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REGULATION AND INHIBITION OF ARACHIDONIC ACID ω-HYDROXYLASES AND 20-HETE FORMATION

Deanna L. Kroetz[1,2] and Fengyun Xu[1]
[1]Department of Biopharmaceutical Sciences and the [2]Liver Center, University of California, San Francisco, California 94143-2911; email: deanna@itsa.ucsf.edu, fengyun@itsa.ucsf.edu

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■ **Abstract** Cytochrome P450–catalyzed metabolism of arachidonic acid is an important pathway for the formation of paracrine and autocrine mediators of numerous biological effects. The ω-hydroxylation of arachidonic acid generates significant levels of 20-hydroxyeicosatetraenoic acid (20-HETE) in numerous tissues, particularly the vasculature and kidney tubules. Members of the cytochrome P450 4A and 4F families are the major ω-hydroxylases, and the substrate selectivity and regulation of these enzymes has been the subject of numerous studies. Altered expression and function of arachidonic acid ω-hydroxylases in models of hypertension, diabetes, inflammation, and pregnancy suggest that 20-HETE may be involved in the pathogenesis of these diseases. Our understanding of the biological significance of 20-HETE has been greatly aided by the development and characterization of selective and potent inhibitors of the arachidonic acid ω-hydroxylases. This review discusses the substrate selectivity and expression of arachidonic acid ω-hydroxylases, regulation of these enzymes during disease, and the application of enzyme inhibitors to study 20-HETE function.

OVERVIEW OF ARACHIDONIC ACID METABOLISM

Arachidonic acid comprises part of the membrane phospholipid pool and is released following activation of phospholipase A_2 by various agonists, such as norepinephrine, angiotensin II, and bradykinin (1). Metabolism of free arachidonic acid by cyclooxygenases and lipoxygenases leads to the formation of prostaglandins, thromboxanes, and leukotrienes with important roles in the regulation of vascular tone, inflammation, and renal and pulmonary function (2). Cyclooxygenase- and lipoxygenase-catalyzed arachidonic acid metabolism is well characterized, and both of these pathways are targets of approved drugs. In contrast, our knowledge of metabolism of arachidonic acid by cytochrome P450 (CYP) enzymes is more

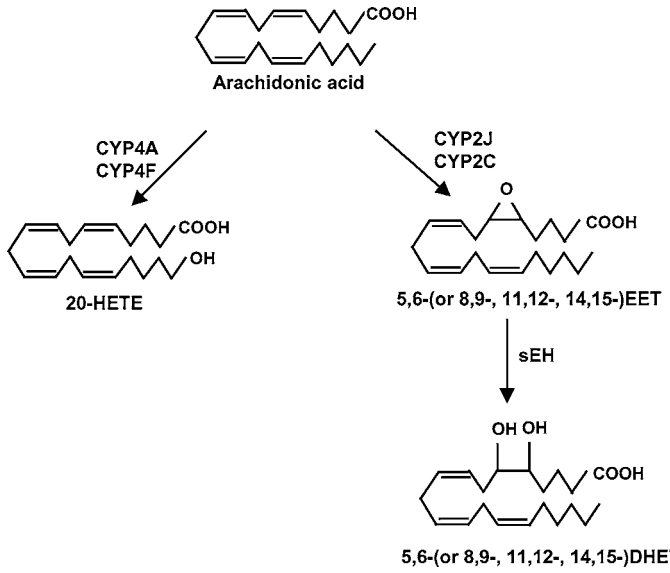


Figure 1 Major pathways of arachidonic acid metabolism catalyzed by cytochrome P450. Arachidonic acid is metabolized into epoxyeicosatrienoic acids (EETs) by CYP2C and CYP2J epoxygenases and into 20-hydroxyeicosatetraenoic acid (20-HETE) by CYP4A and CYP4F ω-hydroxylases. EETs can also be further metabolized by soluble epoxide hydrolase (sEH) into their corresponding dihydroxyeicosatrienoic acids (DHETs).

limited, although recent efforts in this area hold the promise that new drug targets will also emerge from this pathway.

CYP enzymes can metabolize arachidonic acid into numerous eicosanoids with the relative abundance dependent on the tissue and species (Figure 1). The major products in most tissues are the ω-hydroxylated metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) and regio- and stereospecific epoxyeicosatrienoic acids (EETs). Hydroxylation of arachidonic acid at the ω-1 and midchain (carbons 16–18) positions is less common. CYP4A and CYP4F enzymes catalyze the ω- and ω-1 hydroxylation reactions, whereas members of the CYP2C and CYP2J families are responsible for epoxidation (3–5). A novel CYP isoform, CYP2U1, has recently been identified as a human arachidonic acid ω-hydroxylase (6). EETs are efficiently hydrated by soluble epoxide hydrolase (sEH) into the corresponding dihydroxyeicosatrienoic acids (DHETs) (7, 8). CYP eicosanoids can also be further metabolized by CYPs, dehydrogenases, or cyclooxygenases (4, 9–11); β-oxidized (4, 10); or incorporated into membrane phospholipid pools (10, 11). The focus of this review is on pathologic regulation and inhibition of the CYP ω-hydroxylase pathway.

