review

Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase

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Abstract The demonstration of in vivo arachidonic acid epoxidation and ω-hydroxylation established the cytochrome P450 epoxygenase and ω/ω−1 hydroxylase as formal metabolic pathways and as members of the arachidonate metabolic cascade. The characterization of the potent biological activities associated with several of the cytochrome P450-derived eicosanoids suggested new and important functional roles for these enzymes in cellular, organ, and body physiology, including the control of vascular reactivity and systemic blood pressures. Past and current advances in cytochrome P450 biochemistry and molecular biology facilitate the characterization of cytochrome P450 isoforms responsible for tissue/organ specific arachidonic acid epoxidation and ω/ω−1 hydroxylation, and thus, the analysis of cDNA and/or gene specific functional phenotypes. The combined application of physiological, biochemical, molecular, and genetic approaches is beginning to provide new insights into the physiological and/or pathophysiological significance of these enzymes, their endogenous substrates, and products.—Capdevila, J. H., J. R. Falck, and R. C. Harris. **Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase.** ***J. Lipid Res.*** **2000.** 41: **163–181.**

Supplementary key words cytochrome P450 • fatty acid hydroxylase • arachidonic acid • eicosanoids • arachidonic acid monooxygenase • arachidonic acid epoxygenase • EET • HETE • salt sensitivity • hypertension • hyperpolarizing factor • EDHF

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I. INTRODUCTION

The rapid advances in lipid biochemistry and in membrane, cellular, and molecular biology of the last 10–15 years have unraveled a complex array of cell signaling roles played by a variety of lipid molecules. The discovery and functional characterization of these lipid-derived mediators has become an important and growing area of chemical, biochemical, and physiological research, and led to the concept that, in addition to their structural roles, cellular lipids play important functional roles as key components of cell signaling cascades. Considerable efforts are currently being directed towards the identification and the molecular and functional characterization of these mediators, the study of their mechanisms of action, and of the enzymatic pathways responsible for their for-

Abbreviations: AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; RT-PCR, reverse transcriptase polymerase chain reaction; EET, epoxyeicosatrienoic acid; PLA_2, phospholipase A_2.

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mation, bioactivation, and disposition. Thus, for example, enzymatic regio- and stereoselective oxygenations of the arachidonic acid (AA) molecular template are utilized by eukaryotic cells for transduction of chemical information and the generation of cell signaling molecules. The AA metabolic cascade and its products, the eicosanoids, are a prime example of the physiological and biomedical importance of these lipid-derived molecules and of their biosynthetic pathways (1–12).

Most mammalian tissues contain substantial amounts of AA esterified to the *sn*-2 position of cellular glycerophospholipid so that, in the absence of an stimulus, the cellular levels of free AA are nearly undetectable. Upon stimulation, AA selective lipases release the fatty acid from unique, hormonally sensitive glycerolipid pools and make it available for its oxidation by the enzymes of the AA cascade (1–3, 6, 8). The absolute requirement for a free fatty acid, the presence of the AA-selective lipases, and the hormonal regulation of the releasing reaction control the rates of eicosanoid formation and allow for the functional and temporal integration of these bioactive molecules into cell signaling pathways (1–12). The AA metabolic cascade consists of prostaglandin H_2 synthase, lipoxygenases, and, more recently, cytochrome P450 (P450) (1–6, 8). Metabolism by prostaglandin H_2 synthase generates an unstable cyclic endoperoxide (prostaglandin H_2, PGH_2) that rearranges enzymatically or chemically to generate several prostaglandins (PGs), prostacyclin (PGI), or thromboxanes (TX) (1, 2). Metabolism by the lipoxygenases generates several regioisomeric allylic hydroperoxides containing a *cis-trans* conjugated-diene functionality (3). One of these, 5-hydroperoxyeicosatetranoic acid (5-HPETE), is the precursor in the biosynthesis of leukotrienes (3). The reactions catalyzed by prostaglandin H_2 synthase and lipoxygenases are mechanistically similar to those of free radical mediated autooxidation of polyunsaturated fatty acids. Reactions are initiated by regioselective hydrogen atom abstraction from a *bis*-allylic methylene carbon, followed by regio- and enantioselective coupling of the resulting carbon radical to ground state molecular oxygen. Thus, in contradistinction to the P450 monooxygenases, prostaglandin H_2 synthase and lipoxygenases are typical dioxygenases that catalyze substrate carbon activation, instead of oxygen activation (1, 2, 13).

Microsomal P450s comprise a family of membrane-bound hemoproteins that catalyze the redox couple activation of molecular oxygen and the delivery of an active form of atomic oxygen to a ground state substrate carbon acceptor (13). These ubiquitous proteins are widely distributed in plants, insects, and animal tissues, and are expressed in all mammalian cell types so far investigated (14, 15). P450s are typical monooxygenases in that the enzymatic cleavage of molecular oxygen is followed by the insertion of a single atom of oxygen into the substrate, while the remainder is released as water (13). Catalytic turnover requires electron transfer from NADPH to the P450 heme iron, a reaction catalyzed by a membrane-bound flavoprotein, NADPH-cytochrome P450 reductase (13). Microsomal P450s belong to a large gene superfamily coding for protein isoforms that share variable degrees of structural and functional homology and that have in common a cysteinyl-coordinated protoporphyrin IX heme prosthetic group (13–16). In mammals, the nature and the distribution of P450 isoforms is species specific and more or less age, sex, and organ dependent (13–16). Furthermore, the tissue inventory of P450 isoforms is also controlled by the animal hormonal status, diet, and its exposure to a wide variety of foreign chemicals (14–16). **Figure 1** shows an schematic view of the catalytic cycle of P450. The first three sequence of steps shown, i.e., substrate binding (Fe^{+3}-S), one electron reduction of the heme iron (Fe^{+2}-S), oxygen binding and formation of "oxy-P450" ($Fe^{+2}O_2$–S)

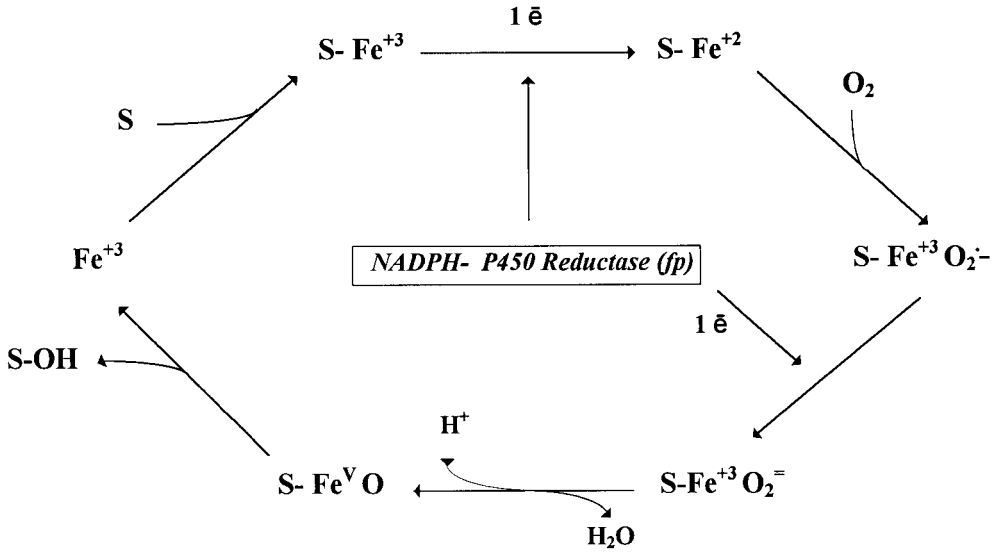

Fig. 1. Catalytic cycle of cytochrome P450 during NADPH-dependent substrate hydroxylation. For clarity, only a single iron resonance is shown; S, substrate; fp, flavoprotein.

complex (13) have been characterized spectrally and are known to follow the indicated sequence. The nature and the exact sequence of events occurring after the one electron reduction of the oxy-P450 complex, i.e., oxygen–oxygen scission, protonation, C–H insertion, and product and water release, are yet to be fully characterized. As indicated in Fig. 1 and during catalytic turnover, oxygen and NADPH are consumed in a 1:1 molar ration to generate equimolar amounts of the hydroxylated substrate and water.

As opposed to the other enzymes of the AA cascade in which, for the most part, functional significance preceded biochemical and molecular characterizations, the extensive biochemical and molecular characterization of microsomal P450s carried out during the last 20 years was stimulated by its toxicological and pharmacological relevance and its roles in chemical carcinogenesis (13–16). Hence, with the exception of P450 isoforms involved in cholesterol and stereoid metabolism, and notwithstanding abundant protein biochemical and molecular knowledge, until recently, few significant functional roles could be attributed to most microsomal P450 isoforms. However, during the last 10 years there has been an increased interest in the study of roles and functional significance of this enzyme system as a participant in the metabolism of various endogenous substrates, including fatty acids, hormones and vitamins. Among these, AA, as the precursor for several important bioactive eicosanoids, served as a focal point for many of these efforts (5–12). We will review past and recent advances in the molecular and enzymatic characterization of the P450 branch of the AA cascade, the biological properties and mechanism of action of its products, and their physiological and pathophysiological relevance. Functional aspects that, in our view, hold the greatest promise for the future will be also highlighted. It is important to recognize that, in addition to the catalysis of AA oxygenation, P450 plays an established role in the oxygenated metabolism of eicosanoids such as prostanoids, thromboxanes, and leukotrienes (16–28) and furthermore, that prostacyclin and thromboxane synthases have been characterized as P450-like peroxide isomerases (28).

