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Cytochromes P450 Catalyze the Reduction of α,β -Unsaturated Aldehydes

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Abstract

The metabolism of α,β -unsaturated aldehydes, e.g. 4-hydroxynonenal, involves oxidation to carboxylic acids, reduction to alcohols, and glutathionylation to eventually form mercapturide conjugates. Recently we demonstrated that P450s can oxidize aldehydes to carboxylic acids, a reaction previously thought to involve aldehyde dehydrogenase. When recombinant cytochrome P450 3A4 was incubated with 4-hydroxynonenal, O₂, and NADPH, several products were produced, including 1,4-dihydroxynonene (DHN), 4-hydroxy-2-nonenic acid (HNA), and an unknown metabolite. Several P450s catalyzed the reduction reaction in the order (human) P450 2B6 $\cong$ P450 3A4 > P450 1A2 > P450 2J2 > (mouse) P450 2c29. Other P450s did not catalyze the reduction reaction (human P450 2E1 & rabbit P450 2B4). Metabolism by isolated rat hepatocytes showed that HNA formation was inhibited by cyanamide, while DHN formation was not affected. Troleandomycin increased HNA production 1.6-fold while inhibiting DHN formation, suggesting that P450 3A11 is a major enzyme involved in rat hepatic clearance of 4-HNE. A fluorescent assay was developed using 9-anthracenealdehyde to measure both reactions. Feeding mice diet containing *t*-butylated hydroxyanisole increased the level of both activities with hepatic microsomal fractions, but not proportionally. Miconazole (0.5 mM) was a potent inhibitor of these microsomal reduction reactions, while phenytoin and α -naphthoflavone (both at 0.5 mM) were partial inhibitors, suggesting the role of multiple P450 enzymes. The oxidative metabolism of these aldehydes was inhibited >90% in an Ar or CO atmosphere, while the reductive reactions were not greatly affected. These results suggest that P450s are significant catalysts of reduction of α,β -unsaturated aldehydes in liver.

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INTRODUCTION

α,β -Unsaturated aldehydes are highly reactive environmental and endogenous compounds formed during combustion, consumption of food-stuffs, metabolism of some drugs and inflammation, respectively^{1–3}. Their chemical reactivity is due to the presence of the unsaturated bond next to the aldehydic functional group, allowing nucleophilic attack at either the double bond or the aldehyde forming a carbinol derivative. Considerable interest in these compounds arises from the production of several lipid aldehydes, the most studied being 4-hydroxy-2-nonenal (4-HNE). Except during chemically-induced lipid peroxidation, the concentrations of 4-HNE attained in tissues are in the low μM concentrations^{4, 5}, but have been associated with the onset of cardiovascular and neurodegenerative diseases^{6, 7}. Modification of low density lipoproteins by 4-HNE apparently makes the lipoprotein more atherogenic, resulting in increased foam cell formation. The proteins associated with atherosclerotic lesions have been shown to be modified by 4-HNE⁸. When cells are exposed to low concentrations of 4-HNE, cell proliferation is stimulated and genotoxic events noted. Other aldehydes like *trans*-2-hexenal and hexenal have been shown to inhibit germination of seeds and as a result have been used as fungicides for plants².

Interest in the biological properties of these aldehydes produced in various pathophysiological processes has stimulated interest in understanding the metabolism and disposition of lipid aldehydes, like 4-HNE. Several groups have characterized the metabolism of 4-HNE to established that reduction to 1,4-dihydroxy-2-nonene (DHN) by cytosolic alcohol dehydrogenase^{9, 99999}, oxidation by aldehyde dehydrogenases to 4-hydroxynonenoic acid (HNA), and glutathione conjugation to form the GSH conjugate (GS-HNE) are major metabolic routes in hepatocytes, enterocytes, and tumor cells^{10, 11}. Studies *in vivo* after i.v. injection of 4-HNE into rats yielded four mercapturic conjugates in the urine, namely 1,4-dihydroxy-2-nonene mercapturic acid, 4-hydroxynonenal mercapturic acid, 4-hydroxynonenoic acid mercapturic acid, and the corresponding lactone mercapturic acid derivatives¹²; GSH conjugates of 4-HNE and DHN were also observed. De Zwart *et al.*¹³ demonstrated that conjugation of 4-HNE with GSH is a high capacity, first-pass metabolic step in the elimination of this aldehyde. A recent study¹⁴ using rat adrenal PC-12 cells demonstrated that miconazole, a P450 inhibitor, decreased HNA formation by 40% and benomyl, an ALDH inhibitor could only inhibit HNA formation by 75%, similar to the results reported by Amunom *et al.*¹⁵ for rat hepatocytes.

