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Liver microsomal cytochrome *P*-450 and the oxidative metabolism of arachidonic acid

(microsomal electron transport/oxygenase/fatty acid metabolism)

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ABSTRACT **Arachidonic acid is oxidatively metabolized by rat liver microsomes at a rate of approximately 5 nmol per min per mg of protein at 25°C. This reaction is dependent on the presence of NADPH and oxygen. Studies with various inhibitors indicate a role for membrane-bound cytochrome *P*-450 in the transformation of arachidonic acid to a mixture of hydroxy acid derivatives. The stoichiometry of the reaction conforms to that of a monooxygenase reaction—i.e., one mole of NADPH is oxidized per mole of oxygen utilized—suggesting a reaction mechanism different from that proposed for lipid peroxidation reactions. No evidence for the formation of prostaglandin-like metabolites was obtained. The diene character of some of the metabolites formed suggests another role for cytochrome *P*-450—i.e., participation in hydrogen abstraction reactions for the activation of various substrates.**

The oxidative metabolism of arachidonic acid can lead to a wide variety of metabolites. Interest in the physiological function of prostaglandins, thromboxanes, leukotrienes, and other oxidative metabolites of arachidonic acid, as well as the reactions of lipid peroxidation, have established the need to better understand the role of the various enzymes responsible for oxygen incorporation during the process of arachidonic acid metabolism—e.g., cyclooxygenase, lipoxygenase, etc. (1, 2).

The microsomal fraction from rat liver, like that from many other tissues, is rich in phospholipids containing arachidonic acid (3). During the NADPH- and oxygen-dependent function of the liver microsomal cytochrome *P*-450-containing electron transport system, a significant portion of electrons is diverted to either the formation of hydrogen peroxide or the oxidative transformation of "endogenous substrates" (4, 5). The latter reaction conforms to the stoichiometry of a monooxygenase reaction in that one mole of NADPH is oxidized for each mole of oxygen consumed. The nature of the endogenous substrate(s) of liver microsomes has eluded characterization, although Schenkman *et al.* (6) have provided evidence that unsaturated fatty acids, in particular oleic acid, might serve in this capacity. In addition, it has been reported that unsaturated fatty acids appear to be released from liver microsomal phospholipids during the course of NADPH oxidation (7, 8). In the presence of an iron chelate, such as the ADP–Fe^{3+} complex, and an electron donor, such as NADPH or ascorbate, malonaldehyde is rapidly formed concomitant with a rapid rate of oxygen utilization (9). It is generally assumed that one source of malonaldehyde is from the oxidation of arachidonic acid (1, 10). Thus, it was of interest to evaluate the oxidative metabolism of arachidonic acid by the electron transport system associated with rat liver microsomes.

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It is the purpose of this communication to describe the role of liver microsomal cytochrome *P*-450 in the oxidation of arachidonic acid. It is suggested that free arachidonic acid, as well as that derived from microsomal phospholipids, may contribute, in part, to the pool of "endogenous substrate(s)" oxidatively transformed by liver microsomal cytochrome *P*-450.

MATERIALS AND METHODS

Microsomal fractions were prepared from homogenates of rat livers by means of differential centrifugation as described (11). Male Sprague–Dawley rats (150- to 200-g body weight) were treated by four daily intraperitoneal injections of phenobarbital (75 mg/kg of body weight) and the animals were starved 18 hr prior to sacrifice.

All reactions were performed in a buffer mixture containing 10 mM $MgCl_2$, 150 mM KCl, and 50 mM Tris·HCl (pH 7.5). Unlabeled arachidonic acid was obtained from Nu-Chek (Elysian, MN), and ^{14}C-labeled arachidonic acid (specific activity of 54.6 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from Amersham. The radiochemical purity of the samples used was routinely analyzed by high-performance liquid chromatography (HPLC) and found to be greater than 99.9%. A stock solution of sodium arachidonate (25 mM) was prepared in deoxygenated 0.1 M Tris buffer with the pH adjusted to 8.5 by the careful addition of 2 M NaOH. Solutions of sodium arachidonate were stored frozen under an atmosphere of nitrogen. NADPH was obtained from Boehringer-Mannheim; metyrapone, from Ciba–Geigy; superoxide dismutase, from Sigma; and catalase, from Worthington. Glass-distilled solvents for HPLC analyses were purchased from Burdick and Jackson (Muskegon, MI). HPLC analysis was carried out by using a μBondapak C_{18} column from Waters Associates (Milford, MA).

