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Microsomal cytochrome P450 and eicosanoid metabolism

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Abstract. The demonstration of a role for microsomal P450 in the metabolism of endogenous pools of arachidonic acid established this enzyme system as a member of the arachidonic acid cascade and characterized a new an important metabolic function for this enzyme system. Studies from several laboratories documenting the powerful biological activities of the P450-derived eicosanoids have suggested important roles for the P450 arachidonic acid monooxygenase in renal and vascular physiology, and in the pathophysiology of experimental hypertension. These studies provide significant evidence to indicate that in addition to its recognized traditional toxicological and pharmacological roles, microsomal P450s also play important physiological roles in the control of tissue and body homeostasis.

Key words. Arachidonic acid monooxygenase; fatty acid hydroxylases; P450 epoxygenases; P450 ω-hydroxylases, EETs; ω-alcohols; P450 eicosanoids.

Introduction

Our understanding of the roles that lipids play in cell and organ biology has changed dramatically since, in addition to their accepted structural importance as the building blocks of cellular membranes, an extensive body of evidence has demonstrated that fatty acids, glycerolipids, glycerophospholipids, ceramides and so on participate as mediators in a variety of transmembrane signaling cascades, as well as in cell differentiation, replication and apoptosis. The functional significance of these mediators, further emphasized by their proposed roles in the pathophysiology of diseases such as inflammation, asthma, cancer, diabetes, and hypertension, has stimulated an intense research of the biochemistry, enzymology and regulation of lipid metabolism. The arachidonic acid (AA) cascade, composed of prostaglandin H2 synthase, lipoxygenases and, more recently, microsomal P450, serves as a premier illustration of the role that lipid-derived mediators play in cell and organ function [1–9].

After phospholipase A_2-catalyzed release, AA is oxidized by the enzymes of the AA cascade. Metabolism by prostaglandin synthase generates an unstable cyclic endoperoxide (prostaglandin H_2) that rearranges enzymatically or chemically to prostaglandins (PGs), prostacyclin (PGI_2) or thromboxane A_2 (TXA_2) [10]. Metabolism by the lipoxidases generates several regioisomeric allylic hydroperoxides containing a cis,trans-conjugated diene functionality. One of these, 5-hydroperoxyeicosatetraenoic acid (5-HPETE), serves as the precursor for the biosynthesis of leukotrienes [11]. The physiological and biomedical significance of prostanoids and leukotrienes has been extensively documented [1–3]. Among these are their critical roles in pulmonary, vascular and renal physiology as well as in the pathophysiology of inflammation, asthma and, more recently, cancer [1–3]. On the other hand, the biological roles of many of the AA metabolites formed by the P450 system, the newest member of the AA metabolic cascade, are currently under extensive investigation [1–9].

A functional implication for P450 in AA metabolism was first suggested in 1976 by the demonstration that the AA-induced aggregation of human platelets could be blocked by known P450 inhibitors [12]. However, the then growing pharmacological and toxicological importance of P450 turned the attention of most investigators towards its roles in drug and xenobiotic metabolism. It was not until 1981 that the role of microsomal P450 in the oxidative metabolism of arachidonic was unequivocally demon-

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strated when microsomal fractions and reconstituted P450 systems were shown to catalyze AA oxidation to products chromatographically distinct from prostanoids [13–17]. Soon after, the structural characterization of most of the products generated from AA by incubates containing liver or kidney microsomal fractions was completed [13–17]. It was evident from those original studies that the physiological importance of the AA substrate made those observations unique and likely to be functionally significant. Two factors contributed to focus attention in the study of chemistry, biochemistry and physiological roles of this branch of the AA cascade: i) the early demonstration of the potent biological activities of its metabolites, the epoxyeicosa trienoic acids (EETs) [18, 19], and ii) the establishment of EETs as endogenous constituents of organs such as liver, kidney, and lung and of human and rat plasma and urine [4]. 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 important, suggested functional roles for this enzyme in the bioactivation of the fatty acid. In recent years, the study of the biochemistry and biological significance of these reactions has developed into an area of intense research. Although the physiological and/or pathophysiological implications of this pathway of AA metabolism remain to be fully understood, work from several laboratories is beginning to establish biochemical and functional correlations that can be interpreted as suggestive of a physiological function [4–9].

