fluid appears to be fast enough to initiate paracrine effects. For example, the incorporation of extracellular 14,15-EET into vascular smooth muscle cell phospholipids was observed within 3 min (41), and the conversion of the newly incorporated 14,15-EET to DHET, which takes place in the cytosol (174), also occurred after only 3 min (41).

Additional studies indicate the EETs can directly interact with cellular proteins. Patch clamp experiments show that EETs can directly interact with myocardial Na^+ and K_{ATP} channels (94, 100), and the latter finding is consistent with the fact that an EET binding site has been detected in the K_{ATP} ion channel (99). EETs also bind to intracellular proteins that contain fatty acid binding sites, including FABPs and PPARγ (97, 161). These findings support the possibility that EETs act through an intracellular mechanism as depicted in Fig. 4, but there is no conclusive evidence indicating that this actually occurs in vivo.

EET ACTIONS

EETs produce a number of diverse actions in a variety of tissues and cells. In vascular smooth muscle, they produce vasorelaxation and anti-migratory effects. The EETs are also quite active in endothelial cells where they have anti-inflammatory, angiogenic, fibrinolytic and Ca^{2+}-signaling effects. In addition, EETs have mitogenic effects in renal tubular and mesangial cells, produce bronchodilation, have anti-adhesive effects in platelets, and effect myocardial preconditioning and polypeptide hormone secretion. There is significant heterogeneity in the intracellular mechanisms that have been associated with these EET actions. Because the initial receptive events in EET signaling remain unknown, it has been often difficult to distinguish primary from secondary events in the cellular actions initiated by EETs. Table 1 summarizes the functions of EETs and the mechanisms reported to mediate these effects.

Vasodilation

Vasodilation is the most extensively studied EET function. The most potent effects of EETs occur in small resistance vessels; for example, 14,15-EET has been observed to produce

relaxation of isolated coronary microvessels at concentrations as low as 10 pM (39). This occurs through hyperpolarization and suggests that the EETs function as an EDHF in a number of vascular beds including the coronary circulation (7, 8, 51). A proposed mechanism is the EET is released by the endothelium and produces hyperpolarization by acting on the vascular smooth muscle (149). Two recent observations in coronary preparations support this mechanism. One is the finding that the EDHF response in bovine coronary arteries is inhibited by the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (61). The other is that EET is the transferable mediator of vasorelaxation in a perfused system consisting of donor and detector coronary arteries (62). An alternative possibility is that the EETs hyperpolarize the endothelium and this is transmitted to the smooth muscle by electrical coupling through myo-endothelial junctions or by release of K^+ into the intercellular space (50, 55). EDHF mechanisms that do not involve EETs also have been proposed, including the release of lipoxygenases products or hydrogen peroxide from the endothelium (50, 182). In addition to the uncertainty regarding mechanism, there also is a question as to whether the EDHF effect is functionally important under normal physiological conditions or only when endothelial nitric oxide and prostacyclin production are compromised.

Ion Channel Activation by EETs

EETs increase the open probability of the BK_{Ca} channel (75). This causes hyperpolarization of the vascular smooth muscle, producing vasorelaxation. An alternative mechanism proposed to underlie vasorelaxation is that EETs activate the TRPV4 Ca^{2+} channel leading to hyperpolarization and vasorelaxation by forming a Ca^{2+} signaling complex (33,55).

Endothelial TRPV4 Ca^{2+} channel also are activated by 5,6-EET and 8,9-EET (156), which may explain the finding that 5,6-EET is a second messenger for Ca^{2+} entry into endothelial cells (65).

Studies in the bovine coronary artery with 11,12-EET indicate that activation of the BK_{Ca} channel is mediated by $G\alpha_s$-protein in a process that involves ADP-ribosylation (95, 96). Likewise, 14,15-EET stimulates ADP-ribosylation in the liver (137), but the functional significance of this process is not known. Additional studies indicate that the $G\alpha_s$-protein also mediates BK_{ca} activation by 11,12-EET in human kidney cells (59). Based on these observations, the receptor-mediated BK_{Ca} activation mechanism illustrated in Fig. 3 includes $G\alpha_S$,but the possibility that EETs directly interact with the BK_{Ca} channel as shown in Fig. 4 cannot be excluded.

EETs activate BK_{Ca} channels in other tissues. This process occurs in platelets, decreasing platelet adhesion to the endothelium (90), and in airway smooth muscle, producing bronchodilation through hyperpolarization. Inhibition of smooth muscle Cl^- channels also is involved in the mechanism through which EETs produce relaxation of the airway smooth muscle (3, 31, 133, 134).

EETs are reported to affect other ion channels, including the K_{ATP}, Na^+, L-type Ca^{2+} channels. EETs bind to the myocardial K_{ATP} channel and thereby reduce its sensitivity to ATP by an allosteric alteration of the ATP binding site (99, 100). Activation of the mitochondrial K_{ATP} channels protects the myocardium against ischemia-reperfusion injury (140), suggests that myocardial preconditioning may occur through a direct interaction between EETs and the channel. However, activation of the p42/p44 MAPK pathway also appears to be involved in myocardial preconditioning (140), and studies in mice with targeted deletion of the sEH support a mechanism involving EET-mediated activation of the PI3K signaling pathways and K^+

channels (139). In addition to activating the myocardial K_{ATP} channel, EETs inhibit the myocardial Na^+ channel by decreasing the probability of channel opening (94). Likewise, the cardiac L-type Ca^{2+} channel reconstituted in a planar phospholipid bilayer is inhibited when PC containing 11,12-EET in the *sn*-2 position is present in the bilayer (13).

Antiinflammatory Effects of EETs

EETs produce an antiinflammatory effect on the endothelium by inhibiting cytokine-induced NF-κB transcription (120). 11,12-EET produces the most potent effect in bovine aortic endothelial cells. It inhibits IKK-mediated phosphorylation of IκBα, maintaining NF-κB in an inactive state (150). 11,12-EET also enhances fibrinolysis by activating t-PA gene expression through a cAMP-driven promoter. This involves a $G\alpha_s$-protein mediated signal transduction mechanism (121). Likewise, a cAMP-PKA signaling pathway mediates the inhibitory effect of 11,12-EET on rat aortic smooth muscle cell migration (151).

Angiogenesis

There is increasing evidence that EETs stimulate angiogenesis (55, 100, 107, 109). However, the signaling pathway that mediates this process appears to differ depending on the species, type of endothelium, and the EET regioisomer that initiates the process. A pathway involving activation of MAPK phosphatase-1 that inactivates c-Jun N-terminal kinase (JNK), leading to up-regulation of cyclin D1 and proliferation, occurs in human umbilical vein endothelial cells that overexpress CYP2C9 or are incubated with 11,12-EET (55, 117). However, other studies with 11,12-EET indicated that the angiogenic process is initiated by PI3K/Akt-

dependent phosphorylation and inactivation of the forkhead transcription factors FOXO1 and FOXO3a, which decreases the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (125). This pathway is activated by phosphorylation of the epidermal growth factor (EGF) receptor (108). Still another angiogenic signal transduction pathway has been reported for human umbilical vein endothelial cells, this one involving cAMP-PKA activation, COX-2 induction and PGI_2 synthesis (101, 106).

A pathway involving ERK1/2 phosphorylation also has been observed in porcine coronary endothelial cells incubated with 11,12-EET (54). This is consistent with the previous finding that 11,12-EET activates tyrosine kinase activity in porcine aortic endothelial cells (72).

A pathway involving MAPK, PI3K and Akt also mediates the angiogenic response in bovine aortic endothelial cells either engineered to overexpress CYP epoxygenases or treated with EETs (157). Likewise, these signaling pathways are involved in the angiogenic response in murine pulmonary endothelial cells, but the results are more complicated. 8,9-EET and 11,12-EET stimulate proliferation of the pulmonary endothelial cells through the p38 MAPK pathway, whereas the response to 5,6-EET and 11,12-EET occurs through PI3K activation (127). To complicate things further, only 5,6-EET and 8,9-EET promote endothelial cell migration, tube formation and in vivo neo-vascularization in mice (127), and though 11,12-EET is effective in stimulating the angiogenic response in most of the endothelial culture systems that have been studied, there is no evidence that it is active in vivo.