Incubations were carried out at 25°C, using open vessels with constant mixing. Samples were removed at the times indicated and added to an equal volume of a mixture of HCl and ethyl acetate so that the pH in the aqueous phase was 3. The samples were extracted three times with equal volumes of ethyl acetate. The organic phase was washed with glass-distilled water to remove excess HCl. After drying with sodium sulfate, the samples were filtered through 0.5-μm pore diameter Millipore filters and then evaporated to dryness by a stream of nitrogen gas. All other experimental conditions are described in the legends to the figures.

RESULTS

Interaction of Arachidonic Acid with Liver Microsomal Cytochrome *P*-450. One criteria for assessing the possible role of cytochrome *P*-450 in the metabolism of a chemical is to deter-

Abbreviation: HPLC, high-performance liquid chromatography.

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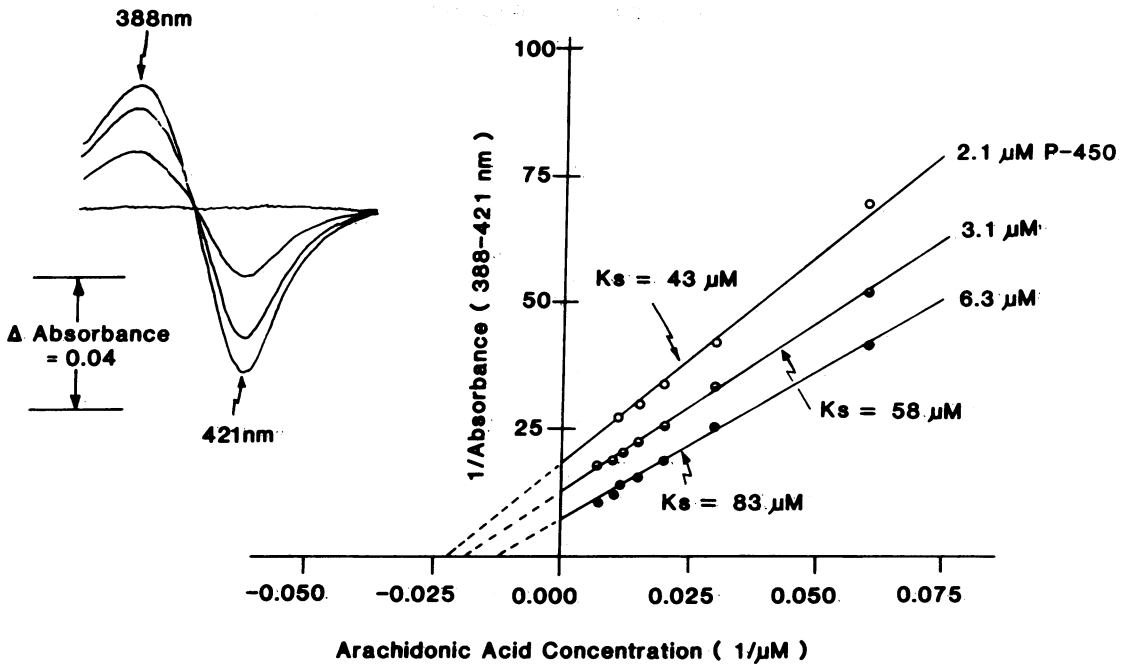

FIG. 1. Interaction of arachidonic acid with liver microsomal cytochrome *P*-450. Microsomes from the livers of phenobarbital-treated animals were diluted in buffer and the magnitude of absorbance change at 388 nm minus 421 nm was determined after the successive additions of small aliquots of 25 mM sodium arachidonate (see the upper left portion of the figure). Difference spectra were recorded at 25°C, using an Aminco DW2A spectrophotometer in the dual beam mode. The specific content of cytochrome *P*-450 was 2.6 nmol/mg of microsomal protein, of which 42% was initially in the high-spin form.

