Oxidative Metabolism of 1-Nitropyrene by Rabbit Liver Microsomes and Purified Microsomal Cytochrome P-450 Isozymes

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ABSTRACT

Rabbit liver (male) microsomal metabolism of 10 μM [4,5,9,10-3H]-1-nitropyrene (1NP) was investigated. The total metabolism was not appreciably different with rates of 4.44 ± 0.45, 3.98 ± 0.19, 3.90 ± 0.16, and 3.75 ± 0.27 nmol/min/mg protein, respectively, for microsomes from phenobarbital, Aroclor-1254, ethanol-treated, and untreated rabbits. However, a more noticeable difference was found in the formation of specific metabolites. Phenobarbital treatment induced changes which favored 1-nitropyrene-3-ol formation, and Aroclor-1254 and ethanol-induced changes which favored 1-nitropyrene-6-ol and 1-nitropyrene-8-ol formation. 1NP was incubated with untreated microsomes and α-naphthoflavone, an inhibitor of rabbit cytochrome P-450 form 450 form 6 at low concentrations (<1 μM), and an activator of form 3c at high concentrations. 1NP had rates about 50- to 300-fold lower than form 3b. High performance liquid chromatography of metabolites while not affecting the total metabolism. Using purified isozymes of rabbit P-450, we found the constitutive form 3b metabolized 1NP at the highest rate with a catalytic activity of 26.8 nmol/min/mg P-450. Forms 2 and 6 exhibited rates of 2 and 2.2 nmol/min/mg P-450. Forms 3a, 3c, and 4 had rates about 50- to 300-fold lower than form 3b. High performance liquid chromatography was used to identify the metabolites when the incubations were carried out in the presence of purified rabbit epoxide hydrolase. With form 6, 54% of the metabolites were accounted for as 1-nitropyrene-3-ol, while with form 3b, 73% of the metabolites were 1-nitropyrene-6-ol and 1-nitropyrene-8-ol. The K-region dihydrodiols were formed by forms 2 and 3b, but not by forms 3c or 6. These results demonstrate that 1NP is a preferential substrate for form 3b, and that a preponderance of the metabolism with untreated rabbit liver microsomes can be attributed to this isozyme.

INTRODUCTION

The nitrated polycyclic aromatic hydrocarbons are a class of chemicals that are present in the environment as the result of incomplete combustion processes. The most abundant of the nitro-PAH are the nitro-PAH, such as 1-nitropyrene, which are activated through oxidation to phenols or diols followed by nitroreduction, or strictly through nitroreduction to the corresponding arylhydroxylamine (6, 7), other nitro-PAH, such as 1-nitrobenzo(a)pyrene, which are activated through aryl oxidation to the corresponding epoxides or dihydrodiol epoxides (8). Most of the mono nitro-PAH studied to date are detoxified through aryl oxidation to epoxides, phenols, dihydrodiols, triols, tetrols, and other products, and excreted as glutathione, sulfate, or glucuronide metabolites (1, 3, 4, 9–12). Therefore, with the nitro-PAH, microsomal-mediated oxidation plays a role both in metabolic activation and in detoxification.

Several studies have indicated that the changes which occur in the microsomal cytochrome P-450 population after pretreatment of animals with cytochrome P-450 inducers, result in changes in the metabolism of several of the nitro-PAH (7, 11–12). In this study, we focus on the oxidative metabolism of 1-nitropyrene with rabbit liver microsomes and purified isozymes of rabbit microsomal cytochrome P-450 in an effort to explain these changes.

MATERIALS AND METHODS

Chemicals. [4,5,9,10-3H]-Nitropyrene (1 Ci/mmol) was obtained from Chemsys, Inc., Lenexa, KS, and was purified to an isotopic purity of >98% by chromatography by using silica (100–200 mesh, Aldrich) with benzene as the eluent. Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane hydrochloride salt, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XII), and NADP were obtained from Sigma Chemical Co. All other chemicals were reagent grade or better.

Purified Liver Microsomal Enzymes. Hepatic microsomes were isolated from untreated adult New Zealand male rabbits, or from animals pretreated with 10% (v/v) ethanol for 2 weeks in the drinking water, Aroclor-1254 administered i.p. at 200 mg/kg in corn oil 3 days prior to sacrifice, or with 0.1% (v/v) PB (adjusted to pH 7) in the drinking water for 7 days prior to sacrifice. Rabbit liver microsomal cytochrome P-450 isoforms 2, 3a, 3b, 3c, 4, and 6 were purified to homogeneity as previously described (13–17). The specific contents ranged from 14 to 16 mmol P-450/mg protein. NADPH-cytochrome P-450 reductase was isolated from microsomes from PB-treated rabbits to a specific activity of 40 to 60 μmol cytochrome c-reduced/min/mg protein (17), and epoxide hydrolase was isolated as described by Koop and Coon (16). Protein concentrations were determined by the method of Lowry et al. (18).