In summary, three signaling pathways appear to play a role in EET-mediated angiogenesis. This is summarized in Table 1 and illustrated schematically in Fig. 3. One is a cAMP-dependent pathway that activates the cyclicAMP response-element binding protein (CREBP) and COX-2 expression. This pathway is activated by EETs produced by CYP2C9, especially 11,12-EET (106). The second pathway that also is activated by EETs produced by

CYP2C9 involves PI3K and Akt, leading to an increase in cyclin D1 expression (55). Tyrosine phosphorylation of the EGF receptor is associated with this mechanism (108), as well as decreased expression of the cyclin D1 inhibitor $p27^{kip1}$ due to Akt-mediated phosphorylation of the forkhead transcription factors FOXO1 and FOXO3a (55). Another contributing factor to the increase in cyclin D1 expression is activation of MAPK phosphatase-1 that decreases JNK activity (55, 126). The third is a p38 MAPK pathway that is activated by 8,9-EET and 11,12-EET (127). Which of these pathways is operative probably depends on the species, type of endothelium, and EET regioisomers produced by the CYP epoxygenase. Furthermore, it is not known whether each of these pathways is activated by EET binding to the putative EET receptor, as depicted in Fig. 3, by direct interaction with EETs or membrane phospholipids as shown in Fig. 4, or as a secondary response to another effect of EET on the endothelium.

Mitogenesis

EETs stimulate mitogenesis of renal epithelial cells through a complex signal transduction mechanism. The most potent regioisomer is 14,15-EET. It activates cleavage of heparin-binding EGF-like growth factor (HB-EGF), which is a ligand for the EGF receptor (13, 16). This activates a tyrosine kinase signaling cascade initiated by Src kinase (14, 17), and metalloproteinases also are activated (16). In addition to stimulating the proliferation of renal cells, 14,15-EET appears to reinforce this response through an inhibitory effect on apoptosis in the renal epithelium. This is indicated by studies with LLCKPc14 cells transfected with a mutant bacterial CYP epoxygenase that produces only 14,15-EET. This prevented apoptosis of the LLCKPc14 cells through a mechanism involving activation of the PI3K/Akt signaling pathway

(15). 14,15-EET and 8,9-EET also stimulate mitogenesis in cultured rat glomerular mesangial cells. However, in these cells, the mechanism involves activation of Na^+/H^+ exchange (69).

Other Functions

EETs stimulate the secretion of several polypeptide hormones. 5,6-EET and 14,15-EET stimulates growth hormone release from somatotrophs (145), and 8,9-EET increases dopamine-stimulated somatostatin release from hypothalamic neurons (81). CYP2J2 and endogenous EETs are present in the endocrine pancreas, suggesting that EETs also might be involved in pancreatic hormone secretion (173). However, the pathways that are involved in producing these effects have not been determined.

FUNCTION OF OMEGA-3 EET ANALOGS

Eicosapentaenoic acid (EPA), the 20-carbon ω-3 analog of arachidonic acid (147), is converted to an epoxide derivative by human recombinant CYP2C epoxygenases with a catalytic efficiency similar to that of arachidonic acid (2). The main EPA epoxide derivative that is formed, 17(R),18(S)-epoxyeicosaquatraenoic acid (17,18-EEQ), is a potent activator of BK_{Ca} channels in arterial smooth muscle cells (93). Likewise, chemically synthesized EEQ regioisomers dilate canine and porcine coronary microvessels with EC_{50} values in the same range as those for the corresponding EET regioisomers (177). Furthermore, 11,12-EEQ was more potent than 11,12-EET in activating the cardiac K_{ATP} channel (102). These findings suggest that some functional effects of ω-3 fatty acids might be due to EEQ synthesis, or alternatively, to a reduction in EET synthesis because of competition between EPA and arachidonic acid for CYP epoxygenases. At present, however, there is no evidence that EEQs are produced in vivo or that

ω-3 fatty acid supplementation has any effect on EET formation either in an intact cell or experimental animal.

Epoxides have been chemically synthesized from docosahexaenoic acid (DHA), a 22-carbon ω-3 fatty acid (168). DHA is the most abundant ω-3 fatty acid in many tissues and accumulates to high levels in the brain (128, 147, 148, 163). The epoxydocosapentaenoic acids (EDPs) synthesized from DHA activate BK_{Ca} channels and dilate preconstricted porcine coronary arterioles (168). The EC_{50} range for vasodilation by the EDP regioisomers was 0.5 – 24 pM, and 13,14-EDP was 100-times more potent than 11,12-EET in activating the BK_{Ca} channels in coronary smooth muscle cells. Another epoxide derivative of DHA, 16,17-epoxy-docosatriene, is an intermediate in the pathway that produces neuroprotectin D1 in human ARPE-19 cells (113). However, 16,17- epoxy-docosatriene is formed as a result of a lipoxygenase reaction, not a CYP reaction, and there is no information as to whether it has vasoactive properties similar to an EDP.

SOLUBLE EPOXIDE HYDROLASE

The enzyme encoded by the *EPXH2* gene, sEH, hydrates the EET epoxide group to form the corresponding diol (117; 175). Fig. 2b illustrates this reaction, showing the conversion of 14,15-EET to 14,15-DHET. 14,15-EET is a better substrate for sEH than either 11,12-EET or 8,9-EET, and 5,6-EET is a poor substrate (174). sEH also exhibits selectivity for the most abundant 14,15-, 11,12- and 8,9-EET enantiomeric forms normally present in tissues, 14(R),15(S)-EET, 11(S), 12(R)-EET and 8(S),9(R)-EET (176). The enzyme functions as a homodimer (1, 63), and each subunit consists of two domains that have different enzymatic activities. The carboxyl-terminal domain contains the epoxide hydrolase activity, while the

amino-terminal domain is a Mg^{2+}-dependent lipid phosphatase (23, 118). Although dihydroxy lipid phosphates and polyisoprenyl phosphates involved in sterol synthesis are good substrates (118, 152), the physiological function of the lipid phosphatase activity is not known. Potent inhibitors of the epoxide hydrolase activity are now available (110), and lipid sulfates and sulfonates are being developed as inhibitors of the lipid phosphatase activity (152). The two domains function independently of one another, and inhibition of one activity does not affect the function of the other.

Linear rates of DHET formation were obtained with recombinant mouse sEH during incubations with racemic 14,15-EET and 11,12-EET for 1 and 4 min , respectively (162). The kinetic data were well fit by a Michaelis-Menten model with K_m = 2.5 μM and V_{max} = 38 μmol min^{-1} for 14,15-EET, and K_m = 0.45 μM and V_{max} = 9.2 μmol min^{-1} for 11,12-EET. K_m values in the range of 3 – 5 μM also have been reported for 14,15- 11,12- and 8,9-EETs (176), and the calculated catalytic efficiencies for 11,12-EET in these studies varied from 0.3 to 21 $\mu M^{-1}s^{-1}$ (162, 176). The higher values were obtained in media containing 100 μg/ml phospholipid vesicles to solubilize the EETs (176), as compared with media containing 30 nM bovine serum albumin (162). Porcine coronary endothelial cells converted 60 % of the available EET to DHET during a 1 h incubation with 2 μM 14,15-EET (39). During incubation of porcine aortic endothelial cells with 0.5 μM 14,15-EET, 50% of the DHET formed was produced in the first 10 min (155). Although all of the available EET was not converted to DHET, the amount of 14,15-DHET produced was linear in a 2 h incubation with 0.25 to 5 μM 14,15-EET (155). Endogenous EET concentrations have not been accurately determined, but the intracellular concentration of EET after exposure of platelets to thrombin has been estimated to reach levels as high as 1 μM (181). Based on this estimate, a simulated analysis using DynaFit with the production of 1 μM

EET at a rate of 0.01 s^{-1} indicated complete conversion to DHET in 6 min (162). This simulation suggests that the sEH activity is sufficient to rapidly hydrate the amounts of EET likely to be generated under physiological conditions.

The addition of H-FABP or liver (L)-FABP to incubations containing recombinant sEH reduces the amount of 11,12- or 14,15-EET converted to DHETs, implying that binding to FABP may protect these EET from catabolism and thereby prolong their intracellular action (162). In this regard the simulation described above indicated that about 35% of the released EET would remain as EET after 10 min if the intracellular H-FABP concentration was 1 μM (162). This suggests that FABP binding also might regulate the availability of EETs to the other intracellular metabolic pathways as illustrated in Fig. 2a, but no information presently is available to indicate that EETs binding to FABPs actually occurs in an intact cell.

Effect of Selective Soluble Epoxide Hydrolase Inhibitors on EET Metabolism and Function

Many potentially beneficial actions of EETs are attenuated when EETs are converted to DHETs. Therefore, as illustrated in Fig. 5, inhibiting sEH causes EETs to accumulate and be retained for longer periods after they are formed (39), presumably enhancing their beneficial autocrine and paracrine effects. Because DHETs have little or no activity as compared with the corresponding EETs in producing a number of functional effects (6, 17, 43, 48, 94, 100, 120, 137, 144, 161), it is generally assumed that inhibiting DHET formation should not impair any vital physiological processes. Consistent with this view, no toxicity was observed in sEH gene-deleted mice (143), and none was reported in hypertensive rats treated with several different sEH inhibitors (78, 171, 180). Pharmacologic inhibitors targeted to reduce DHET formation must be selective for sEH because mammals contain four other epoxide hydrolases that have important

metabolic and protective actions (117). However, this presents no difficulty because potent selective inhibitors are available (110), and they have been structurally refined to increase water solubility so they can be easily utilized for biochemical and animal experiments (86, 111).