mine the extent of substrate-dependent spectral perturbations associated with the low-spin to high-spin transition of the ferric heme protein (12). Although this criteria is not absolute, a positive result is supportive of the hypothesis that metabolism will occur. The relatively large content (13) of membrane-bound, high-spin ferric cytochrome *P*-450 in liver microsomes (i.e.,

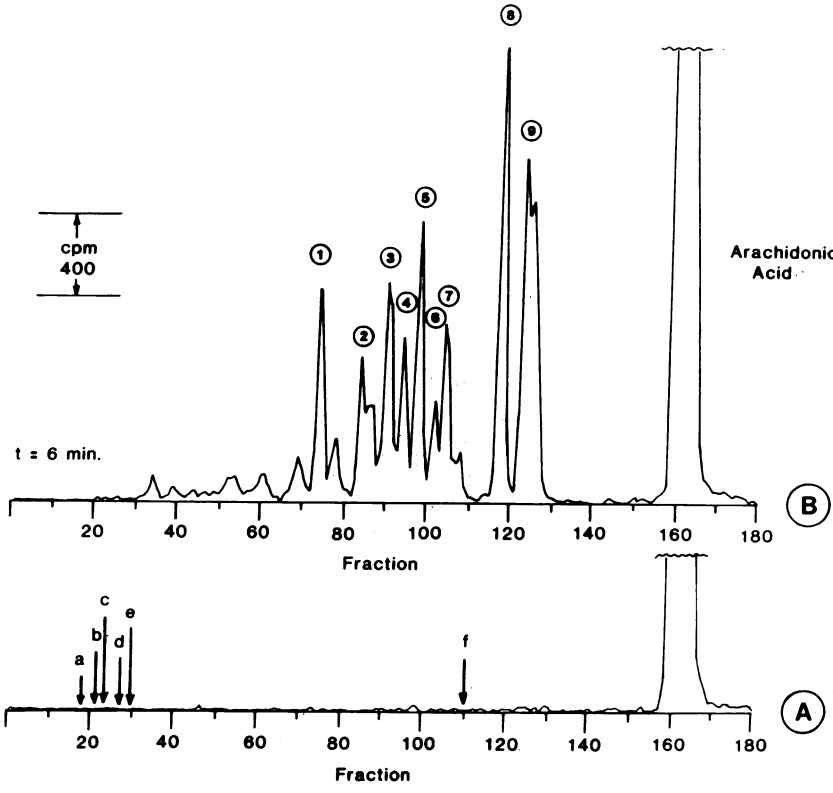

FIG. 2. HPLC profiles of arachidonic acid metabolites. Microsomes from livers of phenobarbital-treated rats were diluted to 0.5 mg of protein per ml in a buffer mixture containing 10 mM $MgCl_2$, 150 mM KCl, 50 mM Tris·HCl (pH 7.5), 0.1 mM arachidonic acid (0.6 mCi/mmol) and a NADPH-regenerating system consisting of 0.25 international unit per ml of isocitrate dehydrogenase and 8 mM sodium isocitrate. The final volume of the reaction mixture was 5 ml. After about 1-min equilibration at 25°C, NADPH (final concentration 1 mM) was added to initiate the reaction. The reaction was terminated after 6 min by the addition of ethyl acetate-containing HCl. The solvent program used for HPLC was a linear gradient ranging from initially equal volumes of water and acetonitrile (containing 0.1% acetic acid) to 100% acetonitrile containing 0.1% acetic acid. The rate of change was 1.25%/min at a flow rate of 1 ml/min. The recovery of radioactivity from the HPLC column was 75%. Curve A shows the HPLC profile from a reaction terminated at 0 min, and curve B represents the products separated from a mixture in which the reaction was terminated after 6 min of incubation. In addition, the arrows on curve A show the relative positions where the following authentic standards would appear in the HPLC analysis (PG, prostaglandin): a, 6-keto-$PGF_{1\alpha}$; b, thromboxane B_2; c, $PGF_{2\alpha}$; d, PGE_2; and e, PGD_2. The arrow at position f on curve A shows the position of elution of the product (15-L-hydroperoxy-5,8,10,14-icosatetraenoic acid) of the soybean lipoxidase-catalyzed oxidation of arachidonic acid. For this experiment, 100 μM sodium arachidonate was added to a buffer mixture containing 10 mM $MgCl_2$, 150 mM KCl, and 50 mM Tris. The reaction was initiated by the addition of soybean lipoxidase (Sigma) at 50 μg/ml. The reaction mixture was incubated for 5 min at 25°C and the reaction was terminated by the addition of ethyl acetate-containing HCl.