BIOLOGICAL SIGNIFICANCE OF 20-HETE

The importance of understanding the molecular mechanisms regulating 20-HETE synthesis is evident from the numerous biological effects attributed to this eicosanoid. Although not the focus of this review, the major biological properties of 20-HETE are summarized below. The reader is directed to several recent reviews that have discussed these properties in detail (4, 5). The main sites of synthesis and action of 20-HETE are the vasculature, kidney, and lung. The formation of 20-HETE has been documented in rat renal microvessels, and expression of CYP ω-hydroxylases in renal, cerebral, pulmonary, mesenteric, and skeletal muscle microvascular beds is consistent with 20-HETE formation throughout the vasculature (12–16). 20-HETE inhibits a large conductance Ca^{2+}-activated K^+ channel, resulting in depolarization of the vascular smooth muscle cell, Ca^{2+} entry, and potent vasoconstriction (17). An ongoing area of investigation focuses on the possibility that the vasoconstrictive effect of 20-HETE is receptor-mediated. The myogenic response to elevations in transmural pressure is also mediated by 20-HETE in cerebral, renal, skeletal muscle, and mesenteric arterioles (13, 15, 18, 19), and 20-HETE plays a role in the autoregulation of renal blood flow and tubuloglomerular feedback in rats (20, 21). 20-HETE can also act as an oxygen sensor in skeletal muscle microcirculation (22). A role for 20-HETE in signaling the mitogenic actions of growth factors and vasoactive agents (23) and in promoting angiogenesis (24, 25) suggests that this eicosanoid is important in regulating vascular cell growth. Arachidonic acid ω-hydroxylation also occurs in the renal proximal tubule and the thick ascending limb of Henle (26–28). In the proximal tubule, 20-HETE inhibits Na^+-K^+-ATPase, whereas in the thick ascending limb, 20-HETE blocks a 70-pS K^+ channel, which limits K^+ availability for transport by a Na^+-K^+-$2Cl^-$ cotransporter (29–31). In the lung microvasculature, 20-HETE has an opposite effect as that seen in other vascular beds, vasodilating pulmonary vessels in an endothelium- and cyclooxygenase-dependent manner (32, 33). In most species, 20-HETE also has bronchodilatory effects (34). The increasing biological properties associated with 20-HETE and the relative abundance of this eicosanoid in the vasculature and renal tubules make it of great importance to understand the molecular mechanisms that regulate 20-HETE synthesis, degradation, and action.

CYP ARACHIDONIC ACID ω-HYDROXYLASES

CYP ω-hydroxylases belong to the CYP4 family, which is evolutionarily one of the oldest members of the CYP superfamily. Isoforms of the CYP4A and CYP4F subfamilies catalyze the ω-, and to a lesser extent the ω-1, hydroxylation of arachidonic acid and other medium- to long-chain fatty acids. In the sections below, the expression and catalytic function of the human, mouse, rat, and rabbit CYP4A and CYP4F enzymes are discussed in more detail.

Catalytic Function of CYP4A and CYP4F Arachidonic Acid ω-Hydroxylases

Members of the CYP4A subfamily were the first characterized arachidonic acid ω-hydroxylases and include four isoforms in rats (35–38), two in humans (39–41), four in rabbits (42–45), and three in mice (46–48). Heterologous expression of the rat CYP4A isoforms in *Escherichia coli* (49) and baculovirus (50–52) revealed that CYP4A1 has the highest catalytic activity toward arachidonic acid ω-hydroxylation, followed by CYP4A2 and CYP4A3 with similar activity. Estimates of k_{cat} for 20-HETE formation were 6 min^{-1} for CYP4A1, 2 min^{-1} for CYP4A2 and CYP4A3, and 1 min^{-1} for CYP4A8 (49). The rat CYP4A enzymes also catalyze the ω-1 hydroxylation of arachidonic acid, with ω:ω-1 ratios ranging from 6–12:1 for CYP4A1 to 2–4:1 for CYP4A2 and CYP4A3 (49, 51). Interestingly, both CYP4A2 and CYP4A3 can also epoxidate arachidonic acid at the 11,12 position (50, 51); however, this effect is likely dependent on the experimental conditions of expression and functional reconstitution of the enzymes, as others have failed to confirm this reaction.

Metabolism of arachidonic acid by recombinant rabbit CYP4A enzymes is highly dependent on the presence of cytochrome b_5. A 2:1 ratio of cytochrome b_5 to CYP catalyzes arachidonic acid ω-hydroxylation with k_{cat} values of 155 min^{-1} and 152 min^{-1} for CYP4A4 and CYP4A7, respectively (53, 54). In contrast, CYP4A5 can efficiently catalyze the ω-hydroxylation of lauric acid but not arachidonic acid (55). The kinetics of arachidonic acid ω-hydroxylation by CYP4A6 have not been described in detail, although this isoform can catalyze 20-HETE formation (56). The substrate selectivity of the mouse Cyp4a enzymes has not been characterized, although high sequence similarity to other members of the CYP4A gene family implicate them in the ω-hydroxylation of arachidonic acid (46–48).

CYP4A11 is the major human CYP4A isoform and purified CYP4A11 from liver and kidney can catalyze the ω- and ω-1 hydroxylation of arachidonic acid (57, 58). In both tissues, CYP4A11-catalzyed 20-HETE formation was quantitatively less important than the corresponding CYP4F2 component, consistent with the low k_{cat} values of 0.4–0.55 min^{-1} for heterologously expressed CYP4A11 (40, 49). In a direct comparison of the four rat CYP4A isoforms and human CYP4A11, the latter had the lowest reported k_{cat} for arachidonic acid ω-hydroxylation (49). Recently, CYP4A22 has been identified as a second human CYP4A isoform (39, 59). Considering the 96% sequence identity with CYP4A11, it is highly likely that CYP4A22 will also catalyze 20-HETE formation, although low expression of this gene may limit its functional impact.

Rat CYP4F1 (60) and human CYP4F3 (61) were the first cloned members of the CYP4F family. To date, there have been four CYP4F members identified in the rat (60, 62), five in the human (61, 63–66), and five in the mouse (67). Initially, the focus of investigations on CYP4F catalytic activity was on ω-hydroxylation of leukotriene B_4 and its importance in controlling inflammation (61, 63, 66, 68–73). More recently, a comparison of catalytic function of the rat CYP4F isoforms

expressed in *E. coli* revealed that CYP4F1 and CYP4F4 could ω-hydroxylate arachidonic acid with k_{cat} values similar to those of CYP4A1 (9 and 11 min^{-1} for CYP4F1 and CYP4F4, respectively; 74). Human CYP4F3B can also metabolize arachidonic acid with a K_m value similar to that for leukotriene B_4 (73). CYP4F2 has been purified from both human liver and kidney and shown to be the major source of 20-HETE in these organs (57, 58). CYP4F12 can also ω-hydroxylate arachidonic acid but with a lower activity than that of CYP4F2 (66). The functional activity of the mouse CYP4F isoforms remains to be studied.