II. CYTOCHROME P450 AND THE NADPH-DEPENDENT METABOLISM OF AA (THE P450 AA MONOOXYGENASE)

Studies of the involvement of microsomal P450 in the metabolism of AA were initiated in 1969 with the demonstration that several polyunsaturated fatty acids interacted with the heme moiety of microsomal P450 and inhibited drug metabolism (29). These studies were confirmed and expanded, nearly 10 years later, by Pessayre et al. (30). The then growing pharmacological and toxicological importance of P450 focused the attention of most researchers on the study of its role in the transformation of xenobiotics and thus, little further work was done in this area. In 1976, Cinti and Feinstein (31) demonstrated the presence of P450 in human platelets and, importantly, showed that AA-induced platelet aggregation could be blocked by known P450 inhibitors. It was not until 1981 that the role of cytochrome P450 in the oxidative metabolism of arachidonic acid was unequivocally demonstrated. Thus, microsomal fractions and/or purified preparations of P450 reconstituted with purified NADPH-P450 reductase were shown to catalyze the metabolism of AA to a group of polar products identified as mixtures of hydroxy- and epoxy-AA derivatives (32–35). It was evident from the outset that the physiological importance of the AA substrate made these observations unique and likely to be functionally significant. Furthermore, interest in these novel P450 reactions was stimulated by *a*) the initial demonstration that some of its products displayed potent biological activities, including the inhibition of distal nephron Na^+ reabsorption (36, 37), and *b*) the documentation of its participation in the in vivo metabolism of endogenous AA pools (38). These earlier studies established the metabolism of AA by P450 as a formal metabolic pathway, P450 as an endogenous member of the AA metabolic cascade and, more importantly, suggested functional roles for this enzyme in the bioactivation of the fatty acid and thus, in cell and organ physiology. In recent years, the study of the biochemistry and biological significance of these reactions has developed into an area of intense research and, as will be discussed below, work from several laboratories is beginning to establish biochemical and functional correlations that can be interpreted as suggestive of a physiological function (5–12).

The early biochemical studies of the P450-dependent AA monooxygenase reaction showed that catalytic turnover was NADPH-dependent, required a functional hemoprotein, and that it was unrelated to NADPH-dependent microsomal lipid peroxidation (16, 32–35). As with most enzymes of the arachidonate cascade, P450 did not metabolize phospholipid bound AA nor its methyl ester (16, 33). The detailed structural characterization of metabolites isolated from incubates containing rat liver or kidney microsomal fractions (6, 8, 16) demonstrated that, under conditions favoring primary metabolism, i.e., low protein concentrations and short incubation times, the microsomal P450 AA monooxygenase metabolized AA by one or more of the following type of reactions: *1*) *Bis*-allylic oxidation (lipoxygenase-like reaction) to generate six regioisomeric hydroxyeicosatetraenoic acids containing a *cis*, *trans*-conjugated dienol functionality (5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acids) (HETEs) (**Fig. 2**); *2*) hydroxylations at or near the terminal sp^3 carbon (the AA 7ω/ω–1 Hydroxylase) affords 16-, 17-, 18-, 19-, and 20-hydroxyeicosatetraenoic acids (16-, 17-, 18-, 19-, and 20-OH-AA)(ω, ω-1, ω-2, ω-3, and ω-4 alcohols) (Fig. 2); *3*) olefin epoxidation (the AA epoxygenase) furnishing four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids) (EETs) (Fig. 2). The EETs are *bis*-allylic epoxides and as such, they are rather resistant to attack by weak nucleophiles such as water and glutathione. On the other hand, cytosolic epoxide hydrolase catalyzes their rapid enzymatic hydration to the corresponding *vic*-dihydroxyeicosatrienoic acids (DHETs) (39, 40).

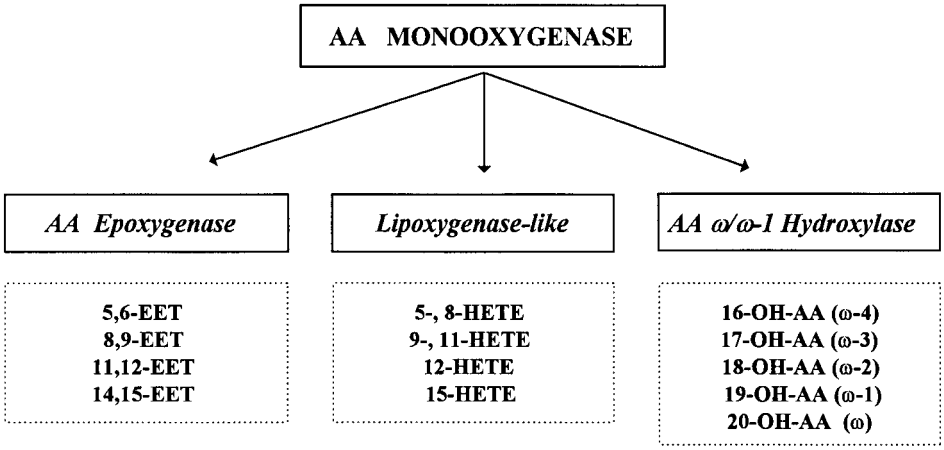

Fig. 2. Reactions catalyzed by the cytochrome P450 monooxygenase pathway of AA metabolism. Only the primary oxygenation products of the AA monooxygenase are shown (products derived from the P450-catalyzed oxygenation of AA).

Inasmuch as single time point organic solvent extracts obtained from incubates containing AA and microsomal or purified P450 isoforms are often utilized as estimates of P450 isoform levels and/or regulation, it is important to indicate that the reiterative oxygenation of primary metabolites by P450s is a well recognized phenomena (13–16, 39). Specifically, P450 AA metabolites, such as for example the EETs, compete efficiently with AA for the enzyme active site and are rapidly further oxidized, even in the presence of excess AA (39). During extended incubation times, the profile of metabolites generated from AA by the P450 enzymes becomes progressively complex due to the formation of complex mixtures of poly-oxidized products, many of which are recovered as water soluble (39). These secondary metabolism effects can severely complicate the estimation of enzyme activities, P450 isoform-specific metabolite formation, and isoform contribution to the overall metabolic capacity.

As with most P450 catalyzed reactions, the type of products generated from AA during metabolism are highly dependent on the tissue source of microsomal enzymes, animal species, sex, age, hormonal status, diet, and exposure to xenobiotics (5–9, 14, 16). For example, metabolism of AA by microsomal fractions isolated from rat liver generated four regioisomeric EETs as the major reaction products (⩾70% of total products) (**Table 1**). Under similar conditions, the products of the reaction catalyzed by rat kidney microsomes corresponded to a mixture of ω/ω-1 alcohols and EETs (77 and 23% of the total products, respectively) (Table 1). On the other hand, microsomal fractions isolated from the anterior lobe of rat pituitaries catalyze the NADPH-dependent and -independent formation of regioisomeric HETEs as the major reaction products (Table 1). Studies utilizing inducers of microsomal P450 or, alternatively, reconstituted systems containing solubilized and purified rat liver P450 isoforms (41) demonstrated that the hemoprotein controls, in an isoform specific fashion, oxygen insertion into the fatty acid template at three different levels: *a*) the type of reaction catalyzed, i.e., olefin epoxidation (EETs), allylic oxidation (HETEs) or hydroxylations at the C_{16}–C_{20} sp^3 carbons (ω, ω-1, ω-2, ω-3, and ω-4 alcohols); *b*) to a lesser extent, the regioselectivity of oxygen insertion, i.e., epoxidation at either of the four olefins, allylic oxidation initiated at any of the three *bis*-allylic methylenes, or hydroxylation at C_{16}–C_{20}; and *c*) the enantiofacial selectivity of the oxygenation step and the generation of chiral products (41).

The classification of the P450 derived arachidonate metabolites proposed in Fig. 2, was based on the chemistry of the reactions catalyzed by the enzyme system (16). Furthermore, this classification has provided a rational and useful framework for most of the subsequent studies of the biochemistry, molecular biology and functional significance of this pathway for arachidonate metabolism. Consequently, our discussion of the involvement of microsomal P450 in the oxygenated, NADPH-dependent, metabolism of AA will be based on that classification.