As functionally important as the many roles attributed to microsomal P450s in the oxidative bioactivation of AA is their documented participation in the metabolic transformation of several oxygenated metabolites of AA (eicosanoids). The microsomal hemoproteins catalyze eicosanoid metabolism via NADPH-dependent or NADPH-independent pathways. This distinction reflects the marked differences in types of oxygen chemistry involved in these reactions, i.e. the NADPH-dependent activation of atmospheric oxygen or the NADPH-independent isomerization of arachidonic acid hydroperoxides. As with AA, the potential functional significance of these reactions has stimulated considerable interest in their enzymology and regulation. For clarity, we will discuss first those reactions associated with the metabolism of oxygenated eicosanoids by P450 and then concentrate on its role(s) in AA metabolism.

NAPH-independent metabolism of eicosanoids

Cytochrome P450 is an active peroxidase that catalyzes the metabolism of a wide variety of organic hydroperoxides, including fatty acid hydroperoxides [20, 21]. This peroxidase activity, initially described by O'Brien and collaborators [20], is associated with the ferric, Fe^{+3}, state of microsomal P450, does not involve electron transfer from NADPH and exhibits high catalytic rates. The mechanism of peroxide O–O bond cleavage, i.e. homolytic or heterolytic scission [20, 21], determines the catalytic outcome of these reactions and is highly dependent on: i) the nature of the P450 isoform, and ii) the chemical properties of the organic hydroperoxide and the oxygen acceptor [21]. A homolytic pathway was proposed to account for the formation, from 15-HPETE, of 11- and 13-hydroxy-14,15-epoxyeicosatrienoic acids by rat liver microsomes [22].

Sequence homology analysis and spectral studies of human lung and platelet thromboxane synthase indicated the presence of structural features, including a heme-thiolate prosthetic group, typical of P450 type hemoproteins [23, 24]. The heterolytic cleavage of the PGH_2 endoperoxide and an oxygen atom transfer or oxenoid mechanism was proposed to account for the enzymatic formation of PGI_2 and TXA_2 from PGH_2 [25]. The complementary DNAs (cDNAs) coding for human thromboxane and bovine prostacyclin synthases were subsequently cloned, sequenced and shown to code for cysteine-heme coordinated hemoproteins with $\leq 35\%$ overall amino acid identity to members of the P450 3 and 7 gene families, respectively [24–26]. Finally, in plants, a flaxseed peroxidase was purified, characterized and its cDNA cloned and expressed [27, 28]. The enzyme catalyzed the heterolytic cleavage of 13-hydroperoxy linoleic acid to the corresponding allene oxide, a precursor in jasmonic acid biosynthesis [27]. Sequence analysis indicated a $\leq 25\%$ overall identity to other P450s and the presence of a conserved cysteine residue involved in heme coordination [28]. The discovery and association of the well-established peroxidase function of P450 to the biosynthesis of autacoids of animal or vegetal origin has opened novel and exciting research areas. The significance of these P450 supported pathways to cell physiology and/or pathophysiology has only begun to be explored. The identification of these enzymes, many of which are members of the arachidonate cascade with established physiological roles, as homologues of the P450 gene family should facilitate studies of their enzymology, molecular properties, and mechanisms and site of action.

NADPH-dependent metabolism of eicosanoids

The oxidative, NADPH-dependent metabolism of several eicosanoids by P450 was described prior to the demonstration of its role in AA bioactivation. The biological importance of these reactions resides in the fact that they i) increase eicosanoid structural diversity, and hence informational content, ii) may alter the pharmacological profile of the substrate and iii) may participate in the

regulation of steady-state and stimulated levels of physiologically relevant molecules. While the consensus is that these reactions, for the most part, attenuate biological activity and initiate eicosanoid catabolism, recent studies have described the unique and potent biological properties of a few ω-oxidized prostanoids [5]. Nonetheless, in most cases, the sequence of steps leading to ω/ω-1 oxidized prostanoids from endogenous AA pools remains to be clarified.