Expression of CYP4A and CYP4F Arachidonic Acid ω-Hydroxylases

Unlike most CYPs, many CYP4A isoforms have their highest level of expression in the kidney. CYP4A mRNA has been localized along the rat nephron and renal vasculature. CYP4A2, CYP4A3, and CYP4A8 can be detected in the glomerulus, proximal tubules, cortical collecting duct, and cortical thick ascending limb of Henle (75). Expression of CYP4A2 and CYP4A3 is also apparent in the medullary regions of the collecting duct and thick ascending limb (75). Consistent with low levels of CYP4A expression by RNase protection assay (76), CYP4A1 mRNA levels were too low to be detected in microdissected tubules (75). Expression of CYP4A1 is more easily detected in the rat renal vasculature, where it is the only CYP4A gene expressed in the aorta and renal artery (77). In contrast, CYP4A1, CYP4A2, CYP4A3, and CYP4A8 were all expressed in interlobar, arcuate, and interlobular arteries (77). In the mouse kidney, Cyp4a10 is ubiquitously expressed throughout the nephron and vasculature, whereas Cyp4A12 and Cyp4A14 expression is limited to the proximal tubule (78).

The rat and human CYP4F genes implicated in arachidonic acid ω-hydroxylation are also highly expressed in the kidney, similar to the corresponding CYP4A genes. CYP4F1 was originally cloned from rat hepatic tumors (60) and was subsequently shown to be expressed in liver, kidney, and brain (79). CYP4F4 is expressed at similar levels in liver and kidney (79). CYP4F2, CYP4F3B, and CYP4F12 are expressed at significant levels in the human kidney (57, 58, 65, 73, 80). In the mouse, Cyp4f mRNA is detected throughout the renal tubule and vasculature (78), making it of interest to characterize its role in 20-HETE formation.

The high degree of amino acid similarity between the rat CYP4A and CYP4F proteins makes it difficult to detect their expression in an isoform-specific manner. Despite these limitations, the expression of CYP4A immunoreactive protein in the proximal tubules, glomerulus, medullary thick ascending limb of Henle, and renal microvessels shows a similar pattern as that of CYP4A mRNA (75, 77, 81–83). In the vasculature, the expression of CYP4A protein is highest in the smaller-diameter vessels, consistent with an important role for 20-HETE in maintaining renal vascular tone. CYP4F expression has been detected at the protein level in the liver, lung, kidney, and brain, but the isoform distribution is unknown (79).

Functional CYP ω-hydroxylase activity has been documented in the nephron and vasculature of the rat kidney, although it is not clear what percentage of this activity is due to CYP4A versus CYP4F isoforms. Detection of 20-HETE following addition of arachidonic acid to homogenates from microdissected nephrons indicates that functional arachidonic acid ω-hydroxylase activity is highest in the proximal tubules, but also detected in the glomerulus (28, 84, 85). In the renal vasculature, arachidonic acid ω-hydroxylase activity is highest in the interlobular artery, with much lower levels detected in the interlobar and arcuate arteries (77). The correlation of arachidonic acid ω-hydroxylase activity with the pattern of CYP4A expression in the vasculature supports a role for the CYP4A enzymes in this catalytic function. Importantly, endogenous 20-HETE levels are detectable in the proximal tubules of Sprague-Dawley rats (28). The detection of endogenous 20-HETE levels in these tissues supports an important biological role for this eicosanoid in controlling renal vascular tone and ion transport.

In the human kidney, both CYP4A11 and CYP4F2 are highly expressed in the proximal tubules, and both enzymes contribute to renal arachidonic acid ω-hydroxylation (58). Immuno- and chemical inhibition studies are consistent with CYP4F2 being the major human kidney microsomal arachidonic acid ω-hydroxylase. A less significant role for CYP4A11 in hepatic 20-HETE formation is suggested from similar inhibition studies in human liver microsomes (57).

Most of the interest in 20-HETE formation focuses on its role in regulating renal vascular tone and ion transport. However, CYP4A and CYP4F isoforms are also expressed outside the kidney and likely have pharmacological significance in these tissues as well. CYP4A protein is found in the brain, prostate, intestine, and lungs (14, 86–88), and CYP4F1 and CYP4F4 are expressed in the brain (79). Alternative splicing of the CYP4F3 gene determines its expression pattern, with CYP4F3B expressed in the liver, kidney, trachea, and ileum (73). In contrast, the leukotriene B_4 ω-hydroxylase CYP4F3A is expressed in myeloid cells in peripheral blood and bone marrow (89). In the rabbit lung, expression of CYP4A protein decreased with increasing arterial size (88). CYP4F12 expression is detected in the small intestine, colon, and urogenital epithelia (66, 80), although a role for 20-HETE in gastrointestinal physiology is not clear. The novel fatty acid ω-hydroxylase CYP2U1 has limited expression in the thymus and cerebellum where its role in mediating 20-HETE synthesis is not yet characterized (6).

REGULATION OF CYP ARACHIDONIC ACID ω-HYDROXYLASES AND 20-HETE SYNTHESIS

Much of what we know about the biological roles of 20-HETE stem from studies of the regulation of the CYP arachidonic acid ω-hydroxylases and the use of disease state and xenobiotic treatment models in which 20-HETE synthesis is altered. The induction of the CYP4A genes by peroxisome proliferators, such as fibric acid hypolipidemic drugs, has been well characterized and is the topic of several reviews

(90, 91). Peroxisome proliferators typically exert their effects through activation of the α isoform of the peroxisome proliferator-activated receptor (PPARα). Unfortunately, the use of these inducers to characterize 20-HETE function is complicated by the fact that the CYP4A isoforms, CYP2C23, and soluble epoxide hydrolase are induced (35, 37, 92), whereas CYP4F and CYP2C11 are downregulated (67, 93) following exposure to peroxisome proliferators. With effects on both the CYP ω-hydroxylase and epoxygenase pathways, often in opposite directions, the interpretation of results using these chemicals should be made with caution. The abundant cellular signaling molecule nitric oxide inhibits CYP4A ω-hydroxylase expression and function (94) and a novel mechanism of regulation involving covalent attachment of the prosthetic heme group through an ester link at a glutamic acid residue conserved in the I-helix of the active site of most CYP4 members has recently been described (95–98). Interestingly, in some cases covalent modification of the enzymes results in increased activity. Heme levels are highest in the liver and vary throughout the body, suggesting that covalent heme binding may influence tissue-specific regulation of CYP4 arachidonic acid ω-hydroxylase activity, a novel mechanism of CYP regulation. Although each of these mechanisms is interesting and of value in the field of eicosanoid biology, the focus of the sections below is on the regulation of arachidonic acid ω-hydroxylation in various disease states and the use of chemical inhibitors to study 20-HETE biology.