Enzymatic Activity of Epoxide Hydrolase. Purified rabbit liver epoxide hydrolase (12 μg/ml) was incubated with a mixture of 5.3 μM 1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide [85:15 (19)] in 0.05 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane hydrochloride salt, pH 8, for 30 min at 37°C. The reaction was terminated by the addition of an equal volume of chloroform:methanol (2:1), and the metabolites were extracted into the chloroform. Following an additional extraction with chloroform, the extracts were combined, dried under N2, and dissolved in methanol. The 4,5- and 9,10-diols were separated from the respective epoxides by HPLC, and enzyme activity was quantitated by both the disappearance of the oxides and by appearance of the dihydrodiols.

Metabolism of 1-Nitropyrene. The metabolism of the [3H]-1-nitropyrene by purified cytochrome P-450s was in 1 ml volume containing: 10 μM [3H]-nitropyrene, 0.05 M potassium phosphate, pH 7.4, 3 mM MgCl2, 1 mM NADP, 2 mM glucose-6-phosphate, 0.1 unit/ml glucose-6-phosphate dehydrogenase, and 0.1 μM P-450, 0.1 μM P-450 reductase, and 30 μg/ml dilauroylglycerol-3-phosphorylcholine. The P-450 and reductase were prepared by mixing concentrated aliquots (10–20 μM) and after at least 5 min on ice, the mixture was diluted with the sonicated lipids. The reactions were started by the addition of the enzymes. Following incubation at 37°C, the reactions were terminated by the addition of an equal volume of chloroform:methanol (2:1, v/v). After centrifugation at 700 × g, the aqueous layer was again extracted with an equal volume of chloroform, and the organic extracts were
combined. The reactions were linear with protein, and were linear with time except where shown.

The metabolism of [3H]-1-nitropyrene by rabbit liver microsomes was essentially as described above except that the protein concentration was 0.1 mg/ml, the total volume was 2 ml in a 25-ml flask, and the incubations were for 15 min at 37°C. The reactions were stopped by addition to an equal volume of chloroform:methanol (2:1). After an additional chloroform extraction, the extracts were combined and stored at −20°C.

Analysis of the Metabolites. The metabolites were separated by injection of 0.05 μCi onto a Varian 5000 or 5500 series HPLC, and eluted with a gradient of 46% water, 27% methanol, 27% acetonitrile to 30% water, 35% methanol, 35% acetonitrile in 36 min, followed by a change to 50% methanol, 50% acetonitrile in 2 min on a Waters C18-Bondapak column (0.39 x 30 cm) with a flow rate of 2 ml/min. Fractions of 0.5 min were collected and the 3H was quantitated by scintillation counting, using Scintiverse-LC (Fisher) as the scintillant, and corrected to dpm, using a Packard 460 liquid scintillation spectrometer. Nonradiolabeled standards were coinjected and monitored at 254 nm by using a Varian UV-200 spectrometer or ISCO UA-5 absorbance monitor (ISCO, Inc.), with a 10-μl flow cell to ensure integrity of the separation (see Fig. 1). The HPLC separation and quantitation of the in vitro and in vivo metabolites of 1-nitropyrene have been previously described (12, 20). Although the HPLC program allowed for base-line separation of the metabolites, 1-nitropyrene-6-ol and 1-nitropyrene-8-ol comigrated under the HPLC conditions and were not further separated by thin layer chromatography as previously described (12, 20).