Fig. 6 illustrates the structures of four urea derivatives that are selective sEH inhibitors and have been shown to be effective in biological systems. Administration of *N,N'*-dicyclohexylurea (DCU) decreased blood pressure in hypertensive rats (171). In endothelial cultures, DCU increased 14,15-EET retention in phospholipids and prevented 14,15-EET conversion to DHET after it was rapidly released from the phospholipids by exposure to Ca^{2+} ionophore A23187 (39). As the incubation with DCU continued, however, the 14,15-EET was progressively catabolized by conversion to chain-shortened β-oxidation products. A DCU derivative, 1-cyclohexyl-3-dodecylurea (CDU), potentiated vasodilation produced by 14,15-EET in human coronary arterioles (92). CDU also protected the kidney vasculature and glomerulus from hypertensive injury in angiotensin-induced hypertension (180), which is consistent with the finding that renal sEH is located primarily in the vasculature (170). Another derivative, 1-cyclohexyl-3-dodecanoylurea (CUDA), inhibited the conversion of 11,12- and 14,15-EET to DHETs in surgical specimens of human saphenous vein, coronary artery and aorta (45). A CUDA derivative in which the cyclohexyl group is replaced by an adamantanyl group, 1-adamantanyl-3-dodecanoylurea (AUDA), lowered blood pressure, increased the urinary EET/DHET ratio, and decreased macrophage infiltration in the kidneys of rats with salt-sensitive hypertension (78). AUDA also lowered blood pressure and increased urinary salt and water excretion in angiotensin-induced hypertension (80), and it decreased cerebral infarct size in spontaneously hypertensive rats following occlusion of the middle cerebral artery (29). Furthermore, AUDA augmented the antiinflammatory effect of EETs in endothelial cells,

probably by increasing EET-induced PPARγ transcriptional activity (97), and AUDA-butyl ester reduced the inflammatory response produced by lipopolysaccharide in mice (135). An AUDA analog, 1-admantanyl-3-cyclohexylurea (AUC), increased the response of the TRPV4 channel to 5,6- and 11,12-EET in mouse aortic endothelial cells (156). Thus, a number of compounds that are effective sEH inhibitors in intact cells, tissue specimens, and experimental animals are now available for investigational studies.

These results provide further evidence that sEH inhibition might be an effective approach for the treatment of hypertension and diseases associated with vascular inflammation (77, 135, 171). There was a concern based on cell culture data that sEH may not be an important pathway for EET metabolism in human tissues (40, 46), but this is much less of a concern because of the recent finding that DHET is the main EET metabolite produced by human blood vessel segments (45). Only one finding in an animal model suggests that sEH inhibition might not produce a beneficial response. When AUDA was injected into the cerebral ventricles of spontaneously hypertensive rats that have high sEH activity in the hypothalamus and brain stem, there was an unexpected substantial increase in blood pressure, and heart rate also increased (138). However, there is no indication that a similar effect would occur if a selective sEH inhibitor was administered systemically.

Recent data indicates that these substituted urea derivatives also produce effects through mechanisms other than sEH inhibition. AUDA has been observed to relax rat mesenteric resistance arteries through a direct action on the vascular smooth muscle that is dependent on the admantanyl group (122). CDU inhibits human aortic smooth muscle cell proliferation through a direct action that is independent of its inhibitory effect on sEH (25,26). In addition, AUDA and

CUDA activate mouse PPARα in a COS-7 cell expression system by a mechanism that is unrelated to sEH inhibition (37).

Functions of DHETs

The perception that DHETs have no vital biological function is supported by the findings that DHETs either are inactive or only minimally active as compared with the corresponding EETs in mediating the following functions: ADP-ribosylation (137), inhibition PGE_2 production in aortic smooth muscle cells (43), mitogenesis in kidney tubular cells (17), VCAM-1 expression in endothelial cells (120), inhibition of myocardial Na^+ channels (94), Ca^{2+} entry into aortic smooth muscle cells (48), activation of myocardial K_{ATP} channels (100), binding to H-FABP (161), and inhibition of cAMP-induced aromatase activity in aortic smooth muscle cells (144).

As opposed to these negative results, DHETs activity has been observed in a number of other biological systems. Bovine endothelial cells took up small amounts of DHETs available in the extracellular fluid and incorporated them into phospholipids, especially PC and PI (154). Furthermore, DHETs at a concentration of 1 μM inhibited the hydroosmotic effect of vasopressin (71), and at concentrations between 1 and 5 μM, they produced relaxation of porcine coronary artery rings constricted with a thromboxane mimetic (42, 158). Although 14,15-DHET also produced relaxation of bovine coronary artery rings, it was only 20 % as potent as 14,15-EET in this preparation (6). In contrast, DHETs produced relaxation of a canine coronary arteriole preparation with EC_{50} values in the range of 0.1 pM (124), and DHETs activated coronary smooth muscle BK_{Ca} channels at concentrations of 1 to 100 nM (101). DHETs, especially 11,12-DHET, produced relaxation in human coronary arterioles through a hyperpolarization mechanism (92). 14,15-DHET at concentrations between 3 and 10 μM

activated PPARα-mediated gene expression in transfected COS-7 cells (38), and all of the DHET isomers at a concentration of 5 μM inhibited the activation of PPARγ by rosiglitazone in transfected endothelial cells (97). Based on these findings, the general perception that DHET are inactive metabolites is incorrect, and it is possible that they might have important effects on vascular tone under conditions where EETs are rapidly converted to DHETs (41, 42, 101, 124, 158). However, there is no evidence that any of these DHET effects are essential for normal physiological function in vivo.

EFFECTS OF EETs AND RELATED CYP PRODUCTS ON PPAR-MEDIATED GENE EXPRESSION

The PPAR transcription factors are members of the nuclear receptor superfamily that are activated by fatty acids and fatty acid derivatives (49). PPARα (NR1C1), which is expressed primarily in liver, heart, skeletal muscle and kidney, regulates lipid utilization. PPARδ (NR1C2, also called PPARβ) is expressed in many tissues and functions in the control of fatty acid oxidation and energy uncoupling. PPARγ (NR1C3), which is expressed mainly in adipose tissue, intestine and macrophages, regulates adipocyte differentiation, lipid storage and insulin sensitivity. In addition, each of the PPARs has specific antiinflammatory properties when they are activated (49). Both PPARα and PPARγ are expressed in endothelial cells and blood vessels (27,28), indicating that they have a role in vascular function.

Polyunsaturated fatty acids, including the most abundant members of the ω-6 and ω-3 classes of essential fatty acids, activate each of the three types of PPARs (30, 32, 58, 64, 88, 116, 169). Saturated fatty acyl coenzyme A derivatives activate PPARα (32, 74). Conjugated linoleic acid also activates PPARα (112), but it can act both as an agonist and antagonist for PPARγ

depending upon the experimental context (66, 129). The synthetic sulfur-containing fatty acid analog, tetradecylthioacetic acid, activates human PPARs in the order of PPARδ > PPARα > PPARγ (160). Expression of either adipocyte-FABP or acylCoA binding protein in CV-1 cells decreased tetradecylthioacetic acid-induced PPAR transactivation, indicating that these binding proteins modulate the access of fatty acids to PPARs (70).

Polyunsaturated fatty acid metabolites produced by the cyclooxygenase and lipoxygenase pathways also function as PPAR ligands and activators. For example, 15-deoxy-$\Delta^{12,14}$-PGJ_2, a derivative of PGD_2, activates PPARγ (57, 87), and 8(S)-HETE is a potent activator of PPARα (32, 58, 169). Likewise, the arachidonic acid lipoxygenase products 12-HETE and 15-HETE, and the linoleic acid lipoxygenase products 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE, activate PPARγ (32). In addition, nitrolinoleic acid, which is formed by reaction of linoleic acid with nitric acid, activates all three PPARs, with the most potent effect being on PPARγ (136). The importance of these polyunsaturated fatty acid products in the activation of the PPARs under physiologic or pathologic conditions is debated.

Because the PPARs bind an assortment of natural lipid products, it has been suggested that they serve as "generic" sensors for fatty acids and related products. However, there is evidence for the existence of high-affinity, as yet unidentified endogenous activators that are essential to some biological processes involving PPARs, such as for PPARγ-mediated adipocyte differentiation (153). Thus, additional naturally occurring lipid metabolites likely function as endogenous PPAR activators. Although EETs are a logical possibility considering their structural similarity to HETEs and HODEs, they have been largely ignored because of the finding that 8,9-EET is only 13% as effective in activating PPARα as pirinixic acid (Wy-14643), the widely used fibrate agonist (58). However, the possibility that EETs might function as endogenous PPAR

activators should be reconsidered in view of recent results demonstrating that EETs and several EET metabolites bind to the isolated ligand-binding domain of PPARs and activate PPAR-mediated gene expression in cultured cell systems (22, 38, 97).