Pathophysiological Regulation of Arachidonic Acid ω-Hydroxylation

The modulation of CYP arachidonic acid ω-hydroxlase expression and function has been described in numerous animal models of disease. In some cases, alterations in 20-HETE formation are hypothesized to play important roles in the pathophysiology of the disease, whereas in other cases, changes in 20-HETE formation are considered an adaptive response to pathologic stimuli. Changes in arachidonic acid ω-hydroxylation in hypertension, pregnancy, inflammation, and diabetes are described below.

HYPERTENSION The ability of 20-HETE to influence vascular reactivity and renal tubular sodium and water transport led to interest in understanding the significance of this eicosanoid in regulating blood pressure. Many studies in this area have used the spontaneously hypertensive rat (SHR) model of essential hypertension. Increased cortical arachidonic acid ω-hydroxylase activity has been documented in the SHR kidney relative to the normotensive Wistar-Kyoto (WKY) rat, suggesting that increased renal 20-HETE levels contribute to the hypertensive phenotype in these rats (76, 99–101). Similarly, endogenous 20-HETE levels are elevated in the SHR mesenteric artery relative to the WKY rat (1.34 ± 0.16 versus 0.27 ± 0.09 pmol/mg; 102). Increased expression of CYP4A mRNA and immunoreactive protein has also been documented in the SHR kidney and this is consistent with the increased arachidonic acid ω-hydroxylase activity. Using differential hybridization

techniques, CYP4A2 was identified as one of three mRNAs differentially expressed in the kidneys of four-week-old SHRs and WKY rats (103). Gene-specific RNA probes were later used to demonstrate differential expression of both CYP4A3 and CYP4A8 in the young SHR kidney (76). In both cases, the increases in CYP4A mRNA were modest (1.4- to 2.0-fold) and only significant between one and four weeks of age. A similar pattern of increased CYP4A immunoreactive protein and arachidonic acid ω-hydroxylase has been found (76). The mechanism by which CYP4A expression and function is altered in the SHR kidney is still not understood.

The function and expression of CYP4A is also altered in other models of hypertension. In contrast to the SHR, in the Dahl hypertension model CYP4A levels and arachidonic acid ω-hydroxylase activity are decreased in the salt-sensitive strain relative to the normotensive salt resistant or Lewis strains, and these differences are restricted to the outer medulla (104–106). Both high-salt and high-fat diets decrease arachidonic acid ω-hydroxylase activity and CYP4A protein levels in the renal tubules of Sprague-Dawley rats (107, 108), whereas induction of hypertension by Angiotensin II treatment decreases CYP4A expression exclusively in the renal microvessels (107).

Genetic deletion of *Cyp4a14* revealed a complex phenotype and evidence that 20-HETE does indeed play a role in the regulation of blood pressure (109). Mice deficient in Cyp4a14 exhibited an androgen-sensitive increase in blood pressure that is normalized by castration. Interestingly, *Cyp4a14−/−* mice have increased renal arachidonic acid ω-hydroxylase activity that corresponds to androgen-mediated induction of the Cyp4a12 isoform. The increased functional Cyp4a activity is consistent with higher renal levels of 20-HETE and the observed increases in blood pressure. Unfortunately, the multiplicity of the Cyp4a and Cyp4f isoforms in the mouse kidney will limit the usefulness of genetic deletion in understanding the physiological and pathophysiological role of 20-HETE.

Although numerous studies in animal models of hypertension have held promise that 20-HETE is important in the regulation of blood pressure in humans, evidence for such an effect has only recently been described. In human essential hypertension, urinary 20-HETE excretion is regulated by salt intake, with distinct relationships between natriuresis and 20-HETE excretion in salt-sensitive and salt-resistant patients (110). The importance of 20-HETE in regulating natriuresis in humans is also supported by studies showing a role for this eicosanoid in mediating the natriuretic properties of furosemide (111). In a population of obese patients with essential hypertension, the urinary excretion of 20-HETE was negatively correlated with insulin levels (112). The negative correlation between urinary 20-HETE levels and insulin suggests that insulin may decrease the expression and function of the CYP ω-hydroxylases, consistent with inhibitory effects of insulin on the rat CYP4A isoforms (113). Although limited in number, these recent studies suggest that regulation of CYP ω-hydroxylase activity in the human kidney is an important determinant of natriuresis and that pharmacological manipulation of this activity may have therapeutic potential in the regulation of blood pressure.

PREGNANCY The first reports of altered CYP4A expression during pregnancy were reported prior to significant interest in the role of these enzymes in arachidonic acid ω-hydroxylation. CYP4A protein levels and increased PGE_1, PGA_1, and $PGF_{2\alpha}$ ω-hydroxylation were found in pregnant rabbit lungs, and this effect was attributed to changes in hormonal levels during pregnancy (114). Despite extensive biochemical and cellular characterization of these functional changes, the physiological significance of altered CYP4A protein and activity in the rabbit lung is still not understood. In the rat, pregnancy-induced changes in CYP4A expression and arachidonic acid ω-hydroxylation show distinct patterns in the tubule and microvessels (115). During early gestation, CYP4A immunoreactive protein levels and arachidonic acid ω-hydroxylase activity in the medullary thick ascending limb are similar to baseline and these values increase at 19 days of gestation. In contrast, both the expression of CYP4A immunoreactive proteins and arachidonic acid ω-hydroxylase activity in renal microvessels are elevated at 6 and 12 days of gestation compared to control, but return to nonpregnant levels at 19 days of gestation. The decrease in microvascular and increase in tubular arachidonic acid ω-hydroxylase activity at 19 days gestation is accompanied by a decrease in blood pressure and elevated urinary excretion of 20-HETE. The vasoconstrictive effects predicted from the increased renal microvascular 20-HETE synthesis during early gestation might act to buffer the increased synthesis of nitric oxide, a potent vasodilator, during this period (116). In later gestation, the increased 20-HETE synthesis in the medullary thick ascending limb may modulate natriuresis and contribute to the decreased blood pressure observed at this time. It will be of interest to define the cellular signals that mediate this unique site- and time-dependent expression of CYP4 arachidonic acid ω-hydroxylases during pregnancy.