A. *bis*-Allylic oxidations (lipoxygenase-like reaction)

This activity of microsomal cytochrome P450 results in the formation of six regioisomeric allylic alcohols containing a characteristic *cis*, *trans*-conjugated dienol functionality (HETEs)(42). While these HETEs are structurally si-

TABLE 1. Reactions catalyzed by the arachidonic acid monooxygenases present in microsomal fractions isolated from rat liver, kidney, and pituitary

Type of Reaction	Liver	Kidney	Pituitary[a]
	% of total microsomal metabolism		
Epoxygenase	70	23	30
ω/ω–1 Hydroxylase	20	77	16
Lipoxygenase-like	9	nd	45
Other[b]	⩽1	⩽1	8
Total rate[c]	2.4	0.34	0.2

[a] Microsomal fractions isolated from anterior pituitaries.
[b] Products that remain to be characterized.
[c] Initial reaction rates in nmol of product/min/mg of microsomal protein at 30°C.

milar to those of mammalian and plant lipoxygenase origin, the requisite fatty acid hydroperoxide intermediary has never been isolated (42). A mechanism for P450-dependent HETE formation that involves *bis*-allylic oxidations at either C7, C10, or C13, followed by acid-catalyzed rearrangements to the corresponding *cis, trans* dienols has been proposed, and the intermediate 7-, 10-, and 13-hydroxyeicosatetraenoic acids have been isolated from incubates containing AA, rat liver microsomes, and NADPH (43, 44). As 12(R)-HETE was the predominant enantiomer generated by the P450 catalyzed reaction (45), and as all mammalian 12-lipoxygenases were known to be selective for 12(S)-HETE, it was thought that mammalian 12(R)-HETE was exclusively a product of the P450 enzyme system (45–47). However, two human regio- and stereoselective 12(R)-lipoxygenases were recently cloned, characterized, and shown to be expressed in human skin (48, 49). These studies have questioned the role(s) that P450 may play in the in vivo generation of 12-HETE and, in particular, during the pathophysiology of human psoriasis (46). It has been proposed that 12(R)-HETE is the product generated from AA by bovine cornea epithelium by a P450-mediated reaction (47). However, more recently, lipoxygenase-derived 12(S)-HETE was shown to be the only 12-HETE eicosanoid generated by that tissue (50). Importantly, in vitro studies have demonstrated that 12(R)-HETE is a powerful and enantioselective inhibitor of Na^+/K^+ ATPase activity (47). Finally, the enzymatic formation of 12(R)-hydroxy-5,8,14-eicosatrienoic acid, a potent ocular pro-inflammatory and vasodilatory substance in rabbits, has been elucidated (51).

The contribution of P450 to the in vivo formation of HETEs, as well as the molecular characterization of P450 isoforms responsible for these reactions, remains to be determined. Nevertheless, the unique chirality of these products and their associated biological activities continue to stimulate interest in their study. Areas to be clarified include: *a*) the role of P450 in the biosynthesis of endogenous HETE pools, *b*) the identification and molecular characterization of the individual P450 isoforms responsible for this reaction and, *c*) the contributions of P450 and 12-lipoxygenases to organ-specific 12(R)-HETE biosynthesis (45–51).

B. Hydroxylations at C_{16}–C_{20} (ω/ω-1 hydroxylase reaction) The AA ω/ω-1 hydroxylase

1. Introduction. The ω and/or ω-1 hydroxylation of saturated, mid-chain, fatty acids is the oldest and best characterized role that microsomal P450 plays in fatty acid metabolism. For instance, lauric acid ω-oxidation was the reaction utilized to demonstrate the first functional reconstitution of a purified liver microsomal P450 (52). It has been the general consensus that these reactions participate in the catabolism of several mid-chain fatty acids prior to degradation by β-oxidation and/or urinary excretion. In 1981, AA joined the list of substrates for these P450-mediated reactions when 19- and 20-OH-AA were isolated from incubates containing AA, NADPH, and rabbit kidney cortex microsomes (32, 34, 35). It is of interest that the microsomal ω and ω-1 hydroxylation of saturated fatty acids, in particular of lauric acid, proceeds at rates that are substantially higher than those obtained with AA. However, regardless of the structure of the fatty acid substrate, i.e., saturated (e.g., laurate) or polyunsaturated (e.g., arachidonate), ω/ω-1 oxidation entails the delivery by P450 of a reactive oxygen to ground state sp^3 carbons. Therefore, it is likely that for these reactions, the oxygen chemistries and the reaction mechanism(s) are similar and independent of the degree of saturation in the fatty acid. Nevertheless, the AA molecular template imposes additional steric requirements on the P450 protein catalyst. Hydroxylation at the thermodynamically less reactive C_{16} through C_{20} and not at the chemically comparable C_2 through C_4 suggest a rigid and highly structured binding site for the AA molecule. This AA binding site must be capable of positioning the acceptor carbon atoms not only in optimal proximity to the heme-bound active oxygen but also with complete segregation of the reactive olefins and *bis*-allylic methylene carbons.

2. Enzymology, isoform specificity. AA ω/ω-1 hydroxylation has been demonstrated in microsomal fractions isolated from organs such as liver, kidney, lung, intestine, olfactory epithelium and anterior pituitaries (5, 6, 8, 16). However, it is in renal tissues that these reactions are best characterized as they are the most prevalent and have several postulated functional roles (5–12). Extensive enzymatic, biochemical and molecular biology evidence shows that P450 4A isoforms are the predominant fatty acid ω/ω-1 hydroxylases in most mammalian tissues (5–12, 15, 16, 53–71). The P450 4A gene subfamily is composed of a group of highly conserved genes coding for proteins that are specialized for fatty acid oxidation and with little or no activity towards the metabolism of most xenobiotics (13–16, 53–71). Fatty acid ω/ω-1 hydroxylations are regulated in vivo by a wide variety of factors including animal age, diet, starvation, administration of fatty acids, hypolipidemic drugs, dioxins, flavonoids, aspirin, steroids, mineralocorticoids, and diabetes (5, 6, 15, 16, 18, 72–77). In rodents, the transcriptional regulation of P450 4A isoforms is under the control of the nuclear receptor peroxisomal proliferator activated receptor alpha ($PPAR_\alpha$) (72, 77). The coordinated, $PPAR_\alpha$-controlled induction of the peroxisomal fatty acid β-oxidation pathway and of P450 4A isoforms has suggested a role for these P450s in lipolysis and fatty acid homeostasis (72, 77). The metabolism of AA to 18(R)-OH-AA by microsomal fractions isolated from monkey seminal vesicles has been shown (19). Finally, purified P450 2E1, an isoform induced in rat liver by diabetes, fasting and alcohol, converted AA to 19(S)- and 18(R)-OH-AA (with 72 and nearly 100% optical purity, respectively) as its major reaction products (78).

Studies with inducers of liver microsomal P450 indicated that AA hydroxylation at C_{16}, C_{17}, C_{18}, and C_{19}, but not at C_{20}, was induced by β-naphthoflavone and dioxin (79, 80) and that liver microsomes isolated from β-naphthoflavone treated rats generated a mixture of the corresponding 16-, 17-, 18-, and 19-OH-AA (or ω-4, ω-3, ω-2,

TABLE 2. Regioselectivity of arachidonic acid hydroxylation by cytochrome P450 1A and 4A isoforms

Regioisomer	1A1	1A2	4A1	4A2
		% distribution		
16-OH-AA	8	47	nd	nd
17-OH-AA	19	20	nd	nd
18-OH-AA	19	13	nd	nd
19-OH-AA	46	20	nd	25
20-OH-AA	nd	nd	100	75
		% of total products		
	87	44	100	100

Purified recombinant cytochrome P450s 1A1, 1A2, 4A1, and 4A2 were reconstituted in the presence of NADPH-cytochrome P450 reductase, and dilauroylphosphatidylcholine as described in references 41 and 71; nd, not detectable.

and ω-1 alcohols, respectively)(79). Reconstitution experiments using purified liver P450 1A1 and 1A2, the two major liver P450 isoforms induced by animal treatment with β-naphthoflavone or dioxins, demonstrated that these isoforms were more or less regioselective for arachidonate oxidation at the C_{16}–C_{19} carbons (87 and 44% of total products for P450 1A1 and 1A2, respectively) (79, 80) (**Table 2**). Furthermore, while P450 1A1 oxidized AA preferentially at C_{19}, oxygenation by P450 1A2 occurred predominantly at C_{16} (79) (Table 2). Whether these P450 1A1 and 1A2 regioselectivities are unique to AA or common to all fatty acids remains to be determined. Furthermore, it is of interest that despite the limited sequence homology that exists between 1A and 4A isoforms, these enzymes show a distinct regioselectivity for the adjacent C_{19} and C_{20} carbons (79).

Several rat, mouse, rabbit, and human members of the P450 4A gene subfamily have been purified and/or cloned and expressed (6, 10, 15, 53–71). P450 4A isoforms appear to be highly specialized for fatty acid and/or prostanoid metabolism (53–71). In rats and rabbits, the 4A gene subfamily is composed of four highly homologous genes (15, 71, 72). Amino acid sequence analysis showed that the rat 4A proteins could be divided into two groups that share ⩾71% overall homology (15, 71). One group is composed of P450s 4A1 and 4A8 (76% sequence identity), and the other is composed of the highly homologous P450s 4A2 and 4A3 (98% sequence identity)(15, 71). The high level of nucleotide sequence identity shared by the P450s 4A2 and 4A3 extends into the corresponding gene intronic areas, and suggests that these genes arose from a relatively recent duplication event (57, 58, 71). Furthermore, the presence of a single murine gene, (P450 4a14) highly homologous to both rat P450s 4A2 and 4A3 (81), suggests that the duplication event occurred after the evolutionary separation of rat and mouse. Studies by Sundseth and Waxman (82) demonstrated sex-specific expression of P450 4A2 in the rat liver and kidney. Recent data from our laboratory confirmed the male-specific expression of this enzyme in rat liver, but showed comparable levels of P450 4A2 transcripts in male and female rat kidney RNAs (71). However, immunoelectrophoresis analysis revealed the presence of anti-P450 4A2 immunoreactive material only in the male rat kidney (71).