For the most part, the NADPH-dependent, P450-catalyzed metabolism of prostanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and EETs result in the hydroxylation of these eicosanoids at the ultimate (C_{20} or ω carbon) or penultimate carbon atoms (C_{19} or ω-1 carbon). However, the epoxidation of infused PGI_2 by a perfused kidney preparation [29], and the metabolism of 5,6- and 8,9-EET by prostaglandin H_2 synthase are published [30, 31]. A detailed study of secondary metabolism of 12(*R*)-HETE and 14,15-EET by P450 has been reported [32, 33]. To date, however, none of the transformations described in this section, with the exception of ω/ω-1 hydroxylation, have been shown to occur in vivo and from endogenous precursors.

Since the demonstration of C_{19}-hydroxylated prostanoids in human semen [34], ω/ω-1 hydroxylation has become a recognized route for prostanoid metabolism. Also, the hydroxylation at the C_{18} (ω-3) carbon of PGE_2 has been reported [35]. Early studies demonstrated that prostanoid ω and ω-1 oxidation was NADPH-dependent, localized to the endoplasmic reticulum [36], and catalyzed by microsomal P450 [37]. Reconstitution studies using purified enzymes or recombinant P450s showed that most of these reactions were catalyzed by members of the P450 4A gene subfamily. At present, approximately 12 4A isoforms have been cloned or isolated and purified from rats (4A1, 4A2, 4A3 and 4A8), mice (4a10, 4a12, and 4a14), rabbits (4A4, 4A5, 4A6, 4A7) and humans (4A11) [38]. Many of these enzymes have been characterized enzymatically [4, 5]. Table 1 summarizes the known substrate specificity and regioselectivity of P450 4A isoforms involved in fatty acid and prostanoid ω/ω-1 hydroxylation [4, 5].

The ω-oxidation of leukotriene B_4 (LTB_4) has been documented in whole animals, isolated cells and subcellular fractions [38–42]. As with prostanoids, these reactions appear to serve mostly catabolic roles. Early biochemical studies suggested that the ω-oxidation of LTB_4 in leukocytes was catalyzed by a unique P450 isoform [40, 41]. Subsequently, a cDNA coding for a novel P450 isoform, Cyp P450 4F3, was cloned form human polymorphonuclear leukocytes and expressed [43]. Recombinant P450 4F3 actively catalyzed the ω-oxidation of LTB_4 with a K_m of 0.71 µM [43]. Finally, the ω-oxidation of 12(*S*)-HETE by polymorphonuclear leukocytes was demonstrated in 1984 by Wong et al. [44] and Marcus et al. [30]. Moreover, the last authors also showed that endogenous AA pools could be converted to 12,20-dihydroxyeicosatetraenoic acid by a coincubated mixture of human platelets and polymorphonuclear leukocytes [30]. Both 5- and 15-HETE are known to undergo ω-oxidation by P450 [45, 46]. A P450 similar to that responsible for LTB_4 metabolism has been implicated in the ω-oxidation of lipoxin A_4 and B_4 by human neutrophils and polymorphonuclear leukocytes [47, 48].

Table 1. Metabolism of fatty acids and prostanoids by P450 4A subfamily isoforms.

Isoform	Species	Enzymatic activities
4A1	rat	ω-oxidation; lauric and AA
4A2	rat	ω/ω-1 oxidation of lauric and AA
4A3	rat	ω/ω-1 oxidation of lauric acid and not AA
4A8	rat	ω/ω-1 oxidation of lauric and AA
4A4	rabbit	ω-oxidation of prostaglandin A, E, D and $F_{2\alpha}$ and palmitic and AA
4A5	rabbit	ω-oxidation of PGA_1, ω/ω-1 oxidation of lauric, palmitic and AA
4A6	rabbit	ω-oxidation of lauric, palmitic and AA; low PGA_1 ω-oxidation
4A7	rabbit	ω-oxidation of PGA_1, lauric, palmitic and AA; inactive towards PGE_1
4a10	mouse	ω-oxidation of lauric and AA
4a12	mouse	unknown
4a14	mouse	ω/ω-1 oxidation of lauric acid and not AA
4A11	human	ω-oxidation of lauric acid and AA

Metabolism of AA acid (the P450 AA monooxygenase pathway)