INFLAMMATION Inflammation and infection are often associated with decreased CYP content and drug clearance (117). However, the CYP4A enzymes are induced in response to inflammation in the rat. Both renal and hepatic levels of CYP4A mRNA, CYP4A immunoreactive protein, and lauric acid ω-hydroxylase activity are elevated following treatment of Fisher rats with lipopolysaccharide (LPS) (118, 119). Studies in PPARα−/− mice indicate that the dependency of these changes on PPARα are gene- and tissue-specific. Both the induction of Cyp4a10 in the kidney and its downregulation in the liver following LPS treatment are PPARα-dependent (120). However, for Cyp4a14, LPS induction is only found in the kidney, and this effect is also PPARα-dependent. An interesting hypothesis is that LPS treatment increases the level of an endogenous eicosanoid or other compound that can activate PPARα. The effects of these inflammatory stimuli on renal 20-HETE synthesis are important to study.

The effects of inflammation on CYP4F expression and activity are also tissue- and gene-specific. Treatment of Fisher rats with LPS decreased hepatic CYP4F4 mRNA levels by 50% and increased CYP4F5 mRNA levels to a similar degree, resulting in no net change in leukotriene B_4 ω-hydroxylation or CYP4F immunoreactive protein levels (121). Differences are also noted between various inflammatory

stimuli, as barium sulfate increased hepatic CYP4F4 mRNA and protein levels as well as corresponding enzyme activity. By contrast, hepatic CYP4F1 and CYP4F6 were unaffected by these inflammatory stimuli. In the kidney, LPS had no effect on CYP4F mRNA levels, but barium sulfate induced CYP4F1 and CYP4F6 up to threefold (121). PPARα plays some role in mediating the inductive effects of LPS on Cyp4f15 in the kidney and the downregulation of Cyp4f15 and Cyp4f16 in the liver (67). Interestingly, a traumatic brain injury model is associated with isoform-dependent changes in both hepatic and renal CYP activity, including a twofold increase in renal CYP4F expression and activity that is sustained for at least two weeks (122). It is tempting to speculate that changes in renal 20-HETE levels resulting from CYP4F activity might contribute to the renal effects associated with head trauma.

DIABETES Alterations in CYP4A expression and function have been noted in animal models of diabetes. Following the induction of diabetes with streptozotocin, CYP4A expression and function are increased in liver and kidney microsomes (113, 123–126). The effect of diabetes can be reversed with insulin treatment (113, 123, 125, 126) or correction of the hyperketonic state (127). An elevation of intracellular fatty acids during diabetes contributes to the effects on CYP4A (128). Activation of PPARα is a necessary step in mediating the effects of diabetes on CYP4A transcription, as streptozotocin treatment has no effect in PPAR$\alpha -/-$ mice (126). It is postulated that levels of an endogenous fatty acid activator of PPARα are increased in the diabetic state, resulting in PPARα activation and CYP4A induction. The effects of streptozotocin-induced diabetes on CYP4A expression appear to be a direct result of the disease state, as similar findings are reported for the *fa/fa* Zucker rat and the *ob/ob* mice (129). The recent report of a negative correlation between insulin levels and urinary 20-HETE excretion in humans (112) suggests that renal CYP4A expression and function is likely altered in human diabetics. Lower renal 20-HETE production in diabetics would provide a protective effect from the vasoconstrictive properties of this eicosanoid but may alter the pressure-natriuresis profile and contribute to the hypertensive complications of diabetes.

Inhibition of CYP ω-Hydroxylases

Important tools for studying the biological role of 20-HETE are potent and selective fatty acid ω-hydroxylase inhibitors. Both small molecules and antisense oligonucleotides have been developed as inhibitors of CYP ω-hydroxylases, and these inhibitors have been used both in vitro and in vivo. A description of the selectivity, potency, and application of mechanism-based and competitive inhibitors, antisense oligonucleotides, and 20-HETE antagonists are described below.

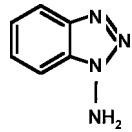
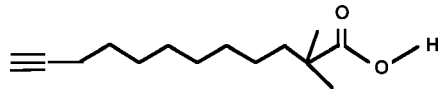
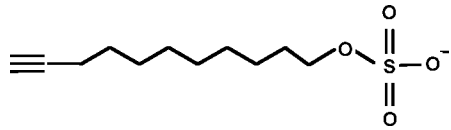
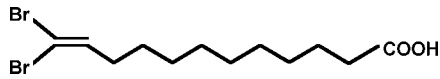
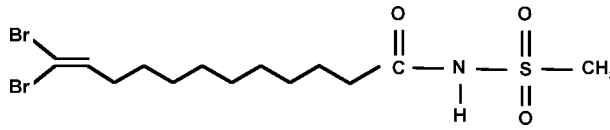
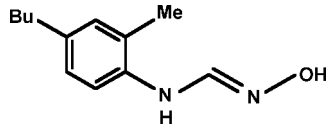
MECHANISM-BASED INHIBITORS One approach to specific inhibitors is the use of mechanism-based inhibitors, also known as suicide substrates, which specifically inactivate enzymes in a catalysis-dependent manner (130). The irreversible nature

of mechanism-based inhibitors makes it easy to document inhibition in tissues by measuring enzyme function following administration of these inhibitors. As a result, mechanism-based inhibitors have been widely used in in vivo and in vitro studies to characterize the biological function of 20-HETE and the substrate specificity of various CYP isoforms.