PPARγ Activation by EETs

PPARγ is activated when bovine aortic endothelial cells are exposed to laminar flow through in a process that is dependent on phospholipase A_2 and CYP epoxygenases (98). This results in suppression of cytokine-induced NF-κB activation and intercellular adhesion molecule (ICAM)-1 expression. A lipid extract of the flow medium also activated PPARγ and suppressed NF-κB activation and ICAM-1 expression. Subsequent results indicated that laminar flow caused a substantial increase in 8,9-, 11,12- and 14,15-EET in the endothelial cells within 15 min (97), suggesting that EETs might be the active component of the lipid extract. Furthermore, addition of the selective sEH inhibitor AUDA to the perfusion medium enhanced PPARγ activity stimulated by laminar flow, while over-expression of sEH reduced PPARγ activity. AUDA also enhanced the inhibitory effect of EETs on TNF-α mediated IκBα degradation (97), which explains the decrease in NF-κB-stimulated expression of ICAM-1. Furthermore, a PPARγ antagonist blocked the antiinflammatory effect of laminar flow to inhibit TNFα mediated IκBα degradation. Additional studies demonstrated that the ligand-binding domain of PPARγ binds 8,9-, 11,12- and 14,15-EET with K_d values between 1.1 and 1.8 μM (97). Taken together, these findings have been interpreted to indicate that EETs mediate the antiinflammatory effect of laminar flow on endothelial cells and that the mechanism may involve EET binding and activation of PPARγ. They also suggest that selective sEH inhibitors will potentiate the anti-

inflammatory effect in the endothelial cells, presumably by increasing the retention of 11,12- and 14,15-EET so that PPARγ activation is prolonged.

PPARα Activation by EETs, EET Derivatives and other CYP Products

Although EETs are weak activators of PPARα, the ω-hydroxylated derivatives of 11,12- and 14,15-EET are potent activators (22). These EETs derivatives are produced by CYP ω-oxidases, another class of CYP monooxygenases that utilize fatty acids as substrates. These enzymes insert a hydroxyl group at or near the methyl-terminal end of the fatty acid chain in a NADPH-dependent reaction (10).

8,9-, 11,12- and 14,15-EET are good substrates for CYP4A1 and CYP4A2 and are converted to 20-OH-EETs by these enzymes (22). The conversion of 14,15-EET to 20-OH-14,15-EET by a CYP ω-oxidase is illustrated in Fig. 2b. In a parinaric acid displacement assay used to measure the relative affinities of various compounds for the ligand-binding domain of PPARα, the K_i values for the EETs were between 22 and 46 nM. In contrast, the K_i for 20-OH-14,15-EET was only 3 nM (22). Furthermore, in RK13 cells that overexpress either the human or mouse PPARα gene, 20-OH-14,15-EET increased PPARα-mediated gene expression to the same extent as Wy-14643, and 20-OH-11,12-EET also increased PPARα-mediated gene expression in these cells (22).

The DHET derivatives of EETs, also activate PPARα. Studies in transiently transfected COS-7 cells containing a luciferase expression system demonstrated that 14,15-DHET at concentrations between 3 and 10 μM was as potent as Wy-14643 in activating mouse PPARα (38). The kinetics of activation produced by 14,15-DHET and Wy-14643 were similar. A 4-fold increase in luciferase activity occurred after 3 h, and this increased to 9-fold after 6 h. 14,15-

DHET was 3- to 4-times more potent than any of the other DHET isomers, and like 20-OH-14,15-EET (22), 14,15-DHET produced a small increase in PPARα-mediated gene expression when the extracellular concentration was as low as 1 μM (38). Small amounts of 14,15-DHET were incorporated into the COS-7 cells, and 14,15-DHET was bound by the ligand binding domain of PPARα with a K_d of 1.4 μM. In addition, incubation of 14,15-DHET with HepG2 cells containing the transfected mouse PPARα gene increased the production of carnitine palmitoyl transferase 1A (CPT1A) mRNA, but the increase was only half as much as that produced by Wy-14643.

These findings suggest that the ω-hydroxy-EET derivatives and 14,15-DHET might be endogenous activators of PPARα (22, 38). The production of these metabolites in the vascular system could contribute to the antiinflammatory effect of EETs because PPARα is expressed in the endothelial and vascular smooth muscle cells (27,28). While this is an attractive hypothesis, it is uncertain whether the intracellular concentrations of either ω-hydroxy-EETs or 14,15-DHET will reach high enough levels to activate PPARα if they are generated endogenously from EETs.

Arachidonic acid also is a substrate for CYP ω-oxidases of the 4A and 4F classes and is converted primarily to 20-HETE (10, 19). Like EETs, 20-HETE functions as a lipid mediator in the vascular and renal systems (10, 34, 82, 89, 104, 131). 20-HETE activates mouse PPARα and mouse PPARγ in a transfected COS-7 cell gene expression system (36, 67). CYP ω-oxidases and alcohol dehydrogenases further oxidize 20-HETE to 20-carboxy-arachidonic acid (20-COOH-AA), and this reaction occurs in endothelial cells, vascular smooth muscle cells and renal tubular epithelial cells (20, 34, 36, 82). 20-COOH-AA also activates PPARα and PPARγ in the COS-7 cell gene expression system (36). EPA, the ω-3 fatty acid analog of arachidonic acid, also is converted to 20-OH-EPA by CYP4F3B, and 20-OH-EPA is 10-times more potent than EPA in

activating PPARα-mediated gene expression in the COS-7 cells (67). However, at concentrations between 1 and 20 μM, 20-OH-EPA was only 20 to 75 % as potent as Wy-14643 in activating PPARα in this system (67). It is not known whether 20-OH-EETs, like 20-HETE, can be converted to 20-carboxy-EET derivatives.

PPARα Activation by Soluble Epoxide Hydrolase Inhibitors

Two of the compounds currently being tested as selective sEH inhibitors recently were found to activate PPARα (37). CUDA stimulated mouse PPARα-mediated gene expression in transiently transfected COS-7 cells, and binding studies indicated that CUDA displaces Wy-14643 from the ligand binding domain of PPARα. CDU, which is structurally similar to CUDA except that it contains a *N*′-dodecyl rather than dodecanoic acid chain (Fig. 6), did not activate PPARα. This implies that a hydrocarbon chain containing a terminal carboxyl group is required for activation, and this conclusion is supported by the finding that AUDA, which also contains a *N*′-dodecanoic acid chain (Fig. 6), stimulated PPARα activity in the COS-7 cell system. However, AUDA was less potent than CUDA, indicating that the *N*-cyclohexyl group is more favorable for interaction with PPARα than the *N*-adamantanyl group. The *N*′-dodecanoic acid chains of CUDA and AUDA are progressively shortened by β-oxidation during incubation with the COS-7 cells, and the potency of CUDA as a PPARα activator decreased substantially when the chain was shortened to 8-carbons, and the intermediate containing a 6-carbon chain was inactive (37). These data indicate that effects mediated by PPARα activation should be excluded before concluding that functional responses produced by either CUDA or AUDA are due to sEH inhibition.

Effects of PPARα Activation on EET Production and Metabolism

Interestingly, activation of PPARα modulates the expression of CYP enzymes that produce and metabolize EETs. However, the effects on the expression of CYP arachidonic acid-epoxygenases appear to vary in different tissues. In rats, treatment with fibrates reduced the level of CYP2C11 expression in the liver (21, 130, 141). This effect could be recapitulated in cultured hepatocytes, but only when the cells were transfected with PPARα. Using reporter plasmids, the segment of the promoter responsible for the effect was identified to the region immediately upstream of CYP2C11 (130). In contrast, the deficient levels of CYP2C11 and CYP2C23 in the kidney microvasculature of obese Zucker rats were restored by administration of the PPARα activator fenofibrate (179). Epoxygenase activity and acetylcholine-induced vasorelaxation in the renal vessels were simultaneously restored, and a CYP epoxygenase inhibitor blocked this effect (179). It is not known whether this effect is mediated by PPARα or by another action of fenofibrate. Fenofibrate also induced expression of CYP2C23 and the ω-hydroxylase CYP4A in the kidney of a transgenic rat model of hypertension (114). CYP4A is a classic target of PPARα, and it is increased by fibrates in the liver as well as in the kidney (22, 80, 114). Accordingly, production of 20-OH-EET was induced in the kidneys of the fenofibrate treated animals (114).