Table 3 summarizes the published fatty acid metabolic properties for several purified and recombinant P450 4A isoforms. As shown in this table, all enzymatically characterized P450 4A proteins (either purified or recombinant proteins) catalyze saturated fatty acid ω-oxidation and most also hydroxylate AA at either the C_{20}, or the C_{19} and C_{20} carbon atoms (Table 3). To date, no member of the P450 4A gene subfamily is known to be selective only for the ω-1 hydroxylation of fatty acids, including AA (Table 3). It is of interest that, despite their high structural homology, P450s 4A2 metabolizes AA while P450 4A3 is either inactive (71), or does it at very low rates (Table 3)(70). On the other hand, both P450 4A2 and 4A3 are active lauric acid ω and ω-1 hydroxylases (71). Recently, a cDNA coding for rat P450 4A2 was cloned by RT-PCR of total kidney RNA and expressed using a baculovirus/*sf*^{9} insect cell expression system. Microsomal fractions isolated from these cells were shown to catalyze the metabolism of AA to mixtures of 19- and 20-OH-AA, as well as small quantities of 11,12-EET (70). On the other hand, the metabolism of AA to solely 19- and 20-OH-AA by purified recombinant P450 4A2 and the presence of an endogenous AA epoxygenase activity in insect cell membranes were reported (71).

TABLE 3. Metabolism of Fatty Acids and Prostanoids by Cytochrome P450 4A Isoforms

4A Isoform	Species	Enzymatic Activities
4A1	rat	ω-oxidation of laurate and arachidonate
4A2	rat	ω/ω-1 oxidation of laurate and arachidonate
4A3	rat	ω/ω-1 oxidation of laurate and arachidonate
4A8	rat	unknown
4A4	rabbit	ω-oxidation of palmitate, arachidonate and of prostaglandins A, E, D and $F_{2\alpha}$
4A5	rabbit	ω/ω-1 oxidation of laurate and palmitate some ω-oxidation of PGA_1 and arachidonate
4A6	rabbit	ω-oxidation of laurate, palmitate and arachidonate. Low PGA_1 ω-oxidation
4A7	rabbit	ω-oxidation of laurate, palmitate, arachidonate, and PGA_1, inactive towards PGE_2
4a10	mouse	ω-oxidation of laurate, arachidonate
4a12	mouse	Unknown
4a14	mouse	ω/ω-1 oxidation of laurate, but not arachidonate
4A9	human	ω-oxidation of laurate
4A11	human	ω-oxidation of laurate

Data presented were compiled from references 52 through 71.

C. Olefin epoxidation (epoxygenase reaction)
The cytochrome P450 AA epoxygenase

1. Introduction. The catalysis of AA epoxidation by P450 was initially suggested by the demonstration that 11,12- and 14,15-dihydroxyeicosatrienoic acids were formed by incubates containing kidney cortex microsomes, AA, and NADPH (83). Soon thereafter, Chacos et al. (84) demonstrated that rat liver microsomal fractions catalyzed the NADPH-dependent formation of 5,6-, 8,9-, 11,12-, and 14,15-EET. These studies unambiguously established microsomal P450 as an active AA epoxygenase and focused attention on its biochemical and physiological implications (5–12). The discovery of several EET-associated biological activities suggested, early on, a potential functional significance for these reactions (36, 37). To date, the catalysis of EET formation by purified P450s, microsomal fractions, or isolated cell preparations has been demonstrated in numerous tissues, including liver, kidney, pituitary, brain, adrenal, endothelium, and ovaries (5, 6, 8, 16). Finally, the establishment of EETs as endogenous constituents of rat liver, human urine, and rabbit kidney move the epoxygenase from the domain of in vitro biochemical reactions to that of an endogenous metabolic pathway (38, 85–89) and instituted microsomal P450 as participant in the metabolism of endogenous fatty acids such as AA.

Only *cis*-epoxides are generated by the P450 system (6, 16, 84). This is consistent with olefin epoxidation via a concerted process or, alternatively, with a protein catalyst imposed restriction in the freedom for C–C rotation in the transition state. In mammals, the epoxidation of polyunsaturated fatty acids to *bis*-allylic, *cis*-epoxides is unique to the P450 enzyme system and, at difference with the fatty acid ω/ω-1 oxygenase, more or less selective for AA (6, 16). Thus, while the enzymatic or non-enzymatic reduction and/or isomerization of polyunsaturated fatty acid hydroperoxides can yield epoxides or epoxy-alcohol derivatives, these products are structurally different than those generated by the P450 enzymes (16, 90).

2. Enzymology, isoform specificity. The multiplicity of P450 isoforms involved in AA epoxidation by liver microsomes was initially suggested by observed changes in EET chirality resulting from animal treatment with P450 inducers (41). For example, phenobarbital treatment increased, in a time-dependent fashion, the regio- and enantiofacial selectivity of the rat liver microsomal epoxygenase(s) (41). The phenobarbital-induced increases in stereoselectivity resulted in a remarkable inversion in absolute configuration of the EETs produced by the liver microsomal enzymes (41). These studies suggested that: *a*) the enantioselectivity of the P450 epoxygenase was variable, and *b*) amongst the enzymes of the arachidonate cascade, the P450 epoxygenase was unique in that its regio- and stereochemical selectivity was under regulatory control and it could be experimentally altered, in vivo, by animal manipulation (41). It was subsequently demonstrated that arachidonate epoxidation is highly enantioselective and that P450 controls, in an isoform specific fashion, the regio- and enantioselectivity of the epoxygenase reaction (6, 8, 16, 41, 80, 89, 91–100). **Table 4** shows a summary of the enantiofacial selectivity of selected rat liver and kidney AA epoxygenases.

TABLE 4. Enantiofacial selectivity of arachidonic acid epoxidation by cytochrome P450 2 gene family isoforms

EET Enantiomer	P450 2B2	P450 2C23	P450 2C24
	enantiomeric composition, %		
8(S),9(R)-EET	90	6	61
8(R),9(S)-EET	10	94	39
11(S),12(R)-EET	84	11	25
11(R),12(S)-EET	16	89	75
14(S),15(R)-EET	35	75	33
14(R),15(S)-EET	65	25	67

Purified recombinant P450s 2B2, 2C23, and 2C24 were reconstituted as described in Table 2 and references 41 and 71.

Reconstitution of the P450 AA monooxygenase activity using purified P450 isoforms and/or recombinant proteins demonstrated that most of the AA epoxygenases were members of the P450 2 gene family (41, 91–100). Isoforms of the 2B and 2C subfamily so far identified as epoxygenases include rat 2B1, 2B2, 2B4, 2B12, 2C11, 2C23, and 2C24 (41, 91, 96, 97, 100); rabbit 2C1 and 2C2 (93), mouse 2b19 (98), 2c37, 2c38, 2c39, and 2c40 (99), and human 2C8, 2C9, and 2C10 (92). While P450s 1A1, 1A2, and 2E1 are active AA ω/ω-1 oxygenases, they also produce low and variable amounts of EETs (⩽20 of total products) (41, 78, 80). More recently, P450s 2J2 and 2J4 have been identified as organ specific epoxygenases (94, 95). A unique case is that of a P450 purified from the livers of dioxin-treated chick embryos (80). This protein has several structural features typical of proteins of the 1A gene subfamily, but metabolizes AA to EETs as the major reaction products (75% of total products) (80).

The structural characterization of the EETs generated by incubates containing purified 2B and 2C P450 epoxygenases showed that, among these proteins, none was selective for the epoxidation of a single fatty acid olefin (**Table 5**). Thus, although highly enantioselective, AA epoxidation showed a more limited regioselectivity (41, 91–97, 100) (Tables 4 and 5). The application of recombinant DNA methods and heterologous protein expression has allowed unequivocal assignments of regio- and enantioselectivity and epoxygenase activity to individual P450 isoforms (91, 92, 94–100). The ability of a single 2C P450 protein to catalyze the enantioselective epoxidation of more than one fatty acid olefin was clearly demonstrated using recombinant proteins (91, 92, 94–100). For example, the first recombinant epoxygenase characterized, rat kidney P450 2C23, catalyzed the enantioselective epoxidation of AA to 5,6-, 8,9-, 11,12-, and 14,15-EET (Table 4), with 11,12-EET as its major product (58% of total; Table 5)(91). Recombinant P450 2C23 generated 8(R),9(S)-, 11(R),12(S)-, and 14(S),15(R)-EETs with optical purities of 94, 89, and 75%, respectively (Table 4) (91). On the other hand, rat liver P450 2B2 also catalyzes the highly asymmetric epoxidation of AA but its enantiofacial selectivity is the opposite of that of P450 2C23 (Table 4). Finally, P450 2C24, an isoform expressed in rat liver and kid-

TABLE 5. Regioselectivity of AA epoxidation by purified rat P450 2 gene family epoxygenases

Product	P450 2B1	P450 2B2	P450 2C11	P450 2C23	P450 2C24
			% of total EETs		
5,6-EET	⩽6	⩽5	⩽1	9	6
8,9-EET	27	27	19	23	16
11,12-EET	37	26	41	58	40
14,15-EET	30	42	39	9	37
			% of total		
AA epoxygenase	100	100	72	98	62

P450s 2B1 and 2B2 were purified from rat livers as described in reference 41. Purified recombinant P450s 2C11, 2C23, and 2C24 were obtained as described in references 91 and 100. The AA epoxygenases were reconstituted as described in Table 2.

ney, shows overall lower stereoselectivity and, while its stereochemical preference for the 11,12-olefin mimics that of P450 2C23, the enzyme epoxidizes the 8,9- and 14,15-olefins with a chiral selectivity similar to that of P450 2B2 (Table 4) (100).