One of the first such inhibitors to be characterized is 1-aminobenzotriazole (ABT) (Table 1). Inhibition of microsomal CYP-dependent activity by ABT is NADPH- and time-dependent and follows pseudo first-order kinetics, a characteristic of mechanism-based CYP inhibitors (131). Inactivation of CYP enzymes by ABT requires catalytic formation of benzyne, which in turn alkylates the prosthetic heme group (132). Arachidonic acid metabolism is inhibited by ABT in both rat renal cortical and hepatic microsomes. The inhibition is dose-dependent and in initial studies showed a fair degree of selectivity for the CYP4A/CYP4F catalyzed formation of 19- and 20-HETE in the cortex (133). A single intraperitoneal injection of ABT (50 mg/kg) to Sprague-Dawley rats selectively inhibits renal cortical and outer medullary 20-HETE formation by 84% and 76%, respectively. In contrast, there is no inhibition of renal epoxygenase activity at this dose (133). However, others have reported that the same dose of ABT completely blocks the formation of 20-HETE and EETs in the kidney within 2 h and reduces the 24-h urinary excretion of 20-HETE by >50% (134). Chronic treatment with ABT (50 mg/kg/day, ip) for 5 days or 2 weeks inhibits renal 20-HETE and EET formation by 80%–90% and 50%–76%, respectively, and urinary 20-HETE excretion falls by 68%–80% (135, 136). Interstudy differences in the selectivity of ABT might reflect strain differences in enzyme sensitivity. Although ABT has broad substrate specificity and at certain doses nonselectively blocks both the ω-hydroxylation and epoxidation of arachidonic acid, its water solubility and lack of toxicity still make it of value for characterizing the biological function of CYP eicosanoids.

Studies with ABT in vivo have focused on the role of arachidonic acid ω-hydroxylation on blood pressure regulation. A single dose of ABT to seven-week-old SHRs causes an acute reduction in MAP of 17–23 mm Hg during the 4- to 12-h period after administration and almost complete inhibition of renal cortical and outer medullary arachidonic acid ω-hydroxylase activity (133). Chronic treatment with ABT inhibits renal arachidonic acid ω-hydroxylase and epoxygenase activity by 80%–90% and 50%–76%, respectively, with corresponding changes in urinary 20-HETE excretion (135–137). The effects of chronic ABT treatment on blood pressure are dependent on the experimental model. Chronic treatment of Sprague-Dawley rats with ABT attenuates the angiotensin II–induced rise in arterial blood pressure by 40% (137) and reduces the blood pressure in rats fed a low-salt diet, while promoting the development of hypertension in rats fed a high-salt diet (136). A five-day course of ABT therapy attenuates pressure-natriuresis by preventing the decrease in Na^+-K^+-ATPase activity and internalization of the sodium-hydrogen exchanger from the brush border of the proximal tubule following an elevation in renal perfusion pressure (135). Collectively, these studies support an important role for 20-HETE in the regulation of renal function and blood pressure. Although acute

TABLE 1 Inhibitors of arachidonic acid ω-hydroxylases

Inhibitor[a]	Structure
ABT	
10-UDYA	
11-DDYA	
17-ODYA	
DMDYA	
10-SUYS	
DBDD	
DDMS	
HET0016	
6(Z),15(Z)-20-HEDE	

[a]ABT: 1-aminobenzotriazole; 10-UDYA: 10-undecynoic acid; 11-DDYA; 11-dodecynoic acid; 17-ODYA: 17-octadecynoic acid; DMDYA: 2,2-dimethyl-11-dodecynoic acid; 10-SUYS: 10-undecynyl sulfate; DBDD: 12,12-dibromododec-11-enoic acid; DDMS: N-methylsulfonyl-12,12-dibromododec-11-enamide; HET0016: *N*-hydroxy-*N*′-(4-*n*-butyl-2-methylphenyl)formamidine; 6(Z),15(Z)-20-HEDE: 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid.

inhibition of CYP arachidonic acid ω-hydroxylase activity has apparent effects on vascular reactivity, chronic inhibition suggests that 20-HETE is more important in maintaining renal tubular transport function.

A series of terminal acetylenic monocarboxylic acid fatty acids varying in length from 11 [10-undecynoic acid (10-UDYA)] to 18 [17-octadecynoic acid

(17-ODYA)] (Table 1) carbons (138, 139) have also been synthesized and characterized as arachidonic acid ω-hydroxylase inhibitors. These compounds are oxidized to ketenes that inactivate the CYP protein instead of alkylating the prosthetic heme group (140). They are highly selective inhibitors of rat liver CYP isoforms that are active toward fatty acid substrates without affecting total P-450 levels or other P-450-dependent activities. 10-UDYA and 11-dodecynoic acid (11-DDYA) (Table 1) specifically inhibit hepatic CYP enzymes that catalyze lauric acid ω- and ω-1 hydroxylation (138). 11-DDYA and 17-ODYA inhibit the lauric acid ω- and ω-1 hydroxylation by microsomes prepared from the lungs of pregnant rabbits and reconstituted P-450 (141). 17-ODYA is a very potent inhibitor toward arachidonic acid metabolism; however, the inhibition is nonspecific (142, 143). It irreversibly inhibits both ω-hydroxylation and epoxidation of arachidonic acid with IC_{50} values of 7 and 5 μM, respectively (142). It also potently inhibits ω- and ω-1 hydroxylation of arachidonic acid catalyzed by recombinant rat CYP4A1, CYP4A2, CYP4A3, CYP4F1, and CYP4F4 with a similar IC_{50} for all isoforms (51, 74).

Despite its lack of selectivity, 17-ODYA has been widely used in in vitro and in situ studies to characterize the biological function of 20-HETE. For example, 17-ODYA has been used to establish the role of 20-HETE in the regulation of renal blood flow and tubuloglomerular feedback and as a K^+ channel inhibitor in rat renal arterioles (20, 21, 143). It also has been used to demonstrate that 20-HETE mediates the vasoconstrictor response to angiotensin II in isolated renal arterioles and the myogenic response of renal, cerebral, and skeletal muscle arteries (19, 137, 144). Intrathecal administration of 17-ODYA prevents the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat (145).