CONCLUSIONS AND FUTURE DIRECTIONS

EETs are one of the substances that function as an EDHF in a number of vascular beds, including the coronary and renal circulations. The EETs produce vasorelaxation by a mechanism that involves hyperpolarization of the smooth muscle through activation of the BK_{Ca} channels. Potent selective sEH inhibitors are available that prolong the action of EETs and decrease blood

pressure in animal models. sEH inhibitors decrease DHET formation, and some functional effects of DHETs have been observed in experimental systems. However, adverse effects with sEH inhibitors have not been observed in vivo, and this approach is progressing to the point where translational studies are being contemplated.

Other potentially beneficial actions of EETs have been noted in cell culture systems. These include antiinflammatory, angiogenic, fibrinolytic and Ca^{2+} signaling effects in endothelium, an anti-migratory effect in vascular smooth muscle, and activation of PPARα and PPARγ in gene expression systems. The evidence supporting many of these actions is less compelling and requires additional confirmation and further exploration of mechanism. Part of the confusion is due to the fact that EETs are comprised of four distinct regioisomers, each with two R/S enantiomeric forms, and that the arachidonic acid CYP epoxygenases produce a mixture of these compounds, many of which likely have different quantitative and qualitative actions. These differences will have to be sorted out by comprehensive investigation. Furthermore, the recent findings regarding the production of ω-3 analogs of EETs and the potency of these compounds open up a new area of investigation that may explain some of the biological actions of dietary ω-3 fatty acid supplements.

It is likely that many of the most potent EET actions occur through a seven transmembrane receptor coupled via $G\alpha_s$ protein to cellular signal transduction systems. Therefore, a pressing need is to identify, clone and express the putative EET receptor and determine its coupling to the intracellular signaling pathways. Because there are four regioisomers, it is possible that more than one EET membrane receptor exists and that each might be coupled to a different signal transduction pathway. Identification of *bona fide* EET receptors will greatly facilitate precise dissection of the proximal events in EET-induced

signaling, help untangle the myriad of signaling events, and potentiate the discovery of EET mimetics with favorable pharmacologic properties.

Other actions are likely to occur through direct interaction of EETs with intracellular effector systems or transcription factors. EETs are rapidly taken up by many different types of cells and incorporated into phospholipids. Further studies are needed to determine which subcellular membranes contain these phospholipids, if they are clustered in domains, and whether they perturb membrane proteins or phospholipid signaling pathways. EETs bind to FABPs, suggesting that possible modulatory effects depending on intracellular free fatty acid availability should be explored. Likewise, the recent findings that EETs and their metabolites are endogenous PPAR ligands are very preliminary and require further studies in more relevant physiological systems.

While the current translational emphasis is on the antihypertensive action of the EETs, the many other functions that have been observed at the cellular and biochemical levels suggest that these biomediators are likely to have beneficial effects on other physiological processes. In particular, the simultaneous vasodilating and antiinflammatory effects of the EETs on the vasculature may hold promise in the prevention or treatment of atherosclerosis, either through sEH inhibition or administration of EET mimetics.

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Figure Legends

Fig. 1. EET regioisomers synthesized from arachidonic acid by CYP epoxygenases. The structure of arachidonic acid shows the conventional numbering of the carbon atoms that form its four double bonds. The main mammalian CYP epoxygenases, which are members of the 2C and 2J classes, can add an oxygen atom across each of the double bonds, producing four separate EET regioisomers. Although these epoxygenases synthesize all four EETs when they oxidize arachidonic acid, most of the enzymes produce substantial amounts of only two or at most three of the regioisomers.

Fig. 2. EET metabolic pathways. The diagram in Fig. 2a provides an overview of EET metabolism, though there are quantitative and qualitative differences among the four regioisomers. The main pathways are (i) incorporation into phospholipids through an acyltransferase reaction requiring ATP and coenzyme A (CoA), (ii) phospholipase A_2 (PLA_2)-catalyzed hydrolysis from phospholipids, and (iii) hydration to form the corresponding diol by soluble epoxide hydrolase (sEH). β-Oxidation and chain-elongation occur to an appreciable extent only when EET begins to accumulate intracellularly because the sEH activity either is inherently low or is inhibited. These two pathways have been demonstrated only with 11,12- and 14,15-EET. 8,9-, 11,12- and 14,15-EET can undergo ω-oxidation, and 5,6- and 8,9-EET are converted to bioactive products by cyclooxygenase (COX). Fig 2b illustrates four of the metabolic products where the EET is structurally modified, using 14,15-EET and its products for illustration. These are chain elongation (16,17-EDT), hydration (14,15-DHET, ω-oxidation (20-OH-14,15-EET) and β-oxidation (10,11-EHD).

Fig. 3. Membrane receptor mechanism of EET action. The key element in this mechanism is EET binding to a putative plasma membrane EET receptor that activates various intracellular signaling pathways to elicit a functional response. The intracellular signaling pathways are shown in different colors ttto indicate that each is active in different tissues under unique conditions. There is evidence that EETs utilize cAMP and tyrosine kinase cascade signal transduction mechanisms. Activation of BK_{Ca} channels occurs through a $G\alpha_s$-protein coupled to the putative receptor. While the cAMP-PKA, PI3K-Akt, MAPK and src-kinase pathways produce responses by activating gene expression, the anti-inflammatory effect produced by the IKK pathway is due to inhibition of cytokine-induced NF-κB activation.

Fig. 4. Intracellular mechanism of EET action. The key element in this mechanism is direct activation of the response by intracellular EET, rather than through cell surface receptor-mediated activation of a second messenger pathway. Autocrine responses are produced by EETs synthesized from arachidonic acid (AA) or released from intracellular phospholipids by phospholipase A_2. Paracrine effects are produced by uptake of the EET released into the extracellular fluid from an adjacent cell. A pool of EET is maintained in the cytosol through binding to FABP and is available for direct interaction with ion channels, components of signal transduction pathways and transcription factors. Alternatively, the EETs are incorporated into phospholipids that interact with ion channels or activate phospholipid-dependent signaling mechanisms. Channel abbreviations: BK_{Ca} - large conductance calcium-activated potassium channel; K_{ATP} - ATP-sensitive potassium channel; TRPV4 - transient receptor potential cation channel, subfamily V, member 4.

Fig. 5. Effects of sEH inhibitors on EET function. Because DHET formation is inhibited, EET produced from arachidonic acid by CYP epoxygenase, released from intracellular phospholipids, or taken up from an extracellular source accumulates intracellularly. As a result, higher concentrations of EET are available for a prolonged period to enhance autocrine or paracrine functional responses.

Fig. 6. Selective soluble epoxide hydrolase inhibitors that are effective in intact cells and experimental animals.

Table 1. *Functions of EETs*

Function	Mechanisms	References
Angiogenesis	p38 MAPK	54, 105, 107
	PI3K, Akt, cyclin D1	55, 109, 125, 126, 127, 157
	cAMP, PKA, COX-2	106
Antiinflammatory	Inhibition of IKK	120
Apoptosis	PI3K, Akt	15
Bronchodilation	BK_{Ca} channel	3, 31
	Cl^- channel	133, 134
Ca^{2+} signaling	TRPV4 channel	156
Fibrinolysis	t-PA expression, $G\alpha_s$, cAMP	121
Mitogenesis	Src kinase, EGFR, MAPK	13, 14
	PI3K, Akt	
Myocardial preconditioning	K_{ATP} channel, p42/p44 MAPK	140
	PI3K	139
Platelet anti-aggregation	BK_{Ca} channel	90
Polypeptide hormone secretion	Not determined	81, 145, 173
Smooth muscle anti-migratory	cAMP, PKA	151
Vasodilation	BK_{Ca} channel	7, 8, 51, 59,
	$G\alpha_s$, ADP-ribosylation	61, 75, 95
	TRPV4 channel	55