A role for P450s in the metabolism of endogenous and exogenous α,β -unsaturated aldehydes has been suggested by Amunom *et al.*¹⁵ due to the wide number of P450s catalyzing the oxidation of α,β -unsaturated aldehydes *in vitro* and in primary cultures of rat hepatocytes. In the current study, we demonstrate that several mammalian P450s, i.e. human P450s 2B6, 1A2, 3A4, and 2J2 and murine P450 2c29 catalyze both the facile oxidation and reduction of α,β -unsaturated aldehydes to their carboxylic acids and alcohol form at low μM concentrations of aldehyde substrate. Using mouse liver microsomes and primary hepatocytes in conjunction with selective P450 inhibitors, we show that—in addition to aldehyde dehydrogenase and aldose reductase—several hepatic P450s (e.g., human P450 2B6, 3A4, and 2J2 and murine P450 2c29) participate significantly in the oxidative and reductive metabolism of these aldehydes. The reactions catalyzed by murine liver microsomal fractions are significantly inhibited by known P450 inhibitors—e.g., miconazole, troleandomycin, phenytoin, and α -naphthoflavone—implicating multiple P450s in these reduction reactions. The P450-dependent reduction of α,β -unsaturated aldehydes occurs in the absence and presence of O_2 and was not affected by substitution of a CO atmosphere, which significantly inhibited oxidative metabolism of these aldehydes by P450s. Reduction of aldehydes by the P450s has not been observed previously and may represent a novel reaction pathway *in vivo*.

Experimental Procedures

Chemicals and plasmids

The expression plasmid, pCW-Cyp2c29, with Cyp2c29 cDNA cloned into *Nde*I and *Hind*III restriction enzyme sites, was provided by J. A. Goldstein, National Institutes of Environmental Health Sciences, Research Triangle Park, NC¹⁶. The NADPH-P450 reductase expression plasmid was provided by M. Doll and D. Hein, Department of Pharmacology and Toxicology, University of Louisville School of Medicine. All plasmids were digested with restriction enzymes to confirm the identity of the cDNA of interest. Preparations of *Escherichia coli* membranes containing recombinant NADPH-P450 reductase and P450 1A2, 2B6, 2E1, or 3A4 were prepared as described¹⁷. In these preparations, the ratio of P450 to NADPH-P450 reductase was measured to be between 0.8–2.0. Human P450 2J2 and NADPH-P450 reductase expressed in insect cells were generously provided by D. Zeldin, National Institutes of Environmental Health Sciences, Research Triangle Park, NC. Anthracene-9-carboxaldehyde (9-AA), 9-hydroxymethyl-anthracene (9-AMeOH), and anthracene-9-carboxylic acid (9-ACA) were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO before use. [³H]-4-HNE was prepared as described previously⁷, and unlabelled 4-HNE [(E)-non-2-ene-1,4-diol] was purchased from Calbiochem (San Diego, CA). HNA and DHN standards were prepared as described by Amunom *et al.*¹⁵ and Srivastava *et al.*^{7, 11, 18}.

Escherichia coli growth conditions

Expressed P450s were obtained by growing them in *E. coli* using various pCW-P450 constructs and pACYC-1 Duet CYPOR (NADPH-P450 reductase) gene in *E. coli* BL21 (DE3) served as inoculum for the expression experiment. Cells were grown and harvested as described by Amunom *et al.*¹⁵. Isolation of bacterial membrane preparations was carried out at 4° C, and the membranes were stored at –80° C until use.

Preparation of Mouse Liver Microsomal Fractions

Mouse liver microsomal preparations were prepared from male C57BL/6J mice (22 to 27 g; Jackson Laboratories, Bar Harbor, MA) fed *ad libitum* for 1 week with AIN-76A diet (Purina Test Diet, Richmond, IN) or AIN-76A diet supplemented with 0.45% *t*-butylated hydroxyanisole (BHA, w/w). All procedures for handling the mice were approved by the University of Louisville IACUC Committee and conformed to the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The procedure for preparation of microsomal samples is described by Remmer *et al.*¹⁹. The final preparation was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 10% glycerol (v/v) and stored at –80° C. Protein concentrations were determined using a bicinchoninic acid method (Pierce Chemical Co., Rockville, IL).

Primary Hepatocyte Cultures

Male Sprague-Dawley rats (180 g–200 g; Hsd:SD, Harlan Indianapolis, IN) were used for liver perfusion as described by Skett and Bayliss²⁰ and modified as described by Amunom *et al.*¹⁵. The cells (in 20 mm dishes) were maintained in a CO₂ incubator prior to treatment with 50 μM 4-HNE, and reactions were terminated at 0, 10, and 20 min with trichloroacetic acid (7.5% final concentration, w/v). The resulting samples were removed from the plates and frozen at –80° C prior to analysis by HPLC, as described below. The inhibitors (0.5 mM miconazole or cyanamide) were added just prior to starting the reactions by addition of 4-HNE. When 0.5 mM troleandomycin was used as an inhibitor in the hepatocytes, the cells were pre-incubated with troleandomycin 10 min prior to adding 4-HNE.