Introduction

It is of interest that, given the by then recognized catalytic versatility of P450 and known roles in saturated fatty acid the ω/ω-1 hydroxylation [37], it was not until 1981 that its participation in AA metabolism was recognized [13–17]. The functional significance of AA stimulated the rapid structural characterization and chemical synthesis of the predominant metabolites isolated from incubates containing liver and kidney microsomal fractions [4, 5]. The access to sufficient quantities of most P450-derived eicosanoids opened then the door to extensive studies of the enzymology and biological significance of these reactions. The early demonstration of the EETs as stimulants for the release of peptide hormones [18] and as inhibitors of Na^+ reabsorption in the distal nephron [19], focused interest on these novel P450 reactions and stimulated their subsequent molecular, biochemical and functional characterization. Soon after, the significance of these observations was reinforced by the gas chromatography – mass spectrometry (GC/MS) documentation of chiral EETs pools in rat, rabbit and human organs, in-

cluding kidney [4], and of EETs, dihydroxyeicosatrienoic acids (DHETs) and 20-hydroxyeicosatetraenoic acid (20-OH-AA) in human and rat urine [4, 5]. These results were important since they demonstrated endogenous biosynthesis of these eicosanoids, established P450 catalyzed AA monooxygenation as a formal metabolic pathway, P450 as a member of the endogenous AA cascade, and suggested functional roles for this enzyme system in cell and organ physiology. The studies of the renal significance of this novel pathway of AA metabolism were initiated soon after its initial biochemical characterization and were stimulated, among other things, by the early demonstration of the potent transport and vasoactive properties of its metabolites [5].

AA is metabolized by liver microsomal P450s at rates that varied from 1 to 6 nmol of product/min/mg of microsomal protein and with apparent K_m values for liver microsomes of 20 to 40 μM [13, 49, 50]. As with most of the enzymes of the arachidonic acid cascade, P450 will not oxidize the fatty acid at significant rates, when esterified to phospholipids [49, 51]. Inasmuch as the cellular concentrations of free, nonesterified AA are usually very low, metabolism and bioactivation by the enzymes of the AA cascade, including P450, is under phospholipase control. It is the presence of AA in selected, hormonally sensitive glycerolipid pools and the existence of AA-specific, hormone-regulated lipases that distinguishes AA from most polyunsaturated fatty acids and endows it with unique functional properties [10, 52]. During AA metabolism, the P450 enzyme system catalyzes the NADPH-dependent, redox-coupled activation of molecular oxygen and its delivery to the substrate ground-state carbon skeleton. This feature, i.e. the NADPH-dependent, redox-coupled activation of molecular oxygen, as opposed to the free radical-mediated activation of carbon atoms, distinguishes the P450 enzyme system from the other enzymes of the arachidonate cascade. Thus, while P450 functions as an active AA monooxygenase, prostaglandin synthase and lipoxygenases are AA dioxygenases.

Reactions catalyzed

The analysis of the reaction products generated by microsomal fractions and purified isoforms [4, 5] demonstrated that the P450 monooxygenase metabolizes AA by one or more of the following reactions: i) allylic and/or bis-allylic oxygenase generating, as final products, six regioisomeric HETEs containing a cis,trans conjugated dienol; ii) ω/ω-1 hydroxylases which functionalyze sp^3 carbons at or near the AA methyl terminus to 16-, 17-, 18-, 19-, or 20-hydroxyeicosatetraenoic acids (16-, 17-, 18-, 19- and 20-OH-AA, respectively) and iii) Epoxygenase or olefin epoxidation to 5, 6–, 8, 9–, 11, 12–, or 14, 15-EET. As with most P450 activities, early studies demonstrated that the type of reaction catalyzed, as well as the profile of metabolites generated, was highly dependent on the animal and tissue source of microsomal enzymes, the nutritional and hormonal state of the animal, its sex, age and prior exposure to drugs, pesticides or to known inducers of microsomal P450 [4, 13, 49, 50, 53]. For example, while rat and rabbit liver microsomal fractions preferentially catalyze AA epoxidation [4, 16, 53, 54], rat and rabbit kidney microsomes metabolize the fatty acid to generate mainly ω and ω-1 alcohols [4, 5, 13, 15, 16, 53, 55, 56]. Based on studies with either microsomal fractions or reconstituted system containing several solubilized and purified P450 isoforms [4, 5], it was concluded that P450 controls, in a protein-specific fashion, the regioselectivity of AA oxidation at two different levels: i) the type of reaction catalyzed, i.e. olefin epoxidation (EETs), allylic oxidation (HETEs) or hydroxylation at the C_{16} C_{20} sp^3 carbons (16-, 17-, 18-, 19-, and 20-OH-AA) and ii) to a lesser extent, in selecting the regioselectivity of oxygen insertion. Inasmuch as the role and relevance of P450 to in vivo HETE formation remains unsubstantiated, most research efforts have concentrated on the epoxygenase and ω/ω-1 hydroxylase branches of the P450 AA monooxygenase pathway, the predominant P450-catalyzed reactions in most organ tissues.