The highest degree of regioselectivity so far documented for a mammalian epoxygenase is that of P450 2B12. This enzyme, a keratinocyte specific rat P450, generates 11,12-EET as its major reaction product (⩾80% of total products)(96). The regioselective formation of 11,12- and 14,15-EET by rabbit P450s 2C2 and 2CAA, purified from rabbit kidney cortex, was reported (93). The role that the chemical and spatial orientation properties of single amino acid residues play in controlling the regio- and stereoselectivity of AA epoxidation was suggested by single amino acid replacements introduced into the primary structures of rat P450 2B1 and bacterial P450 BM3 (101–103). Recombinant P450 2B1 metabolizes AA to predominantly the 11,12- and 14,15-EETs (101). Replacement of isoleucine 114 for alanine changed the regioselectivity of P4502B1 towards epoxidation at the 5,6- and 8,9-olefins and reduced, concomitantly, the extent of 11,12- and 14,15-epoxidation (101). On the other hand, studies with bacterial P450 BM3 demonstrated that a single active site amino acid replacement was sufficient to convert this enzyme from a predominantly ω-3 hydroxylase into a regio- and enantioselective 14,15-epoxygenase (14(R),15(S)-EET ⩾98% of total products) (102, 103). Finally, it is of interest that the degrees of enantiofacial selectivity displayed by most AA epoxygenase isoforms are unusually high for P450-catalyzed oxidations of unbiased, non-cyclic molecules such as AA (16).

Antibody inhibition and product chirality experiments indicate that the majority of the constitutive arachidonate epoxygenases in rat liver and kidney microsomes belong to the P450 2C gene subfamily (41, 89, 91, 100). By analogy, it was concluded that the predominant human epoxygenase(s) are also members of the P450 2C gene subfamily (92, 104). The cDNAs coding for human P450 2C8 and 2C9/2C10 were cloned, expressed and shown to catalyze the regio- and enantioselective epoxidation of AA (92). In rat, rabbit, and humans, the P450 2C gene subfamily codes for a highly homologous group of proteins, many of which are expressed constitutively (15, 16, 77, 105). Additionally, some 2C isoforms are sex-specific and/or expressed under developmental and hormonal control (15, 16, 77, 105). While the members of the 2C gene subfamily share extensive sequence homology, it has been demonstrated that this P450 protein structural homology can be often accompanied by significant catalytic heterogeneity (15, 16, 41, 89, 91–97, 100, 105). For example, while the P450 2C8 and 2C9 proteins are ~90% homologous in their amino acid sequences, recombinant P450s 2C8 and 2C9 epoxidize AA with distinct regio- and stereochemical selectivities (92). As more recombinant 2C proteins become available, reconstitution studies will be useful in defining: *a*) the extent to which these P450 isoforms contribute to the epoxidation of endogenous AA pools, *b*) their tissue and/or organ specific distribution, and *c*) their regulation by physiologically meaningful stimuli.

3. The P450 AA epoxygenase: a member of the endogenous "AA metabolic cascade. Inasmuch as in vitro studies are an indispensable tool for the biochemical, enzymatic and molecular characterization of metabolic pathways, they provide only limited information concerning the in vivo significance of the products and enzymes involved. Additionally, in view of its well known catalytic versatility, the participation of microsomal P450 in the in vitro epoxidation of AA was not completely unexpected. It was therefore apparent that the uniqueness and the significance of the P450 AA epoxygenase reaction were going to be defined by whether or not: *a*) the enzyme system participated in the in vivo metabolism of the fatty acid and, *b*) its products played significant roles in cell and organ physiology. As asymmetric synthesis is an accepted requirement for the biosynthetic origin of most eicosanoids, a method for the quantification and chiral characterization of the EET pools present in biological samples was developed and utilized to show the endogenous biosynthesis in rat liver of 8,9-, 11,12-, and 14,15-EET in a 4:1, 2:1, and 3:1 ratio of antipodes, respectively (85). The analysis of the effects of known P450 inducers in the levels and structural properties of endogenous EETs was used to demonstrate the role of P450 in the in vivo catalysis of AA epoxidation (85). Taken together, these results *a*) demonstrated the enzymatic origin of the EETs present in rat liver, and *b*) documented a new metabolic function for P450 in the epoxidation of endogenous AA pools. Differences between the optical purities of the EETs present endogenously in rat liver and those isolated from in vitro microsomal incubates indicated that: *a*) factors other than rates of biosynthesis control the in vivo steady state concentrations of liver EETs, or *b*) epoxygenase isoforms responsible for endogenous EET biosynthesis are lost during isolation and/or analysis of the microsomal enzymes. At present, the existence of endogenous chiral EETs has been demonstrated in rat liver, lung, kidney, brain, plasma, and urine; in rabbit lung, kidney, and urine; and in human liver, kidney, plasma, and urine (5–12).

A distinctive feature of the endogenous EET pools in rat liver and kidney was their presence esterified to the *sn*-2 position of several cellular glycerophospholipids (⩾92% of the total liver EETs) (106). Chiral analysis of the fatty acids at

sn-2 revealed an enantioselective preference for 8(S),9(R)-, 11(S),12(R)-, and 14(R),15(S)-epoxyeicosatrienoates in all three classes of phospholipids, with 55% of the total liver EETs in phosphatidylcholine, 32% in phosphatidylethanolamine, and 12% in phosphatidylinositols (106). EET-phospholipid formation required a multistep process, initiated by the P450 enantioselective epoxidation of AA, ATP-dependent activation to the corresponding EET-CoA derivatives, and EET enantiomer-selective lysophospholipid acylation (106). The asymmetric nature of the esterified EET cogently demonstrated that rat liver biosynthesized these lipid from endogenous precursors, enzymatically and under normal physiological conditions (106). The observed in vivo EET esterification process appears to be unique as most endogenously formed eicosanoids are either secreted, excreted, or undergo oxidative metabolism and excretion. There are, however, reports of esterification by isolated cells of exogenously added HETEs and EETs (107–109).

The biosynthesis of endogenous pools of phospholipids containing esterified EET moieties in rat liver, kidney, brain, and plasma and in human kidney and plasma (8, 16, 85–88, 106, 109), indicate new and potentially important functional roles for P450. As a participant in the arachidonate cascade, microsomal P450 may play a central role(s) in the biosynthesis of unique cellular glycerolipids and thus, in the control of membrane physicochemical properties and/or the generation of novel lipid-derived mediators. Furthermore these studies also show, in contrast to most eicosanoids, the potential for the cellular generation of preformed bioactive EETs via hydrolytic reactions, thus obviating the need for AA oxidative metabolism.

The lipid bilayer provides the matrix in which structural and functional membrane proteins fold or unfold, oscillate between functionally significant conformational states, rotate or move laterally, interact with each other or with their substrates, and ultimately carry out their cellular functions. Localized changes in membrane lipid composition, as well as the asymmetry in the lipid distribution of many biological membranes, allow cells to manipulate the physicochemical properties of membrane domains (110–115) albeit, in many situations, with severe time and spatial limitations. The capability to enzymatically epoxidize localized areas of the phospholipid bilayer could provide cells with a powerful tool for the rapid and efficient control of the structural properties of individual membrane domains. Based on these studies and, the capacity of synthetic 8,9-epoxyeicosatrienoyl-phosphocholine to alter the Ca^{2+} permeability of synthetic liposomes, we proposed a functional role for microsomal P450 in the control of cell membrane microenvironment structure and, hence its functional properties (106, 115). As illustrated in **Fig. 3**, we envision a process in which enzyme-controlled AA release, epoxidation, and EET acylation will induce localized changes in the fluidity of selected membrane micro-environments. These EET-phospholipid (EET-PL)-induced changes in the physicochemical properties of the membrane lipid bilayer could result in its transition from a semi-crystalline, ordered state to a less ordered, more fluid, state (Fig. 3). Changes in the lipid bilayer order, fluidity, and volume could regulate the flux of ions (for example Ca^{+2}) across the membrane permeability barrier (Fig. 3)(115). The process could then be reversed by a lipase-catalyzed hydrolysis of the acylated EET, followed by enzymatic hydration to the corresponding DHET (Fig. 3). Of interest, under conditions that are optimal for EET esterification, no DHET acylation could be detected (106). Finally, the formation and incorporation of EETs into cellular lipids may provide the molecular basis underlying some of the biological properties attributed to the EETs, many of which can be interpreted in terms of the ability of these compounds to alter cell membrane. In this regard, the inhibition of reconstituted L-type calcium channels by synthetic 1-palmitoyl-2-(11,12)-epoxyeicosatrienoyl phosphatidylcholine was recently reported (116).