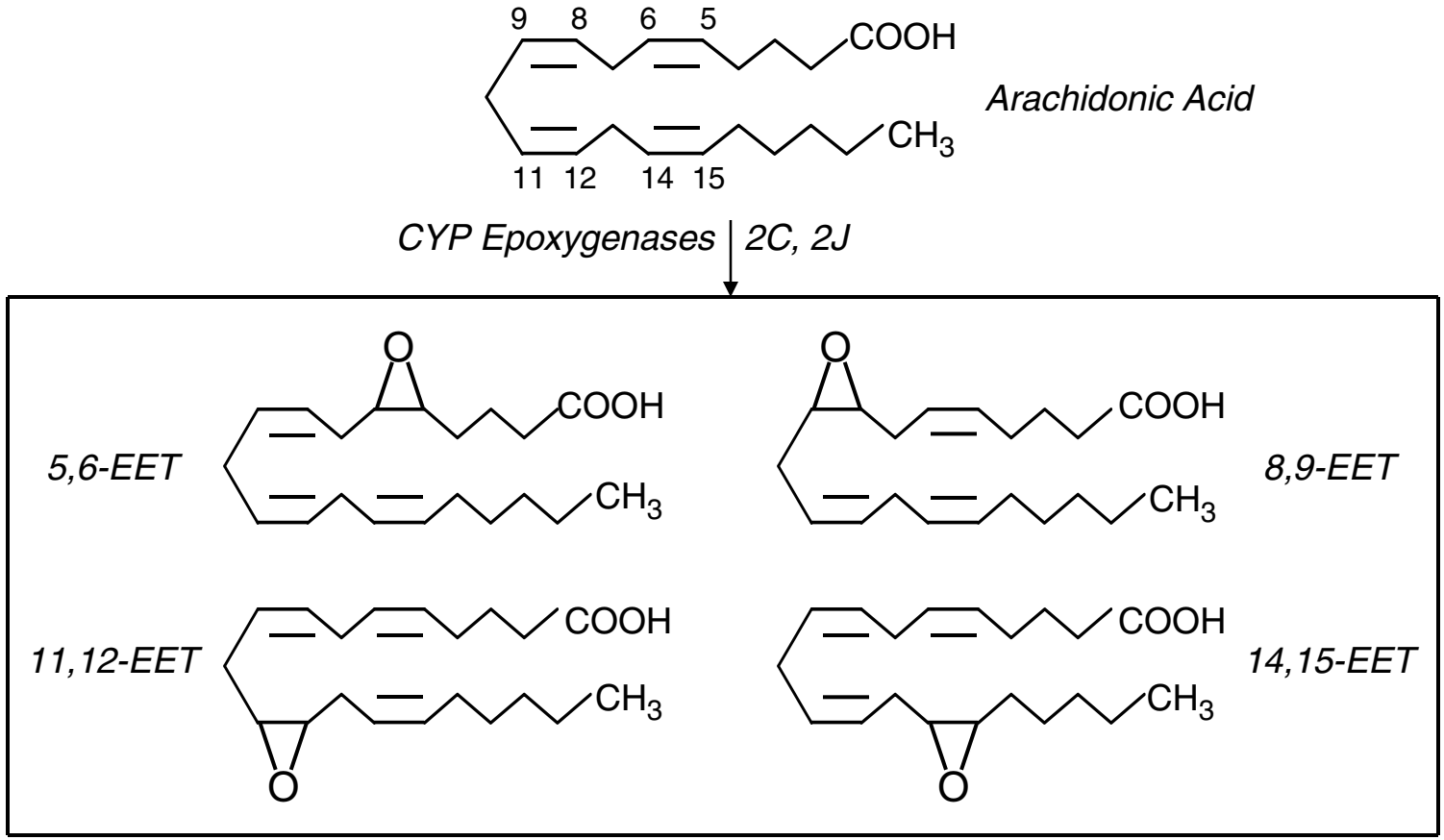

9 8 6 5
COOH
CH3
11 12 14 15
Arachidonic Acid
CYP Epoxygenases
2C, 2J
5,6-EET
8,9-EET
11,12-EET
14,15-EET
O

Figure 1

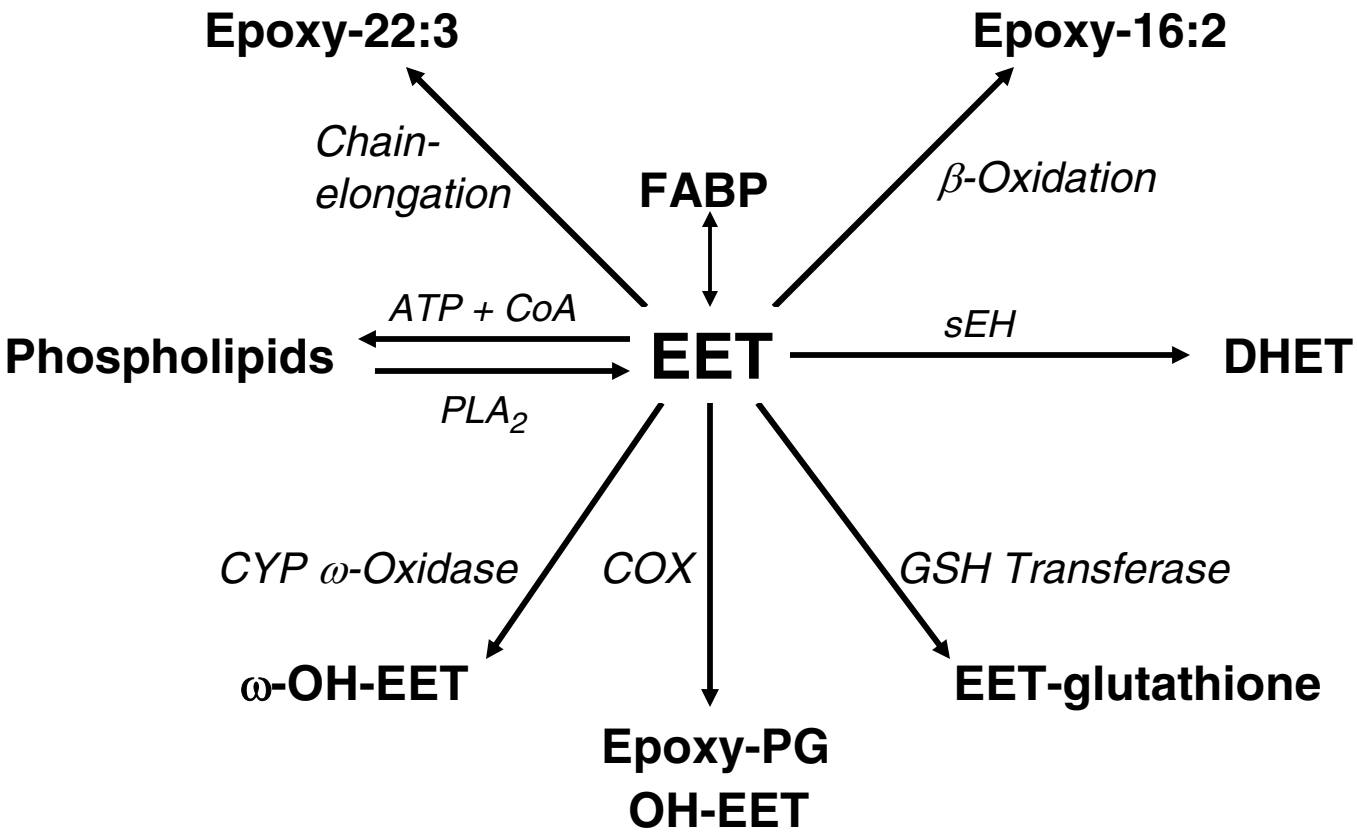
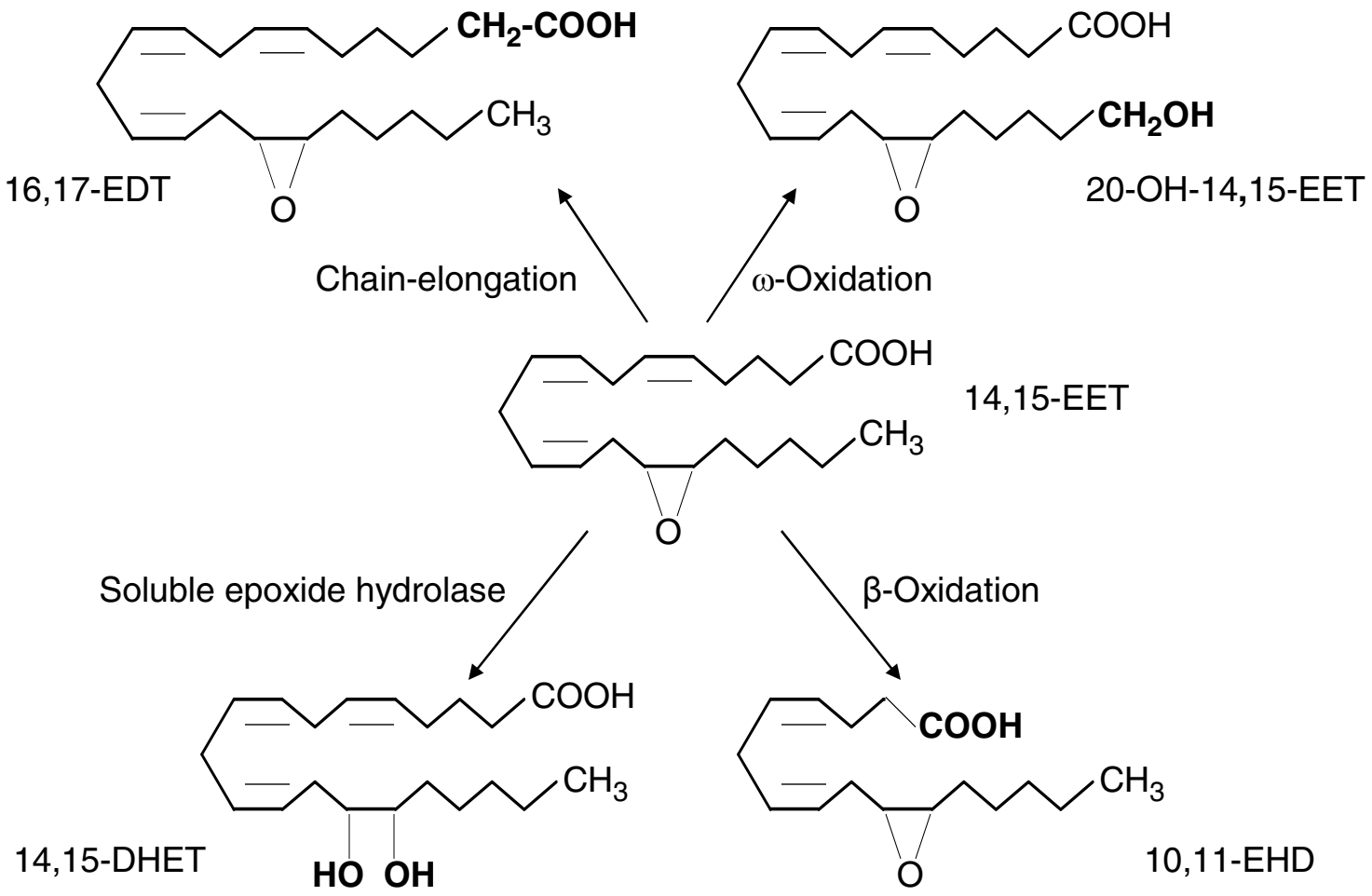