9-AA Oxidation and Reduction Assay

The oxidation of 9-AA by P450 enzymes was determined by measuring the formation of 9-ACA as described by Matsunaga *et al.*²¹ and Marini *et al.*²², as modified by Amunom *et al.*¹⁵. In brief, the incubation mixture included recombinant P450 (50 nM) or mouse liver microsomes (0.25 mg/mL, approximately 50 nM P450), an NADPH-regenerating system consisting of 100 μ M NADPH, 4.25 mM isocitric acid, 50 mM MgCl_2 , and 1.3 Units/mL isocitrate dehydrogenase, 25 μ M 9-AA, and 0.10 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The reaction was carried out at 30° C for 10–30 min and terminated with 1.0 mL of 0.5 M NaOH. Ethyl acetate (4 mL) was added to allow for product separation, using extraction of either alkalized and acidified aqueous reaction mixtures with this organic solvent. The formation of 9-A-MeOH was determined by measuring its fluorescence in the alkaline ethyl acetate organic phase at 255 nm excitation and 411 nm emission wavelength with a spectrofluorimeter (Model LS50B, Perkin Elmer, Waltham, MA. After acidification of the aqueous phase of the reaction, the fluorescence of 9-ACA in the acidic organic phase was subsequently measured at 255 nm excitation and 458 nm emission wavelengths. The fluorescence excitation and emission spectra of the metabolites was nearly identical to authentic 9-ACA and 9-A-MeOH^{15, 23}. The 9-AA metabolism assay was used as an initial indication of aldehyde oxidation or reduction by P450s.

Metabolism of 4-HNE by cytochrome P450s

4-HNE metabolism was performed as described by Amunom *et al.*¹⁵ by incubating 50 μ M [³H]-4-HNE in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA with either *E. coli* –expressed P450 (50 nM), mouse microsomal protein (0.25 mg/mL, ~50 nM P450), or mouse primary hepatocytes as described by Amunom *et al.*¹⁵ except NADPH was used in place of an NADPH-regenerating system. The reaction was initiated by adding 50 μ M [³H]-4-HNE. The reactions were terminated after 20 min incubation by flash freezing sample tubes in liquid N₂. The frozen samples were thawed upon adding trichloroacetic acid (7.5% final concentration), and the denatured protein was sedimented following centrifugation at 13,000 $\times g$ for 5 min at 4 °C. The supernatant was injected onto an HPLC column after an aliquot was removed to measure radioactivity in a Packard Tricarb 2100TR scintillation counter (Packard Instrument Co., Downers Grove, IL) with Ultimagold (Packard) as the scintillation cocktail, prior to separation by HPLC. The recovery of [³H] radioactivity from the reaction mixtures were >98% and the recoveries from HPLC were determined to be >95%. All of the P450s tested (2B6, 3A4, and 2c29) displayed linear reactions to 20 min with 4-HNE.

Inhibition of Aldehyde Metabolism by P450 Inhibitors

Metabolic assays with either 9-AA or 4-HNE were performed with either membranes containing expressed P450s or mouse liver microsomes as described above, except for the addition of selective inhibitors²⁴. Miconazole (0.5 mM) was utilized as a general inhibitor of P450s, and α -naphthoflavone is an inhibitor of the CYP1A enzymes. Phenytoin and troleandomycin (0.5 mM) were used to inhibit mouse P450s 2c29 and 3A, respectively, while cyanamide (0.5 mM) was used as an inhibitor of aldehyde dehydrogenase. Troleandomycin was preincubated with microsomal or expressed P450 fractions and 0.5 mM NADPH for 10 min before 9-AA or [³H]-4-HNE was added. Miconazole, phenytoin, α -naphthoflavone, and cyanamide were added to the reaction immediately prior to the aldehyde substrate.

Anaerobic Metabolism of 9-AA and 4-HNE by Mouse Liver Microsomes

The incubation reaction was prepared as described above for 9-AA and 4-HNE in an Erlenmeyer flask equipped with a serum stopper fitted with syringes. The 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA was bubbled for 5 min with CO or Ar, followed by addition of the P450. A mixture of glucose (74 mM), glucose oxidase (0.33 mg/mL), and catalase (1400 U/mL) was also added. The headspace of the reaction mixture was flushed with either CO or Ar for 3 min, and the reaction was initiated with 100 μ M NADPH. The reaction was conducted for 20 min and during this time, the headspace of the reaction was continuously flushed with either Ar or CO. All procedures were performed at 37° C. The reaction was terminated by adding NaOH (for the 9-AA incubation) or frozen in liquid nitrogen (the 4-HNE incubation). Extraction of products was performed as described above.

HPLC

HPLC separation of HNE products formed from either microsomal fractions, recombinant P450s, or hepatocytes was performed as described by Amunom *et al.*¹⁵. The incubation and HPLC analysis were performed in triplicate.