III. FUNCTIONAL SIGNIFICANCE OF THE P450-EICOSANOIDS

The identification and subsequent chemical synthesis of the P450-eicosanoids opened the door to the study of

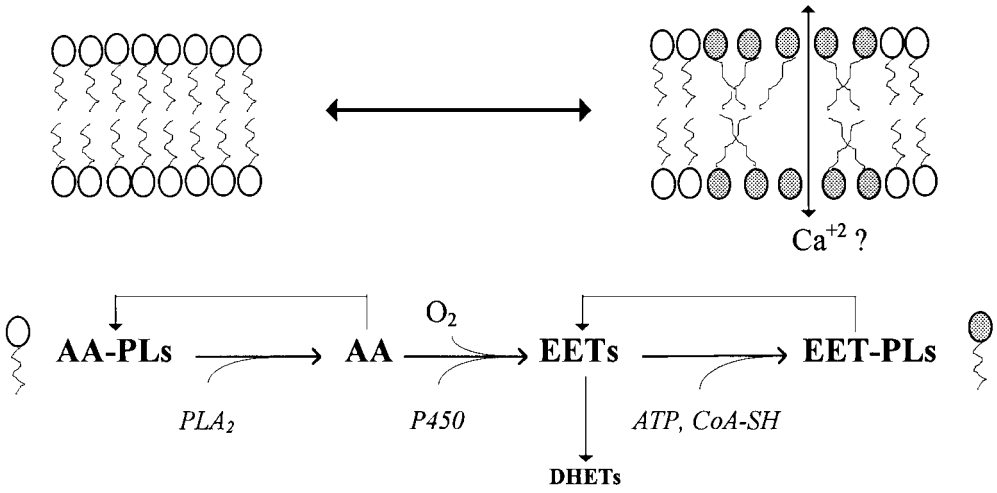

Fig. 3. Cytochrome P450 and membrane micro-environments.

their biological significance. Since then numerous studies have investigated the possibility that the P450 AA metabolites serve physiological and/or pathophysiological roles (5–12). In whole animal physiology, these compounds have been implicated in the mediation of release of peptide hormones, regulation of vascular tone and regulation of volume homeostasis (5–12). On the cellular level, P450 AA metabolites have been proposed to regulate ion channels and transporters and to act as mitogens (5–12).

A. Organ/whole animal functions of the P450-eicosanoids

The first physiologic function attributed to EETs was as secretagogues for peptide hormone release, including hypothalamic release of somatostatin, anterior pituitary release of ACTH, prolactin, luteinizing hormone, and growth hormone, posterior pituitary release of vasopressin and oxytocin, and pancreatic islet release of insulin and glucagon (5, 8, 16). Both 14,15-EET and 12(R)-HETE have also been reported to decrease renal renin release (117, 118). More recently, there has been a wealth of studies indicating that P450 AA metabolites are involved in regulation of vascular tone. However, the results of different investigators have provided conflicting conclusions. Although all of these apparent conflicts cannot yet be resolved, differences in methodology and/or vascular beds studied may be the explanation for some of the observed differences. The vascular bed studied appears to be especially important and, in general, the microvasculature has been reported to utilize P450 AA metabolites in regulation of vascular tone more often than larger vessels (119). Furthermore, a consensus is beginning to arise that 20-OH-AA is a vasoconstrictor and the EETs are vasodilators (5–12, 119–125). 20-OH-AA has been proposed as a mediator of auto-regulation of vascular tone in the renal and cerebral microcirculations (12, 123). However, there remain significant discrepancies in the published literature. Vasoconstriction by 20-OH-AA has been reported to be either cyclooxygenase-dependent (125), or -independent (122, 123). 20-OH-AA has also been reported to be vasodilatory in certain vasculature (coronary and pulmonary vessels), an effect dependent upon cyclooxygenase activity (126–128). Systemic infusion of 20-OH-AA into an anesthetized rat produced natriuresis without alteration of systemic hemodynamics (129). Of the other AA-alcohols produced by P450 metabolism of AA, 12(R)-HETE has been reported to be a vasoconstrictor (130) and 16-, 18- and 19-OH-AA are reported to be vasodilators (131).

Of the four EET regioisomers, 5,6-EET has been most frequently reported to possess vasoactive properties. It has been reported to be vasodilatory (5, 132–135), an effect that certain investigators have reported to be cyclooxygenase-dependent (134, 136) and nitric oxide-dependent (134). Other investigators have determined cyclooxygenase-dependent vasoconstriction (129, 135, 137), with thromboxane A_4 implicated as the vasoconstrictive agent (137). However, as with many studies involving these compounds, certain investigators have been unable to elicit any vasoactive effect with 5,6-EET administration (138, 139). 8,9-EET has also been reported to be a vasodilator (140), a vasoconstrictor (139, 141), or to be without effect (137, 138, 142). Studies by Katoh et al. (141) have determined that the stereoselective enantiomer 8(R),9(S)-EET but not 8(S),9(R)-EET was able to elicit vasoconstriction of the renal vasculature. Similar stereoselectivity has been described for vasodilatation observed by 11,12-EET (137, 143); again, some studies have reported 11,12-EET to be a vasodilator (132, 137, 138, 140, 143), while others have reported vasoconstriction (139) or no effect (142). As with the other EETs, 14,15-EET has also been reported to be a vasodilator (132, 137, 138), a constrictor (137) or ineffective in altering vascular tone (140, 142, 143). A recent report has indicated that, in addition to their vasoactive properties, the EETs may also act as vascular anti-inflammatory molecules by inhibiting the cytokine-induced expression of adhesion molecules in cultured endothelial cells (144).

A number of studies have indicated that EETs may serve as an endothelium-derived hyperpolarizing factor (EDHF) that mediates vasodilatation by activation of smooth muscle voltage-dependent K^+ channels (145–148), although other studies would disagree (149–152). Again, the vascular bed (and perhaps the species) selected for study may be a factor in these disparate results, as Pfister et al. (153) reported that in secondary arteries, such as mesenteric and coronary arteries, EETs serve as an EDHF, while in aorta, the lipoxygenase products, 11,14,15- and 11,12, 15-trihydroxyeicosatrienoic acids, serve as an EDHF (153).

P450 AA metabolites have also been suggested to be mediators of salt and water regulation by the kidney. In this regard, 5,6-EET has been shown to inhibit Na^+ reabsorption in proximal tubule (37, 154) and cortical collecting duct (155), and DHETs have been shown to inhibit vasopressin-stimulated water reabsorption in collecting duct (156). 20-OH-AA has also been shown to inhibit volume reabsorption in proximal tubule (R. Quigley, J. R. Falck, and M. Baum, unpublished results) and to inhibit solute reabsorption in the thick ascending limb of Henle (157–159). In this latter segment, production of 20-OH-AA can be stimulated in response to increases in extracellular calcium by the extracellular calcium-sensing receptor, and 20-OH-AA may then act to inhibit Na/K/Cl cotransport and the renal outer medulla K^+ channel (160).

B. Cellular functions of the P450-eicosanoids

1. Modulation of channel activity. As mentioned above, in vascular smooth muscle, EETs activate Ca^{2+}-activated K^+ channels, which is thought to mediate the vasodilatory properties of these compounds and to underlie their role as an endothelium-derived hyperpolarizing factor (EDHF) (119). Although some investigators have found that all EETs will activate Ca^{2+}-activated K^+ channels in smooth muscle cells (132), Zou et al. (143) have reported stereo- and regiospecificity for 11(R),12(S)-EET. It is of interest that administration of EETs activates calcium-activated K^+ channels in intact vascular smooth muscle cells but has no effects when added directly to cytoplasmic surface of excised inside-out patches (161), and channel activation re-

quires intermediate signaling steps involving G-proteins (162). As indicated, the EETs have been reported to activate Ca^{2+}-activated K^+ channels (143, 161–163). 20-OH-AA activates L-type Ca^{2+} channels in cerebral vessels (121) and inhibits opening of large conductance Ca^+-activated K^+ channels renal arteries and a 70 ps K^+ channel in thick ascending limb of Henle (160). A recent study using antisense oligonucleotide transfections confirmed the proposed EDHF role of 11,12-EET and indicated its biosynthesis by a P450 2C isoform (164).

2. Regulation of transporters. P450 arachidonate metabolites have also been shown to modulate the function of membrane-bound transport proteins. Thus, for example, 14,15-EET stimulated Na^+/H^+ exchange activity in LLC-PK_1 cells (165, 166) and glomerular messangial cells (167). In contrast, 5,6-EET inhibits Na^+ reabsorption in renal collecting duct cells (155) and 20-OH-AA inhibits $Na^+/K^+/Cl^-$ function in thick ascending limb of Henle (157, 159, 160, 168). P450 arachidonate metabolites have also been reported to have direct effects on Na^+/K^+ ATPase, with reports of inhibition of Na^+/K^+ ATPase in proximal tubule and collecting duct by 20-OH-AA, 12(R) HETE, and 11,12-DHET (5, 9) and stimulation of Na^+/K^+ ATPase in rat aortic rings by both 19-OH-AA and 20-OH-AA (169, 170). Of note, this latter stimulation was blocked by cyclooxygenase inhibitors, suggesting a prostanoid intermediate.