40–50%) can obscure or attenuate the magnitude of spectral change seen on binding of a chemical to be metabolized. In the case of arachidonic acid, a significant optical spectral change occurs (14, 15) when increasing concentrations of the unsaturated fatty acid are added to a reaction cuvette containing rat liver microsomes (Fig. 1). Studies of this type demonstrate that cytochrome *P*-450 has a rather high affinity for arachidonic acid (an apparent substrate constant, K_S, of approximately 50 μM). The magnitude of change in the spin state of the membrane-bound cytochrome *P*-450, in this experiment, is from approximately 42% high-spin to 60% high-spin heme protein in the presence of saturating concentrations of arachidonic acid. It should be noted that the binding of arachidonic acid to microsomal cytochrome *P*-450 is dependent on the concentration of membrane protein present in the reaction cuvette (cf. Fig. 1). These differences may reflect the distribution of arachidonic acid in the lipid milieu of the membrane.

Effect of Arachidonic Acid on Microsomal NADPH-Dependent Oxygen Utilization. The addition of NADPH to rat liver microsomes results in the oxidation of NADPH concomitant with a relatively rapid rate of oxygen uptake (approximately 20 nmol of oxygen utilized per min per mg of microsomal protein at 25°C). Recent studies have shown that a significant portion of oxygen reduction results in the formation of hydrogen peroxide (4). This can be directly demonstrated by measuring colorimetrically the extent of hydrogen peroxide generated. Alternatively, the amount of hydrogen peroxide formed can be estimated by use of an oxygen electrode when experiments are carried out in the presence or absence of sodium azide. The addition of sodium arachidonate to a suspension of liver microsomal protein increases the rate of NADPH-dependent oxygen uptake in the presence or absence of sodium azide. Further, it was noted that the addition of arachidonic acid to a suspension of liver microsomal protein does not lead to the utilization of oxygen until NADPH is added to the reaction mixture; i.e., arachidonic acid is not "auto-oxidizable" under these experimental conditions. Second, when limiting amounts of NADPH are added to the reaction system, a stoichiometry of approximately one mole of NADPH oxidized per mole of oxygen utilized is seen in the presence of 1 mM sodium azide and 0.1 mM arachidonic acid; i.e., an "auto-catalytic" reaction of the type normally attributed to lipid peroxidation reactions is not seen (9). Third, the reaction can be repeatedly demonstrated by the sequential addition of limiting concentrations of NADPH; i.e., the accumulation of metabolites of arachidonic acid does not destroy or modify the enzyme system, nor do the metabolites alter the stoichiometry of the reaction.

The colorimetric measurement of peroxide formed during this type of microsomal electron transport reaction shows that the rate of peroxide formation is significantly stimulated during the associated oxidative metabolism of arachidonic acid—i.e., an increase from 9 nmol per min per mg of protein in the absence of arachidonic acid to 16 nmol per min per mg of protein in the presence of arachidonic acid when the experiment is carried out at 25°C. Of interest is the failure to observe the formation of any malonaldehyde during the oxidative metabolism of arachidonic acid in the experiments described.

Metabolites of Arachidonic Acid. The aerobic incubation of ^{14}C-labeled arachidonic acid with rat liver microsomal protein and NADPH, followed by extraction with ethyl acetate and analysis of the extract by HPLC, shows, as illustrated in Fig. 2, the formation of a variety of radioactive metabolites. These metabolites have been arbitrarily grouped into nine fractions. When the extent of increase in radioactive products formed was measured after various times of incubation, no evidence for a precursor–product relationship for any of the metabolites was apparent. In the experiment illustrated in Fig. 2, the rate of decrease of arachidonic acid was approximately 6 nmol per min per mg of protein at 25°C; this rate was equivalent to that calculated from the arachidonic acid-dependent increase in the rate of oxygen utilization (when corrected for the increased rate of peroxide formation) and equal to the sum of the rates of formation of the various metabolites. It should be noted that the rate of arachidonic acid metabolism reported here is 10-fold greater than the rate of oleic acid metabolism by liver microsomal protein as reported by Schenkman *et al.* (6).