GC-electrospray ionization (EI)/MS of 4-HNE Products

For GC-MS analyses, the method of Srivastava *et al.*²⁵ was utilized to determine the identities of products. The samples were dried *in vacuo*, resuspended in 0.5 mL H₂O, and incubated with 5 mg pentafluorobenzyl hydroxylamine (PFBHA) for 30 min at room temperature. CH₃OH (500 μ L) was added, and the samples were extracted with 2 mL hexane. The hexane layer (upper layer) was removed, dried under a stream of N₂, and then derivatized with 20 μ L of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 60° C as described previously (25). The mixture was cooled to room temperature, and 1- μ L aliquots were used for analysis. The GC-EI/MS analysis was performed using an Agilent 6890/5973 GC/MS system (Agilent Technologies) under 70 eV electron ionization conditions. The compounds were separated on a bonded phase capillary column (DB-5MS, 30 m $\times$ 0.25 mm ID $\times$ 0.25 μ m film thickness (J7W Scientific Folsom, CA). The GC injection port and interface temperature were set to 280° C, with He gas (carrier) maintained at 14 psi. Injections were made in the splitless mode with the inlet port purged for 1 min following injection. The GC oven temperature was held initially at 100° C for 1 min and then increased at a rate of 10° C min⁻¹ to 280° C, which was held for 5 min. Under these conditions, the *t*_R for the HNA derivative was 9.67 min.

Statistical analysis

All experiments were conducted in triplicate, and the means and SD values were determined. The rates of metabolism were determined with liver microsomal fractions and primary rat hepatocytes by assaying at 0, 10, and 20 minutes, except for CYP2B6 and CYP3A4 where shorter time courses were utilized. Nearly all of the reactions were linear, so the slope of the plot of metabolite formation vs. time was used to determine the rate of metabolism. Where appropriate, a Student's *t*-Test was used for statistical analysis with *p* $\leq$ 0.05 as the criterion for significance. Alternatively, analysis of variance (ANOVA) was performed when required.

RESULTS

Evaluation of P450s in Oxidation and Reduction of 4-HNE (to HNA and DHN)

In order to study the metabolism of 4-HNE by P450s, 50 nM of each P450 in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA was incubated with 50 μ M [³H]-4-HNE and 100 μ M NADPH for 10 min. The NADPH-regenerating system consisting

of IDH and isocitric acid used by Amunom *et al.*¹⁵ was omitted because we observed a reaction between 4-HNE and a component of that solution formed large amounts of second unknown polar metabolites (possibly due to the glycerol in the isocitric dehydrogenase). The incubation of 4-HNE with recombinant P450 3A4 generated several products that migrated with t_R values of 56, 58 and 62 min upon HPLC analysis with radiometric detection (Figure 1). No other significant analytes were reproducibly observed in samples prepared at 0 minutes of incubation. 4-HNE eluted at $\sim t_R$ 60 min under these conditions, but the conditions used allowed near base-line separation of the metabolites and substrate HNE. The separation is similar to the results shown by Amunom *et al.*¹⁵. The major metabolite (t_R 56 min) migrated at the same position as authentic DHN, the reduced product of 4-HNE, but different than the other products, HNA (t_R 58 min) and an unknown metabolite (t_R 62 min)²⁶.

The identity of the compound migrating at t_R 56 min was determined by GC-EI/MS to be the reduced form of 4-HNE, namely DHN (Figure 2). Amunom *et al.*¹⁵ suggested that DHN was formed from 4-HNE in incubations of primary rat hepatocytes, along with other metabolites presumed to be GSH adducts. The parent peak expected for authentic DHN under these analysis conditions (Figure 2A) was not observed at m/z 318, but major ions were observed at m/z 287 (M-CH₃, due to loss of a methyl group from the trimethylsilyl group) and at m/z 231 (M-C₅H₁₁, due to loss of the 5-carbon alkyl side chain by cleavage between C5 and C6 of 4-HNE). The spectrum of the metabolite formed by P450 3A4 was nearly identical to the authentic standard (Figure 2B). The metabolite eluted from the HPLC and GC at the same retention time as the authentic DHN and all of the major mass peaks for authentic DHN were present in the spectra of the putative DHN metabolite. In addition, incubation of 4-HNE with rat primary hepatocytes resulted in 4-HNE reduction to a metabolite that migrated with authentic DHN, and the metabolite was also shown by GC/MS analysis to be DHN (data not shown). We were not able to ascertain the structure of the unknown metabolite; the δ -lactone was not observed upon acidification of HNA and prolonged periods of storage²⁶.