3. Role in mitogenesis. In rat messangial cells, endogenous non-cyclooxygenase metabolites of AA modulate the proliferative responses to phorbol esters, vasopressin, and epidermal growth factor (EGF), and agonist-induced expression of the immediate early response genes c-fos and Egr-1 is inhibited by ketoconazole or nordihydroguaiaretic acid (NDGA), but not by specific lipoxygenase inhibitors (171). The finding that exogenously applied EETs increase rat messangial cell proliferation was the first direct evidence that P450 AA metabolites are cellular mitogens (172). Subsequent studies have also demonstrated that EETs can stimulate cell proliferation in smooth muscle cells (173) and epithelial cells (174). In cultured rabbit proximal tubule cells, two relatively specific inhibitors of P450, ketoconazole and clotrimazole, inhibited EGF-stimulated [^{3}H]thymidine (175). Utilizing a well-characterized proximal tubule cell line, $LLCPKcl_4$, we determined that all four EET regioisomers stimulated [^{3}H]thymidine incorporation, with 14,15-EET being the most potent. In contrast, no mitogenic effects were seen with AA, other P450 arachidonate metabolites (12(R)-HETE, 14,15-DHET, or 20-OH-AA) or lipoxygenase metabolites (5(S)-HETE, LTB_4, or lipoxin A_4); the mitogenic effect of EETs was blocked by inhibiting either MAP kinase or PI-3 kinase activation (174).

EETs have also been implicated in regulation of proliferation of smooth muscle cells. Depletion of Ca^{2+} pools using the irreversible Ca^{2+} pump inhibitor, thapsigargin, induces smooth muscle cells to enter a stable non-proliferative state (176). 8,9- and 11,12-EETs reverse this state, by inducing new Ca^{2+} pump protein, return of Ca^{2+} pools, and reentry of cells into the cell cycle (176). In addition to the EETs, 20-OH-AA has been shown to increase thymidine incorporation in primary cultures of rat proximal tubule and LLC-PK_1 cells (177) and vascular smooth muscle cells (178), an effect not reproduced by 19(S)- or 19(R)-OH-AA. The mechanisms by which 20-OH-AA induced mitogenesis were not explored in these studies.

4. Gene regulation. P450 AA metabolites have been reported to increase gene transcription, including immediate early genes such as fos, jun, egr-1, and cyclooxygenase-2 (COX-2)(171, 174, 179, 180). For COX-2 activation, 14,15-EET activation of protein kinase C has been implicated (181). One possible mechanism by which P450 arachidonate metabolites may affect gene transcription is via interaction with members of the family of peroxisome proliferator-activated receptors (PPARs)(72). The PPAR alpha subtype (PPARα) stimulates hepatic fatty acid degradation through the activation of peroxisomal fatty acid β-oxidation. In rodents, a PPARα-mediated change in the expression of genes involved in fatty acid metabolism, including the P450 4A gene subfamily of fatty acid ω/ω-2 hydroxylases (72), mediates peroxisome proliferation, a pleiotropic cellular response, mainly limited to liver and kidney. Recent studies using a mouse strain carrying disrupted copies of the genes coding for the $PPAR_\alpha$ and the peroxisomal fatty acyl-CoA oxidase indicate a role for dicarboxylic fatty acids and P450 4a isoforms in the regulation of PPARα- dependent gene transcription and peroxisomal fatty acid β-oxidation (182). Of interest, the PPARα nuclear receptor may interact with other cellular factors to regulate P450 4A gene transcription (72, 182–184).

5. Signaling pathways. The P450-eicosanoids have been reported to activate a variety of intracellular signaling pathways. Given the heterogeneity of the observed signaling responses, it is likely that these are cell-specific responses, although in some cases, the effects attributed to these compounds are in question because of the use of P450 inhibitors of limited selectivity. Older studies suggested that EETs inhibited cAMP production in toad bladder and some, but not all, studies have suggested activation of protein kinase C (185). The involvement of P450 in regulation of cellular Ca^{2+} responses has been suggested by several studies (5–12). Agonist-induced Ca^{2+} and Mn^{2+} entry in human neutrophils (186) and platelets (187) was blocked by P450 inhibitors, possibly via inhibition of the plasma membrane Ca^{2+} permeability regulated by cellular Ca^{2+} stores (188). Voltage-gated Ca^{2+} entry into GH3 pituitary cells and bovine chromaffin cells was inhibited by imidazole antimycotics (189). It has also been suggested that a hemoprotein closely related to cytochrome P-450 might be involved in regulation of voltage-gated Ca^{2+} channels in these cells. In vascular endothelial cells (190) and chromaffin cells (191), EETs increase calcium influx. Similar effects have been noted in cultured rabbit proximal tubule cells in response to EETs, specifically 5,6-EET (175, 192). Although the channel(s) activated are still incompletely described in most of these cells types, Graier, Simecek, and Sturek (190) did describe that in vascular endothelial cells, 5,6- EET-mediated Ca^{2+} entry was sensitive to Ni^{2+}, La^{2+}, and membrane depolarization and was insensitive to removal of extracellular Na^+ or to ni-

trendipine (190). Studies performed with astrocytes have indicated that 5,6-EET may be a component of the calcium-influx factor that links release of calcium from intracellular stores with capacitive calcium influx (193).

More recently EETs have been shown to activate tyrosine kinase cascades in epithelial and smooth muscle cells and activate MAP kinase and PI-3 kinase signaling pathways (174). Immunoprecipitation and immunoblotting revealed that EET administration to $LLCPKcl_4$ cells increased tyrosine phosphorylation of PI-3 kinase and the EGF receptor (EGFR) within 1 min of EET administration. EETs also stimulated association of PI-3 kinase with EGFR. PI-3 kinase inhibitors, wortmannin and LY 294002, markedly inhibited 14,15-EET-stimulated [^{3}H]thymidine incorporation. In addition, 14,15-EET administration stimulated tyrosine phosphorylation of SHC and association of SHC with both GRB2 and EGFR. MAP kinase was also activated within 5 min. Pretreatment of the cells with the MEK inhibitor, PD98059, inhibited the 14,15-EET-stimulated [^{3}H]thymidine incorporation. Moreover, immunobloting indicated that 14,15-EET stimulated tyrosine phosphorylation of the specific $pp60^{c\text{-}src}$ substrate p120 and c-Src association with EGFR. 14,15-EET increased Src kinase activity within 1 min (174).

The mechanism(s) by which P450 AA metabolites activate intracellular second messenger systems is still poorly understood. It has been established that prostaglandins, leukotrienes, and other polar eicosanoids mediate their actions through specific cell surface G-protein-coupled receptors. The less polar EETs and HETEs may exert their biologic effects by incorporation/esterification into cellular phospholipids. However, there have been reports of a specific binding site for 14,15-EET in monocytes (194) and for 12(R)-HETE in microvessel endothelial cells (195). There is also evidence to suggest that the signaling of EETs may be mediated by G protein activation, with the 14,15-EET reported to stimulate ADP-ribosylation of a 52 kDa protein in rat liver cytosol (196). Although this apparent molecular mass suggests the alpha subunit of a heterotrimeric G protein, the identity has not yet been determined, but it was apparently neither Gsa nor Gia (196). Recent patch clamp studies indicated that the EETs activate calcium-activated K^+ channels in vascular smooth muscle cells but have no effects when added directly to cytoplasmic surface of excised inside-out patches (162). These studies have suggested that channel activation requires intermediate signaling steps involving G-proteins (162). Addition of GTP to excised patches restored 11,12-EET activation, which was blocked by GDP-β-S, suggesting a role for G_{sa} in the response (162). Similarly, 14,15-EET binding in U937 cells has been reported to be coupled to Gs and to increase cAMP and activate protein kinase A (197). Finally, recent studies have suggested that NO production by the vasculature may inhibit P450 activity and thereby inhibit production of the vasoconstrictive 20-OH-AA (198).

6. *Second messenger roles.* Given that cyclooxygenase and lipoxygenase metabolites are known to be produced and to act as mediators or modulators of hormonal and growth factor function, it is not surprising that the P450 eicosanoids have also been suggested to be second messengers of hormones and growth factors. We have shown that epidermal growth factor (EGF) stimulates EET production in rat proximal tubule suspensions and primary cultured rabbit proximal tubule cells (175). Omata, Abraham, and Schwartzman (199) have also demonstrated EET production upon stimulation with angiotensin II and 20-OH-AA production with parathyroid hormone or EGF in rat proximal tubules. In addition, Carroll et al. (131) found that intrarenal injection of angiotensin II increases renal production of regioisomeric 16-, 17-, 18-, 19-, and 20-OH-AA)(131).

A common feature of most isolated cell systems is low and/or undetectable levels of bioactive P450. To overcome this limitation to the analysis of cellular mechanisms of action, we developed stable transfectants of the renal epithelial cell line, LLCPKcl4, that expressed the regio- and enantioselective AA 14(S),15(R)-epoxygenase P450 F87V BM3 (103, 200). In these F87V BM3 transfected cells, EGF increased EET production could be abolished by pretreatment with either P450 (17-ODYA) or phospholipase A_2 (PLA_2) inhibitors (quinacrine). Compared to cells transfected with the empty vector, the F87V BM3-transfected cells demonstrated marked increases in both the extent and sensitivity of DNA synthesis in response to EGF. These changes occurred in the absence of significant differences in EGF receptor expression. EGF increased ERK tyrosine phosphorylation to a significantly greater extent in F87V BM3-transfected cells than in the control, vector-transfected, cells. Furthermore, in these control cells, neither 17-ODYA nor quinacrine inhibited EGF-induced ERK tyrosine phosphorylation. On the other hand, in cells expressing the F87V BM3 protein, both inhibitors reduced ERK tyrosine phosphorylation to levels indistinguishable from that seen in cells transfected with vector alone. These studies provide the first unequivocal evidence for a role for the epoxygenase pathway and endogenous EET synthesis in EGF-mediated signaling and mitogenesis and provide compelling evidence for the PLA_2–AA–EET pathway as an important intracellular signaling pathway in cells expressing high levels of P450 epoxygenase (200).