RESULTS

Microsomes from untreated rabbits efficiently catalyze the oxidation of 1-nitropyrene. The rate was at least 2 times that reported for microsomes from rats, guinea pigs, hamsters, and mice (3). HPLC analysis of the metabolites revealed that 6- and 8-ol were the major metabolites (Table 1). Formed in lesser amounts were 3-ol, 4,5-diol, 9,10-diol, and the K-region oxides and their rearrangement products. Under the conditions used, the reduced metabolites of 1-nitropyrene were detected only when the incubations were hypoxic (N2 atmosphere). Pretreatment of the rabbits with ethanol, Aroclor-1254, and phenobarbital had little effect on the overall rate of 1-nitropyrene metabolism with liver microsomes. However, there were significant changes in the formation of specific metabolites. With microsomes from untreated rabbits, the predominant metabolites were the 6- and 8-ol. The ratio of the formation of the 6- and 8-ol to the 3-ol was 3.3. In the incubations with microsomes from ethanol- or Aroclor-1254-treated rabbits, this ratio increased to 4.5 and 5.4, respectively. With microsomes from the ethanol-treated rabbits, this was due to a 23% increase in the formation of the 6- and 8-ol, while with microsomes from the Aroclor-1254-treated animals the increase in the ratio was primarily due to a 50% loss in the formation of the 3-ol. In contrast, PB treatment resulted in a significant decrease in the 6- and 8-ol to 3-ol ratio to 2.1. This was due to a significant increase (154%) in the formation of the 3-ol with no change in the rate of formation of the 6- and 8-ol. This ratio (6- and 8-ol/3-ol) has been previously described as an indicator of alterations in liver microsomal metabolism of 1-nitropyrene (20), and will be used below as an indicator of alterations of the roles of the P-450 isozymes in the metabolism of 1-nitropyrene.

Previous studies with microsomes from other species suggested a major role for the 3-MC-inducible rat isozyme, P-450c, in the metabolism of 1-nitropyrene (7). The participation of the homologous rabbit isozyme form 6 in the metabolism of 1-nitropyrene was investigated by using αNF. This compound exhibits a low $K_I$ for rabbit form 6, and at higher concentrations inhibits isozyme 3b while stimulating the catalytic activity of form 3c (21–23). The effect of αNF on total metabolism of 1-nitropyrene, the distribution of metabolites, and the 6- and 8-ol/3-ol ratio is shown in Fig. 2. While 0.1 μM αNF resulted in a slight stimulation of total metabolism, higher concentrations (1–100 μM) did not significantly affect the overall rate of metabolism, but did affect the distribution of products (Fig. 2b). The increase in the formation of 3-ol and 9,10-diol was accompanied by a concomitant decrease in the formation of 6- and 8-ol, and no change in 4,5-diol formation. This is reflected in a decrease in the 6- and 8-ol/3-ol ratio (Fig. 2c).

Six rabbit hepatic isozymes were reconstituted with purified NADPH-cytochrome P-450 reductase in the presence of lipid, and examined for 1-nitropyrene hydroxylase activity. Isozyme 3b demonstrated the highest rate, 26.8 nmol/min/nmol P-450 (Table 2), and exhibited linearity for approximately 5 min under the assay conditions used (Fig. 3). Forms 2 and 6 demonstrated activities approximately 10-fold lower than isozyme 3b, 2 and 2.2 nmol/min/nmol P-450, respectively (Table 2). Isozymes 3a, 3c, and 4 had catalytic rates which were 60- to 300-fold lower than that exhibited by isozyme 3b.<br>

In order to assess the distribution of metabolites and to compare with the distribution produced by hepatic microsomes, four of the isozymes (2, 3b, 3c, 6) were reconstituted in the presence of purified epoxide hydrolase (Table 3). This concentration of epoxide hydrolase efficiently catalyzed the hydration of 5.3 μM 1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-epoxide at pH 8 in 30 min (results not shown). Incubations with the P-450 and epoxide hydrolase were run for 30 min to ensure optimal reaction of the epoxide hydrolase with the generated epoxides. In the presence of epoxide hydrolase, form 2 catalyzed the formation of the 4,5-diol, and 6- and 8-ol metabolites of 1-nitropyrene, each constituting approximately

Fig. 1. High performance liquid chromatographic separation of the metabolites of [3H]-1-nitropyrene. Approximately 0.05 μCi of metabolites and substrate from the extractions of the microsomal or isozyme incubations were injected onto the HPLC, and identified by chromatography with nonradiolabeled standards (12, 20, 38, 39). The following peaks were indicated: A, 1-nitropyrene-9,10-dihydro-9,10-diol; B, 1-nitropyrene-4,5-dihydro-4,5-diol; C, K-region oxidized derivatives of [3H]-nitropyrene (19, 34); D, 1-nitropyrene-4,5- and -9,10-oxide; E, mixture of 1-nitropyrene-6-ol and 1-nitropyrene-8-ol; F, 1-nitropyrene-3-ol; G, 1-nitropyrene.