Using a fixed concentration of 50 μ M 4-HNE, we examined several P450s as catalysts for oxidation and reduction of this α,β -unsaturated aldehyde to the carboxylic acid (HNA) and the alcohol (DHN) (Table 1). After metabolism, the products were analyzed by HPLC with radiometric detection of product. The reactions with either murine P450 2c29 or human P450 3A4 were shown to be linear to 20 min with 50 μ M 4-HNE substrate (results not presented). In addition to the reconstituted human P450 2B6, several other human and murine P450s catalyzed both the oxidative and reductive transformation of 4-HNE. However, the reduction reaction was highest with human P450s 2B6, 3A4, and 1A2. The rates for both the oxidation and reduction reactions were only high for P450 2B6, while P450s 1A2, 2J2, and 3A4 displayed higher rates of reduction than oxidation. The apparent rates of 4-HNE reduction shown in Table 1 were in the order of P450 2B6 $\cong$ 3A4 $\gg$ 1A2 $>$ 2J2. P450s 2E1 and 2B4 did not appreciably reduce 4-HNE. These results suggest that under aerobic conditions, some P450s both oxidize and reduce 4-HNE to the respective products HNA and DHN, while other P450s show higher levels of formation of the carboxylic acid metabolite than DHN. In addition, some P450s did not catalyze the oxidative or reductive metabolism of these aldehydes (2E1, 2B4).

Effects of ALDH and P450 Inhibitors on 4-HNE Metabolism in Primary Rat Hepatocytes

In order to identify the enzyme systems involved in the oxidative and reductive metabolism of 4-HNE in a cellular model, we utilized inhibitors of ALDH and P450 in primary cultured rat hepatocytes. The HPLC profile for the 4-HNE metabolites formed in rat hepatocytes, polar metabolites (t_R 5 min), GSH conjugates of 4-HNE (t_R 41.5, 45, and 49 min), DHN (t_R 56 min), HNA (t_R 58 min), and the more slowly eluting unknown metabolite (t_R 62 min),

was similar to that reported by Petersen and coworkers²⁷. As reported by Amunom *et al.*¹⁵, HNA was a product of metabolism in intact hepatocytes, as well as several GSH conjugates and DHN. The formation of DHN in rat hepatocytes was significantly inhibited by the P450 inhibitors miconazole (general P450 inhibitor, 57%) and troleandomycin (P450 3A-selective inhibitor, 83%), while the ALDH inhibitor cyanamide had little or no effect on DHN formation (8% inhibition) (Figure 3). A combination of cyanamide and miconazole caused further inhibition (73% of DHN formation). The addition of troleandomycin stimulated HNA formation, but potently suppressed formation of DHN. An apparent competition exists between ALDH and P450 for 4-HNE metabolism, as observed with the increase in DHN formation following inhibition of 4-HNE oxidation by cyanamide and an increase in HNA formation upon treatment with troleandomycin. Taken together, these data suggest that several P450s participate in both the oxidative and reductive metabolism of 4-HNE and that ALDH and P450s compete for utilization of (50 μ M) 4-HNE as substrate in rat hepatocytes.

Fluorescence Spectra of 9-ACA and 9-A-MeOH

In order to study the characteristics of the P450-catalyzed reduction of α,β -unsaturated aldehydes further, we chose a substrate that was more convenient to analyze in higher throughput assays to characterize the reduction of 9-AA. The method for measuring 9-ACA, the oxidized product of 9-AA, has been previously described using fluorescence^{21, 22}. Excitation and emission wavelengths of 255 and 458 nm, respectively, were used to measure 9-ACA formation in ethyl acetate extracts of acidified microsomal reaction mixtures after removal of the substrate. 9-A-MeOH is the reduced product of 9-AA and we observed²³ that it is highly fluorescent, displaying excitation and emission wavelengths at 255 and 411 nm, respectively. The emission wavelengths for both 9-A-MeOH and 9-ACA were strikingly different, thus allowing measurement of these products in organic extracts from either acidified or alkalinized incubation reactions using appropriate standard curves for the two fluorescent products, respectively²³. For example, 9-A-MeOH was measured in the ethyl acetate phase after raising the pH of the microsomal reaction mixture with base. The fluorescence intensity of 9-anthracene methanol was found to be highest in this organic phase after a single extraction step, demonstrating high extraction efficiency of 9-A-MeOH into the ethyl acetate organic phase (data not shown). The substrate 9-AA is extracted into the same ethyl acetate phase as is 9-A-MeOH but does not interfere with the fluorescence of 9-A-MeOH when mixed with 9-A-MeOH when compared to 9-A-MeOH alone (data not shown). 9-AA displayed very weak fluorescence spectra compared to 9-A-MeOH. After acidification of the resulting aqueous phase, 9-ACA was subsequently extracted from the acidified reaction mixture into ethyl acetate as described by Marini *et al.*²². The differences in fluorescence emission wavelengths and the differential extraction with ethyl acetate allows for measurement of both the carboxylic acid and alcohol products in a single assay procedure as described by Amunom *et al.*²³.