EETs, especially 5,6-EET, may mediate the effect of high concentrations (>10–7 M) angiotensin II in increasing cytosolic calcium concentrations and decreasing the activity of Na^+/H^+ exchange activity in cultured rabbit proximal tubule cells and isolated rat proximal tubules (154, 192, 201). 5,6-EET may also mediate the calcium influx in proximal tubules in response to EGF (175). Inhibition of proximal tubule Na-K-ATPase activity by dopamine and parathyroid hormone has also been suggested to be mediated by P450 AA metabolites, specifically 20-OH-AA (170, 202). In the kidney, 20-OH-AA has been suggested as a second messenger for the effects of endothelin in renal blood flow, glomerular filtration rates, and sodium excretion (203). In the vasculature, EETs may be second messengers for acetylcholine and for endothelin, while 20-OH-AA has been shown to be a second messenger mediating norepinephrine-induced activation of MAP kinase

and increase in thymidine incorporation in smooth muscle cells (204). EETs have been reported to increase in response to glutamate receptor activation in astrocytes (205). In the renal thick ascending limb, inhibition of apical K^+ channel activity occurs after activation of a basolateral Ca^{2+}-sensing receptor, and the 20-OH-AA has been implicated in this K^+ channel modulation (206).

IV. CYTOCHROME P450 AND HYPERTENSION

A. The SHR/WKY rat model

A significant contribution to our current understanding of the functional significance of the P450 AA monooxygenase was the early proposal by J. C. McGiff and collaborators of a role for this pathway in the pathophysiology of genetically controlled experimental hypertension (5, 7–12). This proposal served to attract early interest in these reactions and, by providing unique opportunities for the study of genetic, biochemical, and functional correlations indicative of physiological and/or pathophysiological significance, has served as the focal point for many of the biochemical molecular and functional studies of the last 10 years (5, 7–12). Most of the earlier work in this area was done utilizing the SHR/WKY rat model of spontaneous hypertension (5, 9). In SHR animals, systemic blood pressure increases constantly between 6 to 9 weeks of age until it reaches mean arterial blood pressures of 175 to 190 mm of Hg, while under similar conditions, comparable (but not isogenic) WKY rats remain normotensive. Based on: *a*) biochemical and temporal correlates of renal AA monooxygenase gene expression and enzymatic activity with the development of high blood pressure in spontaneously hypertensive (SHR) rats (5, 9), *b*) the prevention of hypertension in SHR rats by $SnCl_2$-mediated depletion of renal AA P450s (5), *c*) the normotensive effects of $SnCl_2$ -mediated renal P450 depletion in hypertensive SHR animals (5), and *d*) the functional effects of 20-OH-AA and its oxygenated metabolites (5, 7–12), kidney P450s were implicated in the development of hypertension in the rat SHR/WKY model of spontaneous hypertension and a pro-hypertensive role was identified for products of the renal AA ω/ω-1 hydroxylase (5, 9). A molecular basis for these observations was provided by the demonstration that P450 4A2 was one of three genes upregulated in hypertensive SHR rats (207). Furthermore, the analysis of AA metabolism by micro-dissected segments of the SHR rat nephron showed the biosynthesis of 20-OH-AA by the S1, S2, and S3 segments of the proximal tubule, and by medullary (mTAL) and cortical thick ascending limb of Henle's loop (199). Transport studies in isolated TAL cells indicated AA-mediated inhibition of ^{86}Rb uptake could be relieved by P450 inhibitors, and that 20-OH-AA was an inhibitor of cellular Rb uptake (157). These furosemide-like effects of 20-OH-AA appear to be related to the inhibition of apical Ca^{2+}-activated in K^+ channels in mTAL segments (5, 7–12, 160, 206). Finally, the normotensive effects of P450 inhibition in the SHR model were more recently confirmed by: *a*) the use of a more selective, mechanism-based, inhibitor of P450 (208), and *b*) antisense nucleotide inhibition of P450 4A isoform biosynthesis (209).

B. The Dahl rat model

Breeding methods and normal Sprague-Dawley stocks were utilized for the development of the Dahl rat model of salt sensitive hypertension (210). After 10–18 days on a high salt diet, Dahl salt-sensitive animals develop hypertension (180–200 mm Hg, mean arterial blood pressure), while comparable Dahl salt-resistant rats remain normotensive (210). Using this rat model of genetic hypertension, a role for 20-OH-AA and P450 4A2 in salt-sensitive hypertension was proposed based on: *a*) inhibitor studies (211), *b*) differences between Dahl salt-sensitive (DS) and salt-resistant rats (DR) in the activity and expression levels of their AA ω/ω-1 hydroxylases (212), and *c*) normalization of Cl^- transport in the TALH segment of DS rats by 20-OH-AA (213). Importantly, alleles at the P450 4A2 locus were found to co-segregate with hypertension in DS rats (214), however the Dahl salt-sensitive phenotype has yet to be associated with either structural or regulatory alterations in P450 4A genes. An opposite, anti-hypertensive, role for the products of the AA epoxygenase was indicated by biochemical and functional correlates of epoxygenase activity, dietary salt intake, and blood pressure (8, 215). In Sprague-Dawley rats, excess dietary salt induces the kidney epoxygenase activity and markedly increases the urinary levels of its metabolites (89). Clotrimazole inhibition of the salt responsive epoxygenase leads to the development of a clotrimazole-dependent, salt-sensitive hypertension (215). Furthermore, high salt diets induce the renal AA epoxygenase in normotensive Dahl salt-resistant (DR) rats (8, 215). In contrast, under similar conditions, hypertensive Dahl salt-sensitive (DS) animals fail to induce their salt-responsive kidney AA epoxygenase (8, 215). Finally, a polymorphism in the gene coding for a P450 2C epoxygenase has been documented in DS rats (8). However, co-segregation studies failed to demonstrate an association between this polymorphism and salt sensitivity in these animals. The differential regulation of renal P450 ω/ω-1 hydroxylases and epoxygenases by NO and NaCl has been documented (216).

CONCLUSION, UNRESOLVED ISSUES, FUTURE PERSPECTIVES

The recognized pharmacological and toxicological importance of microsomal P450 provided the needed rationale for past extensive studies of the biochemistry, chemistry, and molecular biology of this important and ubiquitous hemoprotein catalyst. However, the more recent demonstration of a role for P450 in the endogenous metabolism of AA has opened new opportunities for the understanding of the biological roles of these proteins, as well as their participation in the oxidative metabolism of endogenous molecules. As it has been emphasized through this manuscript,

it is the potential for physiological and/or pathophysiological importance that provides the needed justification for the extensive studies of the enzymes of the arachidonic acid cascade and of their metabolites.

During the past 10–15 years, the contributions of many laboratories have provided an excellent description of many of the biological activities associated with the P450-derived eicosanoids. These studies have contributed to demonstrate the importance and potential functional significance of this metabolic pathway. The next step, i.e., the delineation of the physiological significance and of the mechanisms and site of action of the P450-eicosanoids, is already benefiting from the utilization of molecular approaches for the experimental manipulation of enzyme expression and biosynthetic capabilities at the cell, organ, or whole body levels. Indeed, the last few years have been characterized by advances in the application of bio-molecular approaches to the study of these reactions and their enzymes. As summarized here, several P450 isoforms have been cloned, their cDNAs were expressed and characterized enzymatically, and their tissue specific expression and regulation were studied. Additionally, signal transduction mechanisms for the P450-eicosanoids are beginning to be dissected, and electrophysiology is providing important insights into their ion transport effects and mode of action. In fact, the progress achieved within the last few years places us now at the gateway of an era in which, by the use of molecular genetic techniques such as gene transfection and/or disruption, we will be able to probe P450 isoform-specific, gene-dependent phenotypes and thus, molecular mechanisms of action and physiological roles.

Among the issues that we feel hold the best promises for the future, we include: *a*) the definition of the molecular mechanisms by which P450 eicosanoids control membrane ion permeability and ion channel activity, *b*) the definition of the roles that the fatty acid ω/ω-1 hydroxylases play in body lipid homeostasis, vis à vis their roles in AA bioactivation, *c*) the study of the significance of enzymatic oxidative metabolism in controlling P450 eicosanoid organ and tissue levels and/or biological function, *d*) the biochemical, molecular, and functional characterization of additional organ/tissue/cell specific epoxygenases and/or ω/ω-1 hydroxylases, and of their human homologues, *e*) the study of the role that endogenous EET and/or 20-OH-AA biosynthesis and its hormonal regulation plays in, for example, gene regulation, mitogenesis, and cation and/or anion fluxes, *f*) the characterization of cause–effect relationships between defined physiological stimuli and their functional responses with EET and/or 20-OH-AA organ biosynthesis, levels and biological activities, *g*) the molecular definition of genetically controlled alterations in P450 protein structure, enzymatic activity or regulation, with phenotypic alterations in organ/or body physiology or pathophysiology, and *h*) the analysis of correlations between human pathophysiological conditions and polymorphisms in the genes coding for P450 AA epoxygenases and/or ω/ω-1 hydroxylases.

The authors are grateful to the USPHSNIH NIDDK and NIGMS Institutes for their support of research carried out in their laboratories and cited here. The editorial assistance of Ms. Mary Couey is greatly appreciated.

Manuscript received 1 July 1999 and in revised form 16 November 1999.

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