Epoxy-22:3
Epoxy-16:2
Chain-elongation
FABP
β-Oxidation
ATP + CoA
sEH
Phospholipids
EET
DHET
PLA2
CYP ω-Oxidase
COX
GSH Transferase
ω-OH-EET
EET-glutathione
Epoxy-PG
OH-EET

Figure 2a


CH2-COOH
CH3
16,17-EDT
O
COOH
CH2OH
20-OH-14,15-EET
O
Chain-elongation
ω-Oxidation
COOH
14,15-EET
CH3
O
Soluble epoxide hydrolase
β-Oxidation
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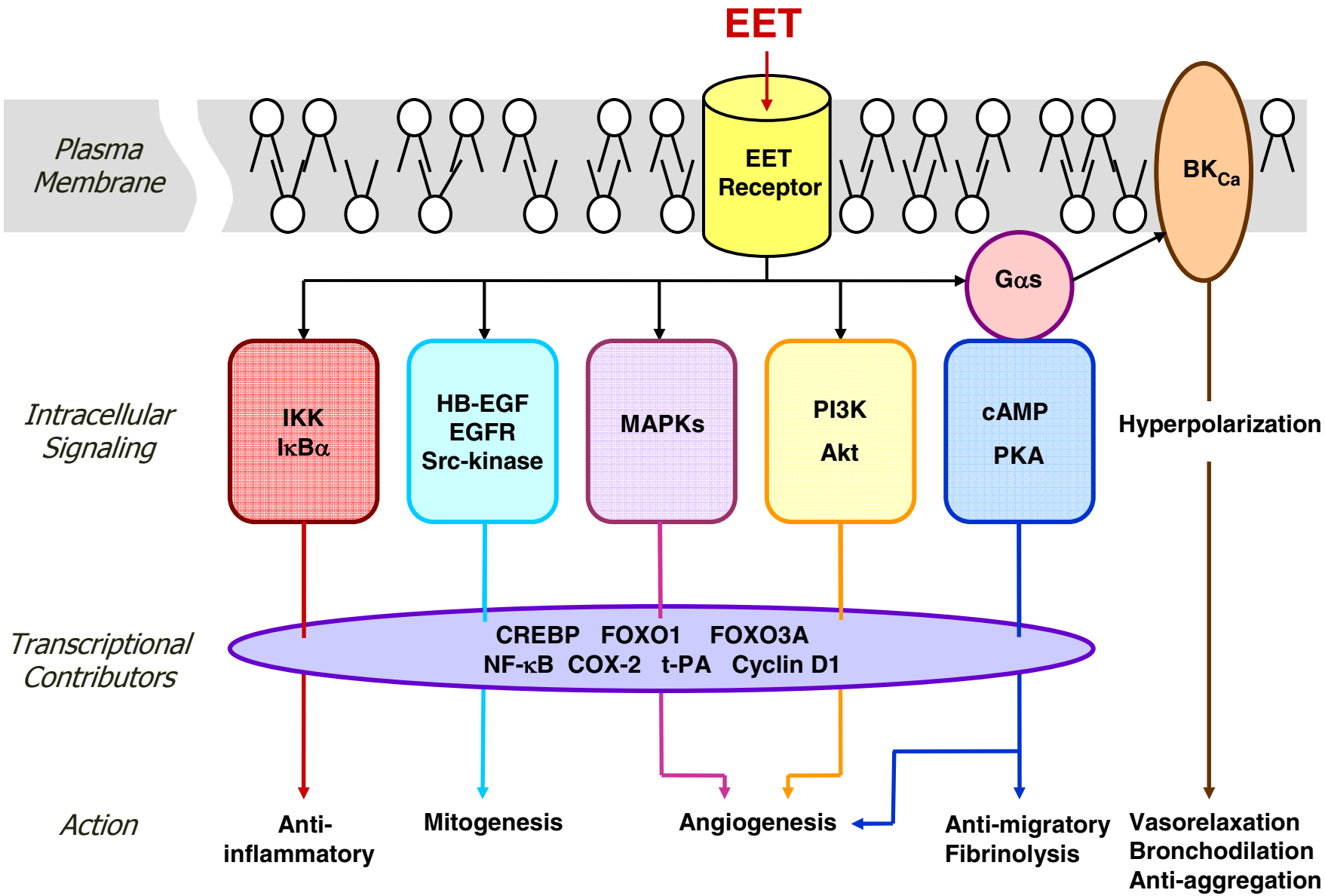
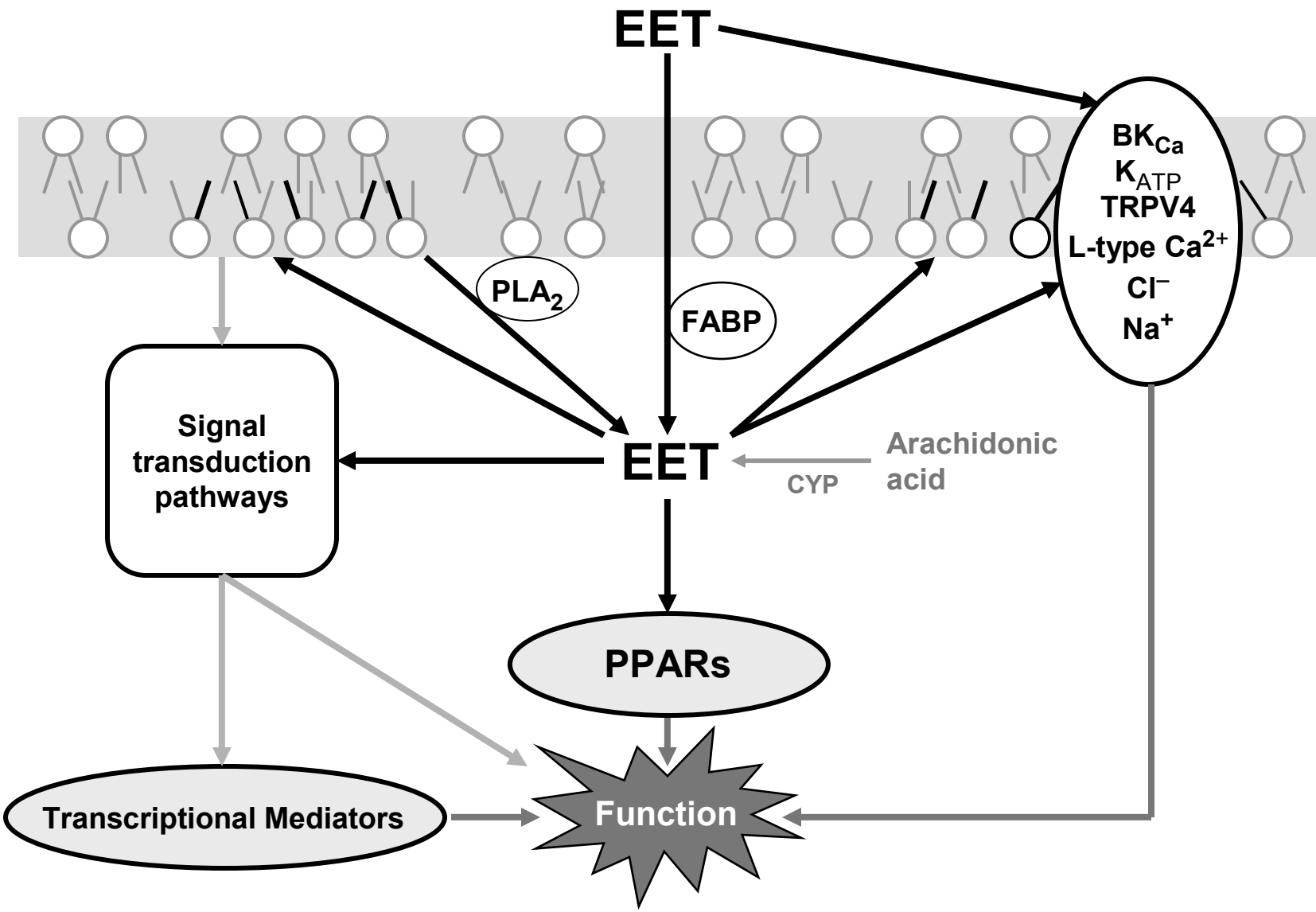
CH3
14,15-DHET
HO OH
COOH
CH3
O
10,11-EHD