Metabolism of 4-HNE in Mouse Liver Microsomes

The mouse microsomal metabolism of the model substrate 9-AA to its oxidized and reduced products was further characterized due to the higher throughput of this assay. Due to interest in the BHA-inducible murine P450 2c29, we first studied the effect of feeding of 0.5% (w/w) *t*-butylated hydroxyanisole (BHA) in the diet and measured 9-AA oxidation and reduction to characterize the two reactions in mouse liver microsomal fractions. The basal expression of the oxidative reaction was 1.5 nmol/min/mg protein and the rate of the reduction reaction was 0.34 nmol/min/mg protein (Figure 4), suggesting that the enzymes in this subcellular fraction displayed higher levels of oxidation than reduction. Both enzyme activities were induced by feeding 0.5% BHA in the diet, but 9-ACA formation was induced 2.8-fold, while 9-A-MeOH formation was only increased 1.8-fold. The difference in the

induction suggests that several distinct P450 enzymes may be involved in the basal and induced activity for the oxidation vs. reduction reactions.

We subsequently used various P450 inhibitors to establish which P450s may be involved in the reduction reaction (Figure 5). Miconazole (0.5 mM, general inhibitor of P450s) potently inhibited both the oxidation and reduction reaction catalyzed by liver microsomal fractions from BHA-fed mice, while troleandomycin (0.5 mM, P450 3A subfamily inhibitor) did not produce statistical differences in either reaction. Phenytoin (0.5 mM, mouse P450 2c29 inhibitor) decreased both the oxidation and reduction reactions, suggesting a role for P450 2c29 in both reactions. The potent affect of α -naphthoflavone (0.5 mM), an inhibitor of Family 1 P450s, suggests a role for P450 1A2, which is expressed in basal conditions. These results suggest that at least two mouse P450s, 1a2 and 2c29, participate in the oxidation and reduction of 9-AA.

Metabolism under Either Ar or CO Inhibits P450-dependent Aldehyde Oxidation but not Reduction

P450s require O₂, NADPH-P450 reductase, and NADPH in order to catalyze monooxygenation reactions. Since P450-mediated reduction of azo dyes are often not O₂ sensitive, we chose to examine the effect of anaerobiosis and exposure to CO to contrast the two reactions. The oxidation of 9-AA to 9-ACA and of 4-HNE to HNA by mouse liver microsomes was significantly inhibited (>90%) under either condition we utilized to achieve anaerobiosis or in the presence of a CO atmosphere (Figure 6). Interestingly, the P450-mediated reduction of 9-AA to 9-A-MeOH and of 4-HNE to DHN was not appreciably affected by the anaerobic environment. The reduction of 4-HNE to DHN was slightly enhanced in the presence of CO, suggesting that while O₂ is required for oxygenation (Figure 6), it is not required for the reduction reaction catalyzed by the P450s. In addition, CO did not inhibit the P450-dependent reduction reaction, suggesting that the substrate may not occupy the area near the 6th coordination position of the heme of P450 3A4 for productive catalysis.

DISCUSSION

A typical reaction catalyzed by P450s is the hydroxylation of an unfunctionalized alkyl group or epoxidation of an unsaturated carbon-carbon bond^{28, 29}. However, examples are also known in which P450s catalyze the oxidation of aldehydes to acids with the incorporation of molecular oxygen into the products. Several critical P450-catalyzed reactions have been shown to involve oxidation of aldehydes or hemiacetals, e.g. the aromatase and sterol 14 α -demethylase reactions. Human liver microsomes possess the ability to oxidize aromatic aldehydes such as 11-oxo- Δ^8 -tetrahydrocannabinol^{30, 31} and tolualdehyde³² to carboxylic acids via P450 systems. Model substrates, e.g. 9-AA and 4-biphenylaldehyde, have been used to measure the aldehyde oxidation activity of several P450s^{21, 22}. These fluorogenic substrates provide simple methods to measure the acid products formed from the P450-dependent oxidation of aromatic aldehydes.

The metabolism of lipid peroxidation-derived metabolites, e.g. 4-HNE, has been shown to involve oxidation, reduction, and GSH conjugation *in vitro* and *in vivo*^{4, 10, 18, 33}. The reduction reactions have been suggested to principally involve aldose reductase or aldo-keto reductases²⁷. During a study with mouse liver microsomes and the different P450s that oxidize 9-AA and 4-HNE, we found that some P450s are able to reduce these compounds to the corresponding alcohols. We developed a method for the screening of P450 reduction enzyme activity involved in aldehyde reduction by using a fluorescence assay for measurement of reduction of 9-AA to 9-A-MeOH. Differential extraction with ethyl acetate allowed separation of the carboxylic acids and the alcohols in the two different organic

phases (acidic and alkaline, respectively). Human recombinant human P450s 2B6, 3A4, and 2J2, murine P450 2c29, and mouse liver microsomes were able to reduce 9-AA to 9-AMeOH. The order of 4-HNE reduction by the individual P450s analyzed was (human) P450 2B6 $\cong$ 3A4 > 1A2 > 2J2 > (mouse) P450 2C29/2c29. The observation of reduction of 4-HNE with the different P450 enzymes in mouse liver microsomes was further confirmed by using P450-selective inhibitors, e.g. miconazole (general P450 inhibitor), troleandomycin (P450 Subfamily 3A-selective inhibitor), phenytoin (P450 2c29-selective inhibitor), and α -naphthoflavone (CYP1A-selective inhibitor).