The pattern of metabolites formed during the oxidative metabolism of arachidonic acid shows no evidence for the formation of prostaglandin or thromboxane derivatives (Fig. 2, curve A). Some of the products formed during the liver microsomal metabolism of arachidonic acid do have retention times on HPLC analysis similar to those noted for the 15-hydroperoxy- and the 15-hydroxy- derivatives seen after the oxidation of arachidonic acid as catalyzed by soybean lipoxidase (Fig. 2, curve A). Preliminary mass spectral analysis of the HPLC-isolated metabolites indicated the presence of hydroxy acids derived from arachidonic acid. Some of these may represent products formed by the ω- and (ω − 1)-hydroxylation of arachidonic acid. The metabolites formed have not yet been identified, but experiments to characterize the isolated metabolites by optical absorbance spectrophotometry indicated the presence of absorbance maxima in the spectral region of 235 nm for four metabolites. This result suggests that these compounds contain a diene conjugation in their structure and that they may be

Table 1. Effect of inhibitors on the rate of arachidonic acid metabolism by rat liver microsomes

Addition	Rate: nmol/min per mg protein	Rate: % of control
None	5.1	100
Metyrapone, 50 μM	1.95	38
Sodium azide, 1 mM	4.5	89
Mannitol, 60 mM	4.55	88
None	4.9	100
Catalase	4.2	85
Superoxide dismutase	4.5	91
None	6.8	100
Benzphetamine, 0.5 mM	1.9	28
NADH (−NADPH)	1.6	24
80% N_2/20% O_2	4.0	100
80% CO/20% O_2	1.05	26

Liver microsomes from phenobarbital-pretreated rats were diluted to 0.5 mg of protein per ml in a buffer mixture containing 10 mM $MgCl_2$, 150 mM KCl, 50 mM Tris·HCl (pH 7.5), a NADPH-regenerating system consisting of 0.25 international unit per ml of isocitrate dehydrogenase, 8 mM sodium isocitrate, and 0.1 mM sodium arachidonate (0.6 mCi/mmol). Where indicated, metyrapone, sodium azide, mannitol, catalase (2600 international units/ml), superoxide dismutase (15 international units/ml), or benzphetamine was added 1 min prior to the initiation of the reaction by the addition of NADPH (1 mM final concentration). In the experiment in which NADH replaced NADPH, the final concentration of NADH was 0.5 mM. The reaction was terminated 3 min after addition of NADPH (or NADH) by the addition of an equal volume of ethyl acetate containing sufficient HCl to adjust the pH of the aqueous phase to 3. For the experiments using gas mixtures, samples were gassed continuously in specially designed vessels, using the gas mixtures designated. After gas equilibration for 9 min, the reaction was initiated by the addition of NADPH. The reaction was stopped after 2 min as described above. The temperature was maintained at 25°C. The samples were analyzed by HPLC.

derived from the reduction of hydroperoxide intermediates (16, 17).

Influence of Inhibitors. To evaluate the role of cytochrome *P*-450 in the metabolism of arachidonic acid, experiments were carried out in the presence of various inhibitors as shown in Table 1. These inhibitors may affect the function of cytochrome *P*-450 directly or interfere with the products of oxygen reduction generated concomitant with the cyclic redox reactions of cytochrome *P*-450. As shown in Table 1, a marked inhibition of arachidonic acid metabolism was observed in the presence of the dipyridyl derivative metyrapone or in the presence of an atmosphere composed of 80% carbon monoxide and 20% (vol/vol) oxygen. Likewise, a dramatic inhibition of arachidonic acid metabolism was noted when a substrate for cytochrome *P*-450, benzphetamine, was included in the reaction medium. Agents that modify the steady-state concentration of oxygen reduction products, such as catalase, superoxide dismutase, sodium azide, or mannitol, were without significant effects on the rate of product formation. Thus, it appears that superoxide anion, hydrogen peroxide, or hydroxyl radicals may not be involved directly in this type of metabolic transformation of arachidonic acid. No significant rate of metabolism was noted when NADH replaced NADPH as the source of reducing equivalents for the function of the microsomal electron transport system.