AA ω/ω-hydroxylase reaction

The catalysis of fatty acid ω/ω-1 oxidation is one of the best-established P450 reactions [37]. The regulation of these reactions by the α subtype of ligand-activated peroxisomal proliferator-activated receptors ($PPAR_\alpha$) [57] suggest that in conjunction with peroxisomal β-oxidation reactions, fatty acid ω/ω-1 oxidation contributes to lipolytic pathways and participates in the control of body fatty acid homeostasis [57]. Fatty acid ω/ω-1 oxidation is regulated in vivo by a variety of factors, including, animal age, diet, starvation, administration of fatty acids, hypolipidemic drugs, aspirin and steroids [4, 5, 57, 58].

The studies of the P450-catalyzed ω/ω-1 hydroxylation of AA were initiated in 1981 when rabbit kidney cortex microsomes were shown to catalyze the NADPH-dependent formation of 19- and 20-hydroxyeicosatetraenoic acids (19-, and 20-OH-AA, respectively) [15, 16]. Since then, these hydroxylation reactions has been demonstrated in several tissues, including human liver and kidney, as well as rat and rabbit liver and kidney [4–7, 9]. More recently, the 16-, 17-, and 18-hydroxyeicosatetraenoic acids have been added to the list of products generated by the P450 arachidonic acid ω/ω-1 hydroxylase activity [4, 5, 50]. While the oxygen chemistry and reaction mechanisms responsible for the ω/ω-1 hydroxylation of AA and of saturated fatty acids are probably similar, for AA these reactions impose 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 highly rigid and structured binding site for the AA molecular template. This 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 5, 6-, 8, 9-, 11, 12-, and 14, 15-olefins and of the bis-allylic C_7, C_{10} and C_{13} methylene carbons. Studies with microsomal fractions, purified and recombinant P450 isoforms demonstrated that i) in most organ tissues fatty acid ω/ω-1 hydroxylations are catalyzed predominantly by members of the P450 4A gene subfamily [4, 5], and ii) with most P450 4A isoforms, the ω/ω-1 hydroxylation of saturated fatty acids proceeds at rates that are substantially faster than those observed with AA [50, 59, 60].

Several rat, rabbit and human members of the P450 4A gene family have been purified or cloned and expressed [4–9, 38]. While individual P450 4A isoforms show regioselectivity for either the ω or the ω-1 hydroxylation of lauric acid or prostanoids [37, 60–70], to date all those P450 4A isoforms characterized that are active towards AA metabolize the fatty acid to either 20-OH-AA or to mixtures of 20-OH-AA and 19-OH-AA; i.e. none of these P450 4A isoforms show exclusive regioselectivity for the fatty acid C_{19} position [4]. Reconstitution of the hydroxylase activity of purified recombinant rat P450 4A1 showed that this enzyme is a highly regioselective laurate and arachidonate ω-hydroxylase [4, 5, 64]. On the other hand, rat P450s 4A2 and 4A3 share extensive sequence homology (≥ 90 amino acid identity) and both catalyze lauric acid ω/ω-1 hydroxylation. However, P450 4A3 is nearly inactive towards AA, while P450 4A2 metabolizes AA to mixtures of 19- and 20-OH-AA [60].

Studies with inducers of microsomal P450, and the reconstitution of the AA monooxygenase using purified isoforms showed that P450s 1A1, 1A2 and 2E1 may be responsible for the hydroxylations occurring at the C_{16} through C_{19} carbon atoms of AA [50, 71]. Thus, while P450 1A1 and 1A2 are more or less selective for hydroxylations at C_{19} and C_{16}, respectively [50], purified P450 2E1 metabolizes AA to 18(*R*)- and 19(*S*)-OH-AA along with other products [71]. Finally, the demonstration of 20-OH-AA excretion in human urine confirmed the participation of the P450 in the ω-hydroxylation of endogenous AA pools [5].