Unfortunately, the terminal acetylenic fatty acid inhibitors are of little value for in vivo inactivation of fatty acid hydroxylases because of their rapid metabolic degradation by β-oxidation, their esterification and storage in the liver, and extensive protein binding (139). Introduction of two methyl groups vicinal to the carboxylic acid group in 10-UDYA yields 2,2-dimethyl-11-dodecynoic acid (DMDYA) (Table 1), and the replacement of the carboxyl group in 10-UDYA with a sulfate yields sodium 10-undecynyl sulfate (10-SUYS) (Table 1). Both of these compounds have in vitro activities similar to that of 10-UDYA and are resistant to β-oxidation and storage and exhibit substantial in vivo activity (146). 10-SUYS selectively inhibits arachidonic acid ω-hydroxylation in rat cortical microsomes (74). The IC_{50} of 10-SUYS for inhibition of 20-HETE formation is 10 μM, whereas epoxygenase activity was not affected at a concentration up to 50 μM. 10-SUYS also shows isoform-specific inhibition of rat recombinant CYP4F1- and CYP4F4-catalyzed 20-HETE formation (74). The IC_{50} of 10-SUYS for inhibition of CYP4F4-catalyzed 20-HETE formation is 25 μM, whereas 10-SUYS has only minimal inhibition toward CYP4F1-catalyzed 20-HETE formation.

Administration of 1–50 mg/kg of 10-SUYS intraperitoneally to SHRs results in a dose-dependent and selective inhibition of renal cortical arachidonic acid ω-hydroxylase activity (147). A single dose of 10-SUYS (5 mg/kg) causes an acute

reduction in mean arterial blood pressure by 18 mm Hg in 8-week-old SHRs 6 h after the treatment. Treatment with 10-SUYS is associated with 66% inhibition of 20-HETE formation and a loss of CYP4A immunoreactive proteins in cortical microsomes, a decrease in urinary 20-HETE formation, and attenuation of the vasoconstrictor response of renal interlobar arteries to angiotensin II in vitro (147). Chronic treatment with 8 mg/kg/day of 10-SUYS for 4 weeks via an osmotic pump results in 51% inhibition of 20-HETE formation in renal cortex without affecting the epoxygenase activity and with no apparent toxicity (F. Xu and & D.L. Kroetz, unpublished data). To date, 10-SUYS is the most potent and selective mechanism-based inhibitor of CYP-mediated 20-HETE formation, with useful properties for in vivo studies. It will no doubt prove beneficial in further characterizing the biological significance of 20-HETE.

COMPETITIVE INHIBITORS In addition to mechanism-based fatty acid CYP ω-hydroxylase inhibitors, the olefinic compounds N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) (Table 1) and 12,12-dibromododec-11-enoic acid (DBDD) (Table 1) have been described as competitive inhibitors of arachidonic acid ω-hydroxylation. DDMS and DBDD exhibit a high degree of selectivity, inhibiting microsomal ω-hydroxylation of arachidonic acid with an IC_{50} value of 2 μM, whereas the IC_{50} values for epoxidation are 60 and 51 μM, respectively (142). However, studies with baculovirus-expressed rat CYP4A isoforms show no selectivity of DDMS between CYP4A1-, CYP4A2-, and CYP4A3-catalyzed 20-HETE formation. A similar IC_{50} (0.8 μM) for DDMS is found for all three isoforms (51).

In contrast to the mechanism-based inhibitors described above, these acyclic dibromide derivatives exhibit a reversible time- and NADPH-independent inhibition. However, the fatty acid structure of these inhibitors imparts a fair degree of selectivity for the CYP fatty acid ω-hydroxylases. DDMS and DBDD are only effective at inhibiting the formation of 20-HETE when added to protein-free solutions in vitro or when directly applied to tissues in vivo. Modification of the carboxyl group in DBDD to a methyl sulfonate in DDMS does not change the potency or selectivity of the inhibitory activity and renders the inhibitor resistant to β-oxidation and of greater utility in vivo (142). Administration of DDMS locally into an isolated perfused renal arteriolar preparation and systemically into anesthetized rats demonstrates a high degree of selectivity for inhibition of 20-HETE formation (148). DDMS has been widely used to selectively inhibit 20-HETE formation and therefore to characterize its biological effects, especially its effect on regulation of vascular tone. DDMS has been used in in vitro studies to establish a role for 20-HETE in the vasoconstrictor response of renal, cerebral, and mesenteric arteries (13, 149, 150); the myogenic response of skeletal muscle resistance arteries (19, 144); and the vasoconstrictor response to elevated PO_2 in skeletal muscle resistance arterioles (16, 151, 152). Chronic intravenous infusion with DDMS (10 mg/kg/day) for five days in Sprague-Dawley rats attenuates the angiotensin II–induced rise in arterial blood pressure by 40% (137).

The most potent and selective inhibitor of 20-HETE formation reported so far is *N*-hydroxy-*N*′-(4-*n*-butyl-2-methylphenyl)formamidine (HET0016) (Table 1) (153). The examination of structure-activity relationships reveals that the unsubstituted hydroxyformamidine moiety and the substituent at the para-position of the *N*-hydroxyformamidine moiety are necessary for the potent activity of HET0016 (154). The IC_{50} value of HET0016 for the formation of 20-HETE by rat renal microsomes is 35 nM, whereas its IC_{50} value for inhibition of the formation of EETs is 2800 nM. In human renal microsomes, HET0016 potently inhibits the formation of 20-HETE with an IC_{50} value of 9 nM (153). The IC_{50} values of HET0016 for the formation of 20-HETE by human recombinant CYP4A11, CYP4F2, and CYP4F3 enzymes are 42, 125, and 100 nM, respectively (145). HET0016 has very little effect on the activities of cyclooxygenase or other CYP enzymes (153).

HET0016 has been applied in some in vivo and ex vivo studies to characterize the biological effects of 20-HETE. Chronic treatment with HET0016 (10 mg/kg per day iv) for 10 days in Sprague-Dawley rats potently and selectively inhibits the formation of 20-HETE in renal cortical homogenates and the urinary excretion of 20-HETE by 90%, whereas renal epoxygenase activity was not significantly altered. However, chronic treatment with HET0016 had no effect on blood pressure in the Sprague-Dawley rats fed a low-salt diet, and the blood pressure rose by 18 mm Hg after the rats are fed a high salt diet (136). Chronic treatment with HET0016 also blocks the increase in 20-HETE formation and angiogenesis induced by electrical stimulation in skeletal muscle (24). The angiogenic activity in rat renal interlobar arteries transduced with adenovirus expressing the CYP4A1 cDNA is fully blocked by treatment with HET0016 and is reversed by addition of a 20-HETE agonist (25). A single dose of HET0016 (10 mg/kg iv) reduces 20-HETE from 199 to 39 ng/ml in the cerebrospinal fluid and prevents the acute fall in cerebral blood flow in the rat following subarachnoid hemorrhage (145, 155).