Reductive catalysis by P450s is not a common reaction, except for substrates like azo, nitro, and quinone containing compounds where the reduction reaction is not easily observed in ambient air and often requires low oxygen tension to measure the reactions. These reduced products are apparently not stable as 1-electron- or 2-electron-reduced intermediates in the presence of molecular oxygen³⁴. Azo dyes are widely used in cosmetics, food, textiles, and drugs and several (e.g., sulfonazo III and aramanth) are reduced by rat hepatic microsomes³⁵. The hepatocarcinogen *N,N*-dimethylamino-azobenzene is reduced by rat liver microsomes in an oxygen- and CO-insensitive manner³⁶. Levine³⁷ has described these oxygen- and CO-insensitive reactions, that the reduction of α,β -unsaturated aldehydes resembles. The bioreductive activation of 2,3,5,6-tetramethyl-1,4-benzoquinone is catalyzed by (rat) P450 2B1³⁸.

P450s require NADPH and oxygen for oxidative metabolism. Therefore in the absence of oxygen, P450-dependent monooxygenation reactions are inhibited. In addition to the use of chemical inhibitors, we were able to demonstrate that the lack of oxygen inhibited the P450-dependent oxidation of both 9-AA and 4-HNE but not the reduction of either compound. By using argon and CO to inhibit oxidative metabolism, we demonstrated the role of P450s in lipid aldehyde reductive metabolism is not altered in the presence of oxygen, suggesting a stable form of the reduced hemoprotein which does not rely upon formation of the perferryl-oxygen intermediate serve as the reducing species of the enzyme³⁹.

A proposed pathway for 4-HNE metabolism by P450s is shown in Figure 7. During the metabolism of substrate, P450s exist in different oxidation states. These oxidation states can influence the product generated by the P450. The oxidation state of the P450 that generates HNA is presumed to be the perferryl (Fe-O^{3+}) or possibly ferric peroxide (Fe-O_2^{-1}), requiring oxygen and electrons from NADPH: P450 oxidoreductase. Hydrogen is abstracted from 4-HNE forming a carbonyl carbon radical (or possibly the carbon radical of the hydrated gem-diol, e.g. see Guengerich *et al.*⁴⁰ that can then react with the iron-bound hydroxyl radical generating 4-hydroxy-2-nonenic acid. The P450-dependent reduction reactions are apparently catalyzed by the ferrous (Fe(II)) P450, with the electrons being transferred from NADPH to the P450 by NADPH-P450 reductase⁴¹. It has been noted that the P450 Fe(II) state can occur during conditions of low oxygen tension in certain tissues³⁹ allowing for facile reduction of compounds like azo dyes, unsaturated aldehydes, etc. This and the Fe(II)-CO P450 form may be the oxidation states of the P450 that generates DHN or 9-A-MeOH, suggesting that the site where substrate binds may not be affected by formation of the ferrous-CO complex.

In conclusion, our results demonstrate that several P450s are efficient catalysts in both the oxidative and reductive transformation of lipid-derived aldehydes to carboxylic acids and alcohols and adds a new facet to the biological activity of these metabolites. Our studies also suggest that P450-mediated metabolism operates in parallel with other metabolic transformations of aldehydes; hence, the P450s could serve as reserve or compensatory mechanisms when other high capacity pathways of aldehyde elimination are compromised due to disease or toxicity. For example, during myocardial infarction, the activity of

aldehyde dehydrogenase is inhibited due to the lack of NAD⁺. In addition, P450s expressed in the liver—e.g. 2B6, 3A4, 2J2—may play major roles in 4-HNE reduction. The role of P450 in vascular metabolism is unclear, in that the aldo-keto reductases and aldehyde dehydrogenases are expressed at relatively high levels relative to the P450s. Finally, because other unsaturated aldehydes, —e.g., acrolein, trans-2-hexenal, and crotonaldehyde—are also food constituents or environmental pollutants, P450s may be significant regulators of toxicity due to their possible roles in oxidation and reduction of xenobiotic aldehydes as well.