The AA epoxygenase reaction

The catalysis of AA epoxidation by P450 was inferred by the isolation of 11, 12- and 14, 15-DHETs from incubates containing kidney cortex microsomes, AA and NADPH [55]. Soon after, rat liver microsomes were shown to catalyze the NADPH-dependent epoxidation of AA to 5, 6-, 8, 9-, 11, 12- and 14, 15-EET [54]. To date, the catalysis of EET formation by purified P450s, microsomal fractions and isolated cell preparations has been demonstrated in numerous tissues, including liver, kidney, pituitary, brain, adrenal, endothelium, pancreas and ovaries [4–9]. 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 hydroxylase, more or less selective for AA. Finally, the fact that the P450-dependent epoxidation of AA generates only *cis*-epoxides suggest that epoxidation proceeds by a concerted pathway or that, alternatively, a rigid active-site binding geometry restricts the freedom of C–C rotation for the transition state.

The P450 isoform heterogeneity of the microsomal epoxygenases was first indicated by studies showing that the regioselectivity of AA epoxidation was more or less organ specific. Thus, while brain microsomes form 5, 6-EET as their major epoxygenase product, the 11, 12-EET is the predominant regioisomer formed by rat liver and kidney microsomal fractions [4, 5]. The changes in EET chirality that resulted after animal treatment with selected P450 inducers further demonstrated the molecular heterogeneity of the AA epoxygenase (table 2) [49]. For example, phenobarbital treatment increased the regio- and enantiofacial selectivity of the rat liver microsomal epoxygenase(s), in a time-dependent fashion [49]. The phenobarbital-induced increases in stereoselectivity resulted in a remarkable inversion in absolute configuration of the EETs produced by the microsomal enzymes (table 2) [49]. It was subsequently shown that AA epoxidation is highly enantioselective and that P450 controls the regio- and enantioselectivity of the epoxygenase reaction in an isoform-specific fashion [4]. These results established that in contrast to the cyclooxygenase and lipoxygenases enzymes, the regio- and enantioselectivity of the AA epoxygenase was variable and P450 isoform specific [49]. Furthermore, amongst the enzymes of the arachidonate cascade, the P450 epoxygenase is unique in that its regio- and stereochemical selectivity is under regulatory control and can be experimentally altered, in vivo, by animal manipulation [4, 49]. Reconstitution of the P450 AA monooxygenase activity using purified P450 isoforms and recombinant proteins demonstrated that members of the P450 2 gene family were the only iso-

Table 2. Effects of animal treatment on the enantiofacial selectivity of the rat liver microsomal arachidonic acid epoxygenase.

Enantiomeric composition (%) EET regioisomer	Animal treatment	
	None	Phenobarbital
8(*R*), 9(*S*)-EET	68	22
8(*S*), 9(*R*)-EET	32	78
11(*R*), 12(S)-EET	81	17
11(*S*), 12(*R*)-EET	19	83
14(*R*), 15(*S*)-EET	33	76
14(*S*), 15(*R*)-EET	67	24

forms that metabolized the fatty acid predominantly by enantioselective epoxidation [4]. Isoforms of the 2B and 2C subfamily so far identified as epoxygenases include rat 2B1, 2B2, 2B4, 2B12, 2C11, 2C23, and 2C24 [4, 49, 72]; rabbit 2B4, 2C1 and 2C2 [72]; mouse 2c37, 2c38, 2c39, and 2c40 [73]; and human 2C8 and 2C9/2C10 [74]. More recently, P450s 2J2 and 2J4 have been identified as organ-specific epoxygenases [75, 76]. Importantly, P450s 2C23, 2C24 and 2C11, the three major 2C subfamily isoforms expressed in the rat kidney, were cloned, expressed, identified as active epoxygenases and shown to account for most of the rat kidney epoxygenase activity [4, 56, 77]. Furthermore, P450 2C8 is the predominant epoxygenase expressed in the human kidney [74]. Finally, while P450s 1A1, 1A2 and 2E1 are active AA ω/ω-1 hydroxylases, they also produce low and variable amounts of EETs ($\leq$20 of total products) [4]. A unique case is that of a P450 purified from the livers of dioxin-treated chick embryos [78]. 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) [78].