4 Unpublished data.
P-450 METABOLISM OF 1-NITROPYRENE

Table 1 Rabbit liver microsomal metabolism of 1-nitropyrene

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>4,5-diol</th>
<th>Oxidizeda</th>
<th>6- and 8-olb</th>
<th>3-ol</th>
<th>6- and 8-ol/3-ol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3.75 ± 0.27</td>
<td>0.65 ± 0.11</td>
<td>0.30 ± 0.13</td>
<td>1.83 ± 0.05</td>
<td>0.54 ± 0.08</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.90 ± 0.16</td>
<td>0.39 ± 0.04</td>
<td>0.29 ± 0.05</td>
<td>2.25 ± 0.18</td>
<td>0.50 ± 0.04</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>Aroclor-1254</td>
<td>3.98 ± 0.19</td>
<td>0.55 ± 0.05</td>
<td>1.12 ± 0.13</td>
<td>1.51 ± 0.04</td>
<td>0.28 ± 0.02</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>4.44 ± 0.45</td>
<td>1.00 ± 0.16</td>
<td>0.58 ± 0.04</td>
<td>1.78 ± 0.22</td>
<td>0.83 ± 0.10</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

* The 4,5- and 9,10-K-region epoxides (19) and lactone rearrangements of the epoxides (37).
* Mixture of 1-nitropyren-6-ol and l-nitropyren-8-ol.
* Mean ± SD.

Table 2 Metabolism of [3H]l-nitropyrene by purified rabbit liver microsomal cytochrome P-450 isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>9,10-diol</th>
<th>4,5-diol</th>
<th>Oxidesa</th>
<th>6- and 8-olb</th>
<th>3-ol</th>
<th>6- and 8-ol/3-ol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form 2</td>
<td>1.48</td>
<td>8.01</td>
<td>1.60</td>
<td>7.86</td>
<td>4.52</td>
<td></td>
</tr>
<tr>
<td>Form 3b</td>
<td>2.61</td>
<td>1.59</td>
<td>7.99</td>
<td>51.37</td>
<td>7.63</td>
<td></td>
</tr>
<tr>
<td>Form 3c</td>
<td>0</td>
<td>1.03</td>
<td>0.91</td>
<td>4.65</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>Form 4</td>
<td>0</td>
<td>0.09</td>
<td>0.83</td>
<td>4.36</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>Form 6</td>
<td>0</td>
<td>0</td>
<td>0.91</td>
<td>14.94</td>
<td>1.96</td>
<td></td>
</tr>
</tbody>
</table>

* Combination of 1-nitropyren-6-ol and 1-nitropyren-8-ol.

DISCUSSION

The metabolism of 1-nitropyrene had been extensively studied by using hepatic S-9 and microsomal preparations from a variety of species, especially the rat. The results of induction studies suggest that rat P-450b and P-450c may be involved in the metabolism of 1-nitropyrene (7, 12). While the role of specific isozymes in the metabolism of some polycyclic aromatic hydrocarbons has been determined, no studies have demonstrated the participation of specific P-450 isozymes in the oxidative metabolism of 1-nitropyrene, although a role for P-450 and the reductase was implicated in the nitrerducive metabolism (24).

Studies over the past several years have established the presence of homologous isozymes of P-450 between species (25, 26). For example rabbit isozyme 6 has 72% sequence homology to rat P-450c in the P-4501A1 subfamily, while isozyme 2 is 77% identical to rat P-450b in the P-4501B1B subfamily (26). The catalytic preference of the homologous isozymes is also maintained. For example, P-450c and form 6 both effectively metabolize benzo(a)pyrene, P-450b and isozyme 2 catalyze benzphetamine N-demethylation, and P-450j and isozyme 3a catalyze aniline hydroxylation (27-32). However, the metab-
Catalyze the reaction.