DISCUSSION

Multiple pathways of metabolism have been shown for the oxidative transformation of arachidonic acid. In recent years, great interest has focused on the role of those metabolites related to the prostaglandins and to some of the hydroperoxide derivatives because these products have been demonstrated to possess profound pharmacological or physiological action (1, 2, 18, 19). The present study describes yet another pathway for the metabolism of arachidonic acid. Of interest is the role of the heme protein cytochrome *P*-450 in this reaction, because it is known that cytochrome *P*-450 is widely distributed in different body tissues and participates in the oxidative metabolism of many drugs, steroids, and carcinogenic chemicals (20). The question of the role of cytochrome *P*-450 in the metabolism of "endogenous substrates" has been frequently posed. Beyond its known activity for the metabolic oxidation of cholesterol and other similar steroid-like compounds (21), it now appears certain that cytochrome *P*-450 can contribute to the metabolic breakdown of many other lipids, in particular fatty acids. This may occur either by ω- or (ω − 1)-oxidation (22, 23) or by a lipoxygenase-like function of cytochrome *P*-450 as described in this paper.

Because of the ease of oxidation of arachidonic acid by oxygen radicals (24, 25), it was necessary to determine whether the contribution of cytochrome *P*-450 to the oxidation of arachidonic acid was a direct or an indirect event. As shown by the scheme presented in Fig. 3, it is concluded that the oxidation of arachidonic acid, observed when rat liver microsomes are incubated aerobically with NADPH, is the result of a direct oxidation by cytochrome *P*-450 (such as reactions 1–3 of Fig. 3) rather than the consequences of a derived oxygen reduction product (reactions 4–6 of Fig. 3), such as the superoxide anion or the hydroxyl radical. This conclusion is based on the failure to observe a significant inhibition of product formation or any change in the pattern of metabolites generated when the reaction was carried out in the presence of mannitol, catalase, or superoxide dismutase (Table 1). Likewise, the failure to note any stimulation of the reaction in the presence of sodium azide, an agent that inhibits catalase and results in a progressive increase in the content of hydrogen peroxide in the reaction medium, supports the conclusion that the results obtained are not the consequence of oxygen reduction products.

The direct involvement of cytochrome *P*-450 in the oxidation of arachidonic acid is supported by the observation that arachidonic acid can perturb the spin-state equilibrium of the membrane-bound heme protein and that inhibitors of cytochrome *P*-450 action, such as metyrapone and carbon monoxide, decrease effectively the rate of arachidonic acid metabolism. In