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Abbreviations

9-AA	9-anthracene aldehyde
9-ACA	9-anthracene carboxylic acid
9-A-MeOH	9-hydroxymethyl-anthracene
BHA	<i>t</i> -butylated hydroxyanisole
DHN	1,4-dihydroxy-2-nonene [(E)-non-2-ene-1,4-diol]
HNA	4-hydroxy-2-nonenic acid
4-HNE	4-hydroxy-2-nonenal

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Figure 1.

HPLC profile of 4-HNE products formed by recombinant P450 3A4. The metabolic assay was performed in triplicate with 2 mL of reaction mixture containing *E. coli*-expressed recombinant P450 3A4 (50 nM) and 50 μ M [3 H]-4-HNE at 37 $^{\circ}$ C for 20 min in a shaking water bath. The reaction was terminated by flash freezing in liquid nitrogen. The reaction mixture was thawed upon adding 1% CF₃CO₂H (w/v), and after centrifugation to remove particulate materials, the supernatant was injected into a C₁₈ column and measured with a radiometric detector. DHN eluted at t_R 56 min and HNA eluted at t_R 58 min. A. Reaction terminated at 0 minutes, B. Reaction terminated at 20 minutes.

Relative Abundance

Figure 2.

Mass spectrum of DHN generated from P450 3A4-dependent metabolism of [^3H]-4-HNE. After metabolism, the samples were analyzed by HPLC and fractions coeluting with a DHN standard at t_R 56 min were collected and subjected to mass spectral analysis. A. Authentic DHN, B. DHN produced by P450 3A4.

Figure 3.

Use of specific inhibitors of ALDH (cyanamide) and P450 (miconazole and troleandomycin) on 4-HNE metabolism in primary hepatocytes (Sprague-Dawley rats). Primary rat hepatocytes were prepared as described in Methods with a viability of >95% and 4-HNE was immediately added to the cell culture media. After 20 min, the reactions were quenched by adding 1% CF₃CO₂H (w/v) and frozen at -80 °C. The products were measured by HPLC and radioactivity determined using an in-line radiomatic detector. The effects of 0.5 mM of cyanamide (ALDH), miconazole (P450), and troleandomycin (P450 3A subfamily) on formation of oxidative and reductive metabolites of 4-HNE was determined. Four preparations of rat hepatocytes were used to obtain statistical significance: * $p \leq 0.05$; ** $p \leq 0.001$.

Figure 4.

Relative enzyme 9-AA oxidative and reductive metabolism of liver microsomal fractions from mice fed normal AIN76A diet or diet containing 0.5% BHA (w/w) for 7 days. Microsomal protein was isolated and the enzyme assays performed as described in Methods. Control liver microsomal fraction from untreated mice fed AIN76A diet (open bar) and liver microsomal fractions from mice fed AIN76A diet containing 0.5% BHA (hatched bar).

Figure 5.

Effects of P450 inhibitors on 9-AA transformation to oxidized and reduced products. Liver microsomal fractions from mice fed 0.5% BHA (w/w) in AIN76A diet were treated with either 0.5 mM miconazole, troleandomycin, phenytoin, or α -naphthoflavone. The control activity was 1.05 ± 0.1 nmol 9-A-MeOH produced per minute per mg microsomal protein.

Figure 6.

Effect of anaerobiosis or carbon monoxide on the oxidative and reductive metabolism of 9-AA. The reaction mixtures were purged with Ar or CO prior to initiating the reaction. Oxygen-depletion systems were included to achieve anaerobiosis as described in Methods. The control activity was 1.05 ± 0.1 nmol 9-A-MeOH produced per minute per mg microsomal protein.

Figure 7.
Scheme for oxidative and reductive transformation of α,β -unsaturated aldehydes, 4-hydroxy-2-nonenal, by P450s.

Table 1Catalytic activities of mouse and human P450s expressed in *E. coli*^a

P450 Form	4-HNE oxidation activity ^b	DHN formation
	(nmol/min/nmol P450)	(nmol/min/nmol P450)
(mouse) 2c29	0.96	0.30
(human) 1A2	<0.1	7.4
(human) 2B6	2.4	25
(human) 2E1	<0.1	<0.1
(human) 2J2	0.95	1.7
(human) 3A4	2.4	25
(rabbit) 2B4	0.36	<0.1
mouse liver microsomes	0.37	1.0

^aThe enzyme activities for *E. coli* expressed P450s and NADPH-cytochrome P450 oxidoreductase are expressed as turnover number and were the average of four assays (SD < 10% of mean). The activity for rabbit P450 2B4 was obtained upon reconstitution of purified hemeprotein with NADPH-cytochrome P450 oxidoreductase and dilaurylphosphatidyl choline. Mouse liver microsomal fractions were prepared from animals fed AIN-76A diet containing 0.5% BHA (w/w) for 7 days. The substrate concentration of 9-AA used was 25 μ M and of 4-HNE was 50 μ M.

^bResults from Amunom et al. (2007).