We found that rabbit isozyme 3b exhibited the greatest activity for 1-nitropyrene oxidation, followed by forms 2 and 6, with form 4 exhibiting almost negligible activity. A homologous isozyme to rabbit 3b has not been reported in other species, although human form 1 and human P-450mp exhibit 65% and 68% sequence homology (26). The specific activity for form 3b toward 1-nitropyrene is high relative to the oxidation of other aromatic compounds and is nearly the same as the N-demethylation of aminopyrine at 28.4 nmol/min/nmol P-450 (30). Form 3b catalyzes the 7-hydroxylation of 2-acetylaminofluorene at a rate of about 1 nmol/min/nmol P-450 (33), and oxidizes cyclopentapyrene and benzo(a)pyrene with rates of 1.4 and 0.3 nmol/min/nmol P-450, respectively. Micsromes from untreated rabbits have a specific P-450 content of approximately 2 nmol/mg protein. If form 3b represents about 10% of the total isozyme as suggested from preliminary immunoblot and electrophoretic studies, and if form 3b is the only catalyst and 100% functional in the microsomes, one would expect a rate of about 19 nmol/min/nmol P-450 for purified form 3b. This value is approximately 70% of that observed with the purified isozyme, and may reflect a limitation of the reductase activity as recently reported by Domin and Philpot for isozyme 6 in the lung (23). The high activity of form 3b and the 10-fold lower activity of forms 2 and 6 is consistent with the lack of a change in the overall rate of 1-nitropyrene metabolism when comparing microsomes from untreated, Aroclor-1254-, ethanoll-, and PB-treated rabbits (Table 1). Even though form 2 is induced about 5-fold by PB (34), if the concentration of form 3b remains unchanged, the overall rate of 1-nitropyrene metabolism would not be significantly altered. However, the participation of form 2 in the microsomal metabolism is indicated by the decrease in the 6- and 8-ol/3-ol ratio, and the increase in the formation of 4,5-diol (Table 1). Unlike P-450c in the rat, form 6 is not extensively induced in adult rabbit liver by Aroclor-1254. Serabjit-Singh et al. (35) reported that Aroclor-1260 induced form 5 in the liver. However, a primary role for the participation of this isozyme in the C-oxidative metabolism of 1-nitropyrene is not probable since neither PB nor Aroclor administration to the animals significantly induced the total metabolic activity of the microsomes. Immunoblot experiments with anti-form 6 antibody, indicate that isozyme 6 was induced about 3.5-fold (results not shown). Again, the increase in the ratio of the 6- and 8-ol/3-ol is consistent with the participation of form 6.

Pretreatment of rats with either PB or 3-MC results in the induction of primarily rat isozymes P-450b and P-450c, respectively (36). Isozyme P-450b and P-450c are homologous to rabbit forms 2 and 6, respectively. In the studies of Howard et al. (12) and Djuric et al. (7), liver microsomes from rats pretreated with PB resulted in increases in the formation of the 4,5-diol, and a lowering of the 6- and 8-ol/3-ol ratio through increases in the formation of the 3-ol. Alternatively, with 3-MC pretreatment, the formation of 4,5-diol, 9,10-diol, and 6- and 8-ol were increased, resulting in an increase in the 6- and 8-ol/3-ol ratio. These observations complement the results described herein where PB treatment results in a lower ratio as a function of the lower 6- and 8-ol/3-ol ratio for form 2 when compared to forms 3b and 6. Similarly, the results from the 3-MC-induced rats would suggest that rat P-450c has a high 6- and 8-ol/3-ol ratio similar to its homologue, form 6.

1-Nitropyrene and other nitro-PAH are metabolically activated by nitroreduction to aryldihydroxylamines. However, in the case of 1- and 3-nitrobenzo(a)pyrene, activation is through aryl oxidation to epoxides or vicinal dihydrodiol epoxides (1, 8). With 1- and 3-nitrobenzo(a)pyrene this activation occurs at the C-7-C-10 carbons, which are distal to the nitro group. Considering that the 6- and 8-ol formation is distal to the nitro group on 1-nitropyrene and the 3-ol is proximal, isozymes 3b and 6 would be candidates for the preferential activation of 1- and 3-nitrobenzo(a)pyrene. In support of this, Domin and Philpot (23) demonstrated an increase in benzo(a)pyrene C-7-C-10 oxidation with whole lung microsomes or distinct cell types from 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced rabbits, which are elevated in form 6.

With most polycyclic aromatic hydrocarbons the formation of a phenolic metabolite results in detoxification through the glucuronide or sulfate conjugation pathways. However, with 1-nitropyrene, while 1-nitropyren-6-ol and 1-nitropyren-8-ol are not mutagenic in the Salmonella typhimurium histidine reversion assay, 1-nitropyren-3-ol is mutagenic (10). Thus, the formation of specific metabolites of 1-nitropyrene and their role in the tumorigenicity of this compound needs to be investigated.

Isozyme 3b in the rabbit is of critical importance in the metabolism of 1-nitropyrene. A homologous isozyme in other species may be important in nitro-PAH oxidation and could play a critical role in the balance between detoxification and activation of nitro-PAH.

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