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 [4]. For example, the first recombinant epoxygenase characterized, P450 2C23, catalyzed the enantioselective epoxidation of AA to 8, 9-, 11, 12- and 14, 15-EET 27, 54 and 19% of total products, respectively) [79]. The recombinant protein generated 8(*R*), 9(*S*)-, 11(*R*), 12(*S*), and 14(*S*), 15(*R*)-EETs with optical purities of 95, 85 and 75%, respectively [79]. The structural analysis of the EETs generated by purified 2B and 2C P450 epoxygenases showed that among these proteins, none was selective for the epoxidation of a single fatty acid olefin. Thus, although highly enantioselective, AA epoxidation shows a more limited regioselectivity [49, 79]. It is of significance that the degrees of stereochemical selectivity displayed by AA epoxygenase isoforms are unusually high for P450-catalyzed oxidations of unbiased, noncyclic molecules such as AA.

In view of its well-known catalytic versatility, the in vitro catalysis of AA epoxidation by microsomal P450s was not completely unexpected. It was apparent then that the uniqueness and significance of these reactions was going to be defined by whether or not the enzyme system participated in the in vivo metabolism of the fatty acid. Since asymmetric synthesis is an accepted requirement for the biosynthetic origin of most eicosanoids, this issue was resolved by the demonstration of the presence of chiral EETs in samples extracted from rat and human organs [4]. 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 ($\geq$ 92% of the total liver EETs) [51]. EET-glycerophospholipid formation required a multistep process, initiated by the P450 enantioselective epoxidation of AA, ATP-dependent activation to the corresponding EET-coenzyme A (CoA) derivatives, and EET enantiomer-selective lysolipid acylation [51]. The observed in vivo EET esterification process appears to be unique since most endogenously formed eicosanoids are either secreted, excreted or undergo oxidative metabolism and excretion.

The biosynthesis of endogenous pools of phospholipids containing esterified EET moieties in rat liver, kidney, brain and plasma, and in human kidney and plasma [4], indicate new and potentially important functional roles for P450 in the generation of cellular glycerophospholipid pools containing oxidized fatty acid moieties. Furthermore, these studies also show, in contrast to most eicosanoids, the potential for the cellular generation of performed bioactive EETs via hydrolytic reactions, thus obviating the need for AA oxidative metabolism.

Functional significance of the AA monooxygenase metabolites

The analysis of functional roles for the metabolites of the P450 AA monooxygenase has developed into an area of intense research, and the list of biological activities attributed to these metabolites has grown considerably during the last few years. Among these, we emphasize: 1) vasoactive properties: the EETs are in vitro systemic vasodilators with 5,6-EET as the most potent regioisomer [4–9]. However, 8(*S*), 9(*R*)-EET, the endogenous enantiomer in rat kidney, is a stereoselective renal vasoconstrictor [4–9]. On the other hand, 20-OH-AA, or products of its oxidative metabolism, are powerful vasoconstrictors [5–7], while 19-OH-AA is a stereospecific renal vasodilator [5], 2) Ion transport: the EETs, and 5,6-EET in particular, increase cytosolic Ca^{++} concentrations in several cell systems [4–9], as well as the single channel open probability of Ca^{++}-activated K^+ channels [4–9]. A role for the EETs in mediating the natriuretic and mitogenic responses to angiotensin II and epidermal growth factor (EGF) has been proposed [4, 5]. Both natriuretic and diuretic effects have been described for 20-OH-AA [5–7, 9]. The stimulation and inhibition of renal Na^+, K^+/ATPase by 19- and 20-OH-AA, respectively, has been reported [5]. In rabbit mTALH cells, 20-OH-AA and the corresponding 1,20-dicarboxylic acid blocked the Na^+, K^+, 2Cl-cotransporter and a Na^+/K^+ATPase [5]. Based on endothelial cell synthesis and release [80], EET-mediated relaxation of preconstricted arteries [80], and G-protein-mediated EET opening of vascular smooth cell Ca^{++}-activated K^+ channels [81, 82], the EETs were identified as endothelium-derived hyperpolarizing factors (EDHFs) [81, 82]. These results, with their potential to provide a molecular understanding of EET vasoactivity, are con-