Figure 2b

Figure 3


EET
Plasma Membrane
EET Receptor
BKCa
Gαs
Intracellular Signaling
IKK IκBα
HB-EGF EGFR Src-kinase
MAPKs
PI3K Akt
cAMP PKA
Hyperpolarization
Transcriptional Contributors
CREBP FOXO1 FOXO3A
NF-κB COX-2 t-PA Cyclin D1
Action
Anti-inflammatory
Mitogenesis
Angiogenesis
Anti-migratory Fibrinolysis
Vasorelaxation Bronchodilation Anti-aggregation


EET
BKCa
KATP
TRPV4
L-type Ca2+
Cl−
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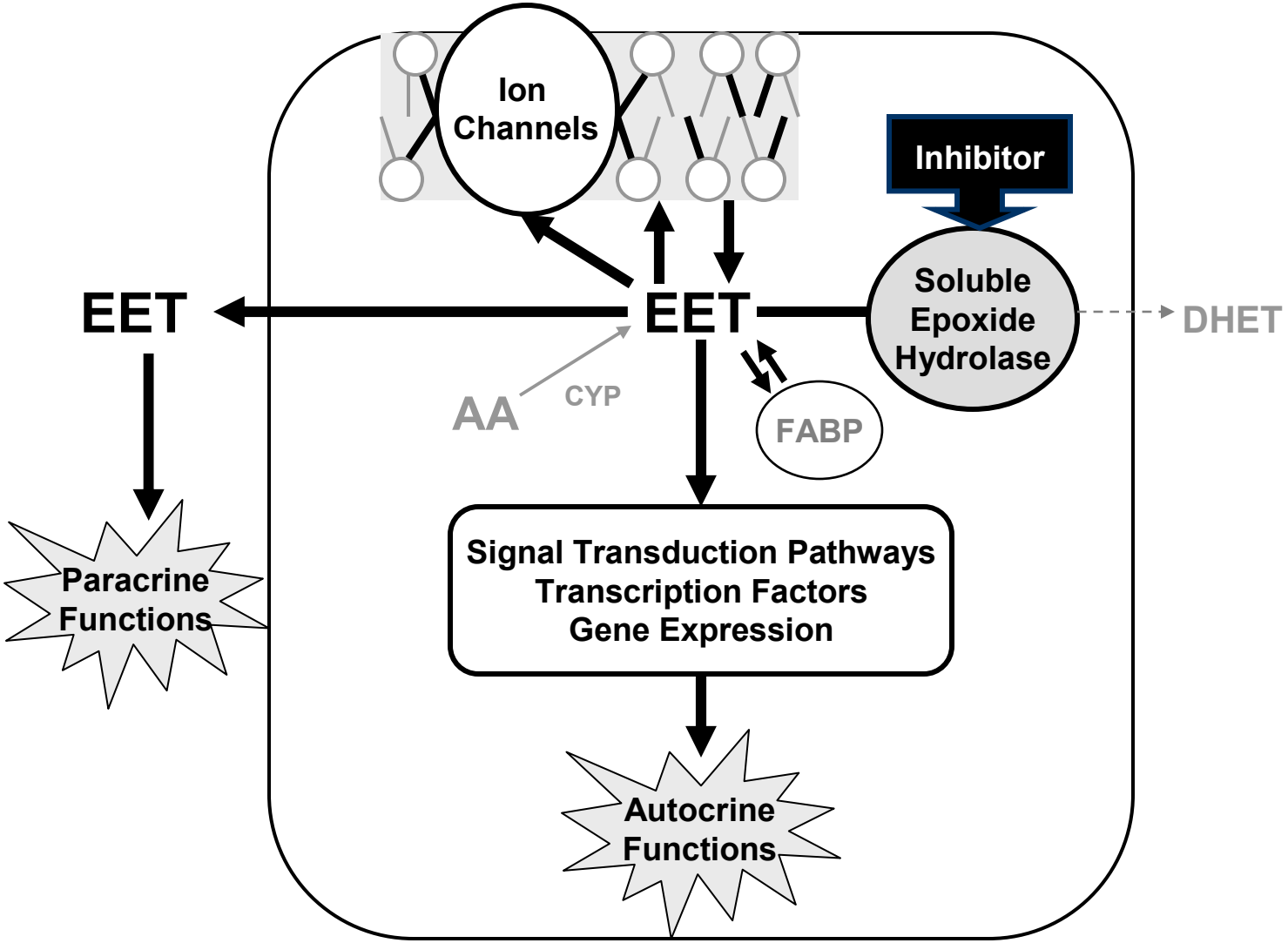
FABP
Signal transduction pathways
EET
CYP
Arachidonic acid
PPARs
Transcriptional Mediators
Function

Figure 4


Ion Channels
Inhibitor
EET
EET
Soluble Epoxide Hydrolase
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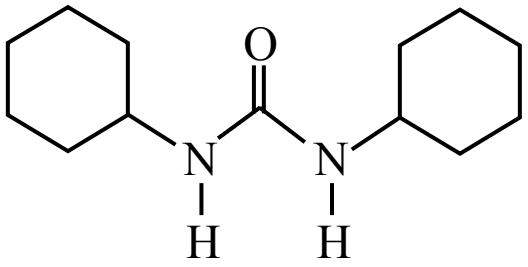
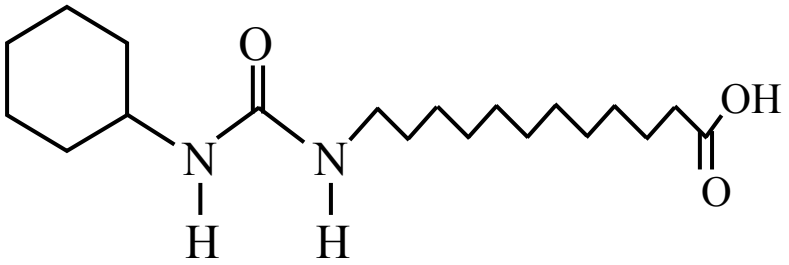
Paracrine Functions
Signal Transduction Pathways
Transcription Factors
Gene Expression
Autocrine Functions

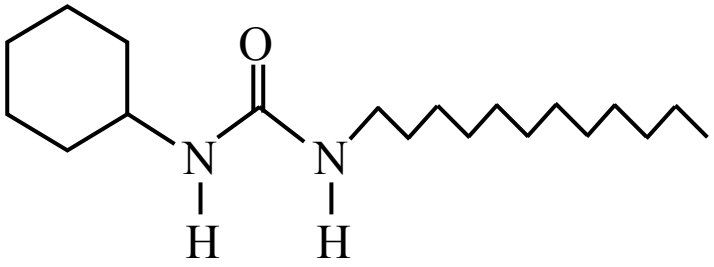
Figure 5



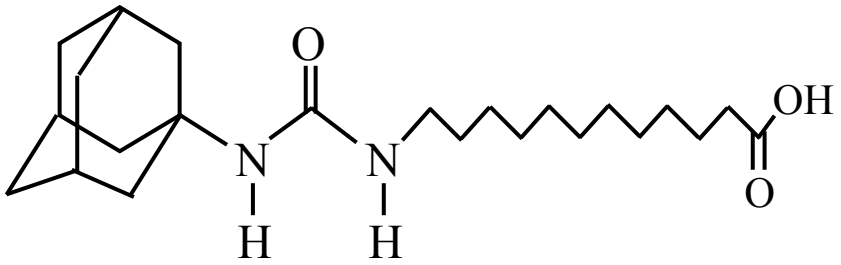
1,3-Dicyclohexylurea (DCU)



1-Cyclohexyl-3-dodecanoylurea (CUDA)



1-Cyclohexyl-3-dodecylurea (CDU)



1-Adamantanyl-3-dodecanoylurea (AUDA)

Figure 6