Despite its promising pharmacological properties, the preparation of an injectable formulation of HET0016 is limited by its poor solubility under neutral conditions and instability under acidic conditions owing to the *N*-hydroxyformamidine moiety, an essential feature for potent and selective activity. A more recent study shows that the activity is maintained when the *N*-hydroxyformamidine moiety is replaced by isoxazole or pyrazole, and these derivatives have improved stability (156). The biologic effects of these second-generation HET0016 derivatives and their potential side effects have yet to be characterized.

ANTISENSE OLIGONUCLEOTIDES In addition to CYP inhibitors, another approach to block the formation of 20-HETE has been the use of antisense cDNA oligonucleotides (ODNs). Antisense ODNs offer the possibility of blocking the expression of a particular CYP gene without any changes in the function of other genes, provided there is enough difference in the sequence of the targeted region between CYP isoforms. CYP4A1- and CYP4A2/4A3-specific antisense ODNs can inhibit protein expression and the corresponding catalytic activity (15, 157). Daily intravenous injections of an antisense CYP4A1 and CYP4A2/4A3 ODN for five days

reduces the expression of CYP4A-immunoreactive proteins and the production of 20-HETE by 52% and 48%, respectively, in renal arterioles of Sprague-Dawley rats. Blockade of this pathway is associated with a reduction in arterial blood pressure by 16 and 17 mm Hg, respectively (157). Administration of CYP4A1 antisense ODN for five days in the SHR also decreases the arterial blood pressure by 16 mm Hg. Treatment with CYP4A1 antisense ODN reduces the level of CYP4A-immunoreactive proteins along with 20-HETE synthesis in mesenteric arteries in the SHR. Mesenteric arteries from rats treated with CYP4A1 antisense oligonucleotides exhibit decreased sensitivity to the constrictor action of phenylephrine and decreased intensity of myogenic constrictor response to elevation in transmural pressure (15). These studies suggest that CYP4A antisense ODNs can provide the specificity needed for evaluating the contribution of each CYP4A isoform to the endogenous production of 20-HETE and thereby can be used to examine the physiological role of 20-HETE.

20-HETE ANTAGONISTS A series of 20-HETE derivatives have been synthesized and examined to determine the structural requirements of the vasoconstrictor response to 20-HETE. In renal arterioles, 5(*S*)-, 15(*S*)-, and 19(*S*)-HETE; a C_{19} analog; and 20-hydroxyeicosa-6(*Z*),15(*Z*)-dienoic acid [6(*Z*),15(*Z*)-20-HEDE or WIT-002] (Table 1) block the vasoconstrictor actions of 20-HETE (158). The strongest antagonist of 20-HETE is 6(*Z*),15(*Z*)-20-HEDE, which completely blocks the vasoconstrictor response to 20-HETE in renal (158), cerebral (13, 159), and skeletal muscle (152, 160) arterioles. These antagonists are increasingly important in investigating the role of 20-HETE in the regulation of vascular tone but are somewhat limited by their high plasma protein binding and their actions as competitive inhibitors of the synthesis of 20-HETE and EETs at high concentrations. These properties limit their use in vivo.

SUMMARY AND FUTURE PERSPECTIVES

Significant progress has been made in the past decade in understanding the biological function of 20-HETE and the molecular mechanisms that determine the intracellular levels of this eicosanoid. The increasing body of literature describing altered CYP arachidonic acid ω-hydroxylase expression and function in various disease models supports an important role for this metabolic pathway in vascular reactivity, renal tubular ion and water transport, organ blood flow, cell growth, and inflammation. Likewise, the development and characterization of selective and potent inhibitors of arachidonic acid ω-hydroxylase have greatly enhanced our understanding of the role of 20-HETE in physiology and pathophysiology. The multiplicity of subfamilies and individual members of CYP arachidonic acid ω-hydroxylases remains a challenge and limits the use of genetic deletion and antisense techniques. Distinct patterns and levels of expression, as well as differences in functional activity between the various members of the CYP4A and CYP4F families, suggest unique roles for individual enzymes in mediating

20-HETE effects. To further delineate the role of individual ω-hydroxylases, additional isoform-specific inhibitors will need to be characterized. The plausibility of such an approach is supported by our recent studies that indicate isoform-specific inhibition of the CYP4F enzymes by 10-SUYS and DDMS (74). Another challenge will be the continued improvement of the biopharmaceutical properties of these inhibitors. Stability and appropriate pharmacokinetic properties are essential for the application of inhibitors in vivo.

Future studies will begin to focus more on the application of our current knowledge of 20-HETE effects and CYP arachidonic acid ω-hydroxylase regulation in human biology and disease. Sensitive LC/MS/MS and GC/MS assays are becoming more widely available for the quantitation of 20-HETE in urine, plasma, and other biological samples. This technology will no doubt prove useful in exploring the hypothesis that arachidonic acid ω-hydroxylation is altered in human disease, e.g., diabetes, as well as hypertension, pregnancy, and inflammation. The recent reports of 20-HETE urinary excretion patterns correlating with insulin levels and natriuresis (110–112) provide promise that therapeutic modulation of CYP arachidonic acid ω-hydroxylase may prove useful in the management of human disease. Another exciting area that should be explored is genetic variability in 20-HETE synthesis and the importance of this variation in eicosanoid function and disease susceptibility. The clinical significance of drug-induced alterations in CYP arachidonic acid ω-hydroxylase activity should also be studied. Although much remains unknown about the role of 20-HETE in human disease, the wealth of information in animal models will no doubt stimulate increasing interest in this field of study, with the hope that new drug targets might be identified for the management of vascular reactivity, renal function, and likely additional clinical conditions that remain to be discovered.

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