tributing to integrate EET transport and vasoactive properties into a coherent mechanistic description, amenable to experimental analysis. Importantly, 20-OH-AA, recently identified as an endogenous inhibitor of this type of Ca^{++}-activated K^+ channels [83] is also a powerful vasoconstrictor [5] and behaves, therefore, as an EET functional antagonist, 3) Gene regulation: the potential for eicosanoid participation as ligands for orphan receptors is an area of intense current interest [113]. The mitogenic activities of 14,15-EET and 20-OH-AA have been reported [4, 5]. Finally, other physiological functions attributed to the EETs include a role as secretagogues for peptide hormone release, including hypothalamic release of somatostatin, anterior pituitary release of adrenocorticotropin, prolactin, luteinizing hormone and growth hormone, posterior pituitary release of vasopressin and oxytocin, pancreatic release of insulin and glucagon, and renal release of renin [4].

The early proposal by J. C. McGiff and collaborators of a role for the kidney P450 AA monooxygenase in the pathophysiology of genetically controlled experimental hypertension [5] provided new and unique opportunities for the study of genetic, biochemical and functional correlations indicative of physiological and pathophysiological significance. Thus, based on i) 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], ii) the prevention of hypertension in SHR rats by SnCl2-mediated depletion of renal AA P450s [5], iii) the normotensive effects of SnCl2-mediated renal P450 depletion in hypertensive SHR animals [5] and iv) the functional effects of 20-OH-AA and its oxygenated metabolites [5–7, 9], kidney P450s were implicated in the development of hypertension in the rat SHR/WKY model of spontaneous hypertension, and a prohypertensive role was identified for products of the renal AA ω/ω-1 hydroxylase [5–7, 9]. Similarly, a role for 20-OH-AA and P450 4A2 in salt-sensitive hypertension was subsequently proposed based on i) inhibitor studies [84], ii) differences between Dahl salt-sensitive (DS) and salt-resistant rats (DR) in the activity and expression levels of their AA ω/ω-1 hydroxylases [85], and iii) normalization of Cl^- transport in the TALH segment of DS rats by 20-OH-AA [86]. On the other hand, an opposite, antihypertensive, role for the products of the AA epoxygenase was indicated by biochemical and functional correlates of epoxygenase activity, dietary salt intake and blood pressure [87, 88]. In Sprague-Dawley rats, excess dietary salt induces kidney epoxygenase activity and markedly increases the urinary levels of its metabolites [56]. Clotrimazole inhibition of the salt-responsive epoxygenase leads to the development of a clotrimazole-dependent, salt-sensitive hypertension [87]. Furthermore, high-salt diets induce the renal AA epoxygenase in normotensive DR rats [87]. In contrast, under similar conditions, hypertensive DS animals fail to induced their salt-responsive kidney AA epoxygenase [87].

Conclusion, unresolved issues, future perspectives

As has been emphasized, the potential for physiological and pathophysiological function provides the needed justification for intensive studies of the role of P450 in AA metabolism and bioactivation. The contributions of many laboratories have provided extensive enumeration of the biological activities associated with P450-derived eicosanoids [4–9]. While exogenously added eicosanoids are useful tools for the analysis of biological function, this type of approach may result in limited and artificial descriptions of eicosanoid roles and biological significance. Often, bioactive eicosanoids are but one component of what are usually complex, multistep signaling cascades that may include, among other things, coordinated, time-dependent changes in cytosolic ion, cyclic nucleotide and phosphoinositide concentrations, changes in the regulation of phosphorylation and dephosphorylation cascades, phospholipase(s) activation and changes in the turnover rate of selected glycerophospholipid pools. On the other hand, the last 5 years have been characterized by the application of molecular approaches to the studies of the biochemical and functional significance of the P450-dependent AA epoxygenase(s) and ω/ω-1 oxygenase(s). As summarized, several P450 isoforms have been either cloned and/or their cDNAs expressed, and their enzymatic activities, tissue and/or organ-specific expression, regulation in salt-sensitive and spontaneous hypertension characterized. Additionally, signal transduction pathways for several P450-derived eicosanoids are beginning to be dissected, and electrophysiology is providing important molecular insights into their ion-transport effects and mode of action. Indeed, the progress achieved during the last few years situates us now on the threshold of an era in which, by the use of molecular genetic techniques, including gene disruption and transfection, we will be able to probe P450-isoform-specific, gene-dependent phenotypes and thus their functional roles.

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