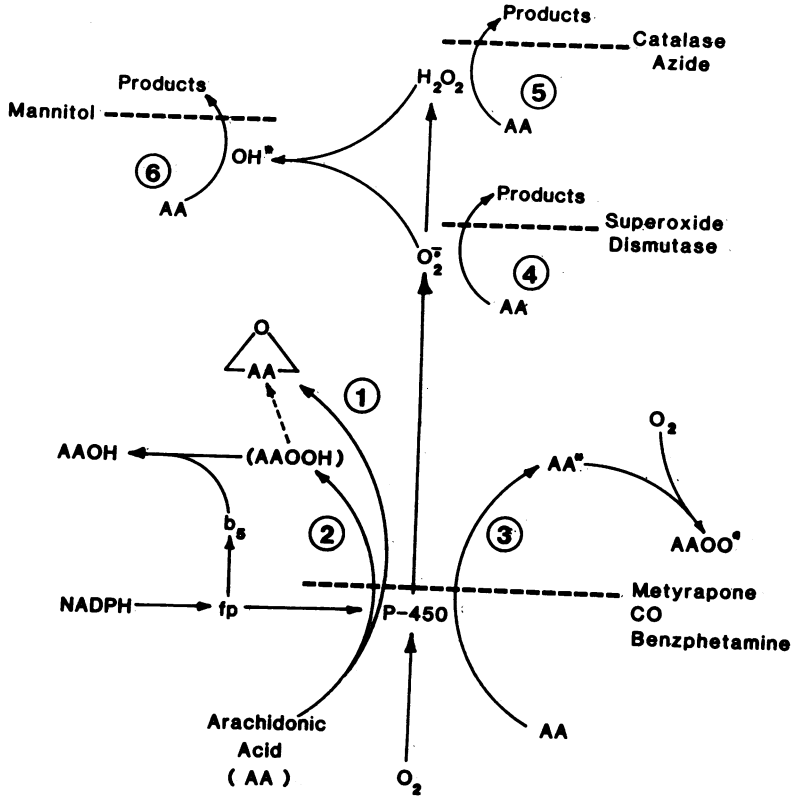

FIG. 3. Schematic representation of the possible reaction pathways associated with the function of microsomal cytochrome *P*-450 for the metabolism of arachidonic acid (AA). The sites of influence of various inhibitors are designated. AAOOH, the postulated arachidonic acid hydroperoxide; AAOH, the hydroxy acid; fp, flavoprotein; b_5, cytochrome b_5.

addition, substitution of the reduced pyridine nucleotide NADH for NADPH results in only a slow rate of metabolism; an observation comparable to that noted with other cytochrome *P*-450-dependent substrates (26). The addition of a known substrate for liver microsomal cytochrome *P*-450, benzphetamine, markedly suppressed the rate of arachidonic acid metabolism. Benzphetamine was chosen for these experiments because it is known that benzphetamine can serve as an "uncoupler" of cytochrome *P*-450 and thereby significantly stimulate the rate of breakdown of oxycytochrome *P*-450 concomitant with an increase in the rate of synthesis of hydrogen peroxide (4). If any oxygen reduction products were responsible for the observed metabolism of arachidonic acid, then one would expect a stimulation of arachidonic acid metabolism, rather than the observed inhibition when benzphetamine was included in the reaction medium.

The mechanism of arachidonic acid metabolism catalyzed by cytochrome *P*-450 remains unknown. Studies with soybean lipoxidase suggest that arachidonic acid undergoes a hydrogen abstraction to form a free radical species that can then interact with molecular oxygen to form a peroxy radical (2). As a result of the hydrogen abstraction, a conjugated diene structure is formed.

Recent studies (27–29) on the metabolism of benzo[*a*]pyrene to quinones, as catalyzed by microsomal cytochrome *P*-450, suggests the operation of a comparable type of reaction for the metabolism of this precarcinogen. It is therefore intriguing to speculate that oxycytochrome *P*-450 may function as the acceptor for the hydrogen atom donated by compounds such as arachidonic acid or benzo[*a*]pyrene. As reported here, an increase in the rate of hydrogen peroxide formation accompanies the liver microsomal cytochrome *P*-450-dependent oxidation of arachidonic acid. It is of interest that the stoichiometry for this effect is the appearance of approximately one mole of additional hydrogen peroxide for each mole of arachidonic acid metabolized. The suggestion that cytochrome *P*-450 may serve as an acceptor of reducing equivalents arising from the hydrogen abstraction of arachidonic acid or other substrates now places this hemeprotein in the role of "substrate activation" as well as "oxygen activation." Because some of the products formed during the liver microsomal metabolism of arachidonic acid show spectral characteristics of diene conjugates (i.e., absorbance maxima around 235 nm), this suggests that they are formed by hydrogen abstraction with the formation of a carbon-centered free radical prior to oxygen insertion. The oxygenation step may be the result of the reaction between a carbon-centered free radical with oxygen to form a hydroperoxide radical. Multiple pathways exist for the reduction of this radical product to form the observed hydroxy acid. In this way cytochrome *P*-450 of liver microsomes functions in a manner comparable to the initial reaction of a lipoxygenase. It must be emphasized that the reaction mechanism described here differs significantly from that proposed for the reactions of lipid peroxidation. In the present study, no cascade of radical-mediated reactions occurs and no malonaldehyde is formed during the cytochrome *P*-450-dependent metabolism of archidonic acid. Indeed, the stoichiometry of a monooxygenase reaction is maintained—i.e., approximately one mole of NADPH is oxidized for each mole of oxygen utilized.

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