Identification of the cytochrome P450 isozymes involved in the metabolism of N-nitrosodipropyl-, N-nitrosodibutyl- and N-nitroso-n-butyl-n-propylamine

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The metabolism of N-nitrosodipropylamine (NDPA), N-nitrosodibutylamine (NDBA) and N-nitroso-n-butyl-n-propylamine (NBPA) was investigated in vitro using liver microsomes and purified isoforms of cytochrome P450 in a reconstituted system. Liver microsomes were prepared from rats pretreated with phenobarbital (PB), pyridine (PYR), β-naphthoflavone (BNF), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), clofibrate (CLO) or from untreated rats. The purified cytochrome P450s used in the reconstituted system were rat 1A1 and 2B1 and rabbit 2E1. The rates of metabolism and the product profiles for NDPA, NDBA and NBPA changed significantly depending on the pretreatment of the rats or the identity of the purified cytochrome P450 isoforms. Induction by PB dramatically increased cleavage of NDPA, NDBA and NBPA at C-N bonds, leading to substantial increases in formation of the respective aldehydes and the overall metabolic rates. Microsomes from PYR-pretreated rats exhibited increased activities for formation of formaldehyde and propionaldehyde from NDPA and NBPA. Microsomes from BHT-pretreated rats showed a slight increase in activity for N-dealkylation of NDPA and NDBA. Treatment with BHA decreased the overall metabolism of NDBA, but slightly increased N-dealkylation of NBPA. Microsomal metabolism of NDPA, NDBA and NBPA was decreased by pretreatment with BNF and CLO. Results from studies using the reconstituted system with purified cytochrome P450 isoforms demonstrated that cytochrome P450 2B1 specifically catalyzed α-hydroxylation of these three long chain nitrosamines with high activity. Cytochrome P450 2E1 catalyzed formation of formaldehyde and propionaldehyde from NDPA and NBPA, but did not catalyze formation of acetaldehyde or butyraldehyde. Cytochrome P450 1A1 exhibited no activity for metabolism of NDPA, NDBA and NBPA. The contributions of cytochrome P450 2B1 and 2E1 to N-dealkylation reactions were determined using inhibitory monoclonal antibodies (mAb). With microsomes from PB-pretreated rats, inhibition by mAb-2B1 indicated a 62% contribution by cytochrome P450 2B1 to debutylation of NDBA and 65% to depropylation of NDPA. In microsomes from PYR-pretreated rats inhibition by mAbs also showed a role for cytochrome P450 2E1 in depropylation of NDPA. These studies provide a better understanding of the role of various forms of cytochrome P450 in metabolic activation of these long chain N-nitrosodialkylamines to potentially toxic, mutagenic and carcinoenic intermediates.

Introduction
N-nitrosodipropylamine (NDPA*), found in herbicides and colored alcoholic beverages (1,2), and N-nitrosodibutylamine (NDBA), a contaminant in industrial rubber products and rubber toys (3), are among some of the most potent carcinogens and mutagens tested in experimental animals (4,5). Although NDBA is thought to be a relatively selective bladder carcinogen in rats (6) and NDPA induces esophageal tumors in rats and pancreatic cancer in hamsters (7), the liver is a common target tissue for carcinogenesis by both NDPA and NDBA in rats (8–10). N-nitroso-n-butyl-n-propylamine (NBPA) is a synthetic asymmetric nitrosodialkylamine and its carcinogenic activity and organotropic effects have not yet been investigated. Since N-nitrosodialkylamines are 'indirect acting' carcinogens, bioactivation is considered to be essential for their genotoxic actions (11). One widely accepted pathway (11) for metabolic activation of short chain N-nitrosodialkylamines such as N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) involves hydroxylation at the α-carbon atom followed by spontaneous cleavage of the C-N bond of the α-hydroxynitrosamine to release an aldehyde and generate an alkylating agent, which can then react with DNA or protein to form a variety of DNA adducts and alkylated proteins (12–14). However, the metabolic routes for biotransformation of long chain nitrosodialkylamines such as NDPA or NDBA to genotoxic or cytotoxic derivatives and the identity of the enzymes involved remain unclear.

Investigations of the metabolism of NDPA and NDBA have suggested that, in addition to α-carbon hydroxylation, β-, γ- and ω-hydroxylation pathways may also be involved in their metabolic activation or detoxification (15,16). In relation to the enzymatic nature of metabolic activation of these nitrosamines, Janzowski et al. (17) have demonstrated that ATP-dependent mitochondrial enzymes involved in fatty acid degradation are responsible for the metabolic transformation of ω-carboxyaldehyde- and ω-carboxyalkyl-nitrosamines to the β-oxopropyl metabolites. These 2-oxopropyl derivatives are good substrates for cytochrome P450-dependent mixed function oxidases (17). It has also been demonstrated that cytosolic enzymes such as alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are involved in the further metabolism of ω-hydroxy derivatives of NDBA to give the ω-carboxylated products (18). Although there is good evidence to indicate that cytochrome P450 may play a more important role in bioactivation of long chain nitrosodialkylamines (8,9–21), the substrate specificity, carbon cleavage selectivity and catalytic efficiency of the individual cytochrome P450 isoforms in metabolizing NDPA, NDBA and NBPA are still not clearly understood.

*Abbreviations: NDPA, N-nitrosodipropylamine; NDBA, N-nitrosodibutylamine; NBPA, N-nitroso-n-butyl-n-propylamine; NDMA, N-nitrosodimethylamine; NDEA, N-nitrosodiethylamine; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; mAb, monoclonal antibody; pAb, polyclonal antibody; DNPH, 2,4-dinitrophenyldiazidine; PB, phenobarbital; PYR, pyridine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BNF, β-naphthoflavone; CLO, clofibrate; DLPC, dilauroylphosphatidylcholine.

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Several different isoforms of cytochrome P450 may catalyze metabolism of a long chain nitrosodialkylamine, but selectively catalyze reactions at different positions on the carbon chain (20,22). Since selective carbon hydroxylation may be dependent on a specific cytochrome P450 isoform, induction of a particular form of cytochrome P450 may lead to profound biological consequences, because oxidation at different carbon positions on a given nitrosodialkylamine can alter its mutagenic potency or carcinogenic organotropy (23). For NBDA ω-oxidation of one butyl chain of NBDA was reported to be a prerequisite for induction of urinary bladder cancer in the rat (24). Interestingly, when NBDA was oxidized only at the α-carbon position it became a potent liver carcinogen in the rat (24). NBDA was also shown to be a powerful liver carcinogen in the rat when activated via α-hydroxylation. However, NBDA was non-carcinogenic when all of the α-carbon hydrogens were substituted by methyl groups (25,26). Variations in the cytochrome P450 composition of different tissues may result in metabolism of these nitrosamines via variant pathways in different tissues. Therefore, it is important to identify the specific cytochrome P450 isoforms involved in metabolism of NDPA, NBDA and NBPA in the target tissues in order to gain a better understanding of the possible bioactivation mechanisms of these carcinogens in target tissues.

In the present study the roles of several isozymes of cytochrome P450 in metabolism of NDPA, NBDA and NBPA were examined in detail. The activities of selected isoforms of cytochrome P450 were induced in rat liver by pretreatment of rats with various inducing agents. The formation of metabolic products from NDPA, NBDA and NBPA was analyzed by HPLC. The specific isoforms of cytochrome P450 responsible for catalyzing dealkylation of these three long chain nitrosamines were characterized using the liver microsomal system and a reconstituted system. The contributions of individual isoforms of cytochrome P450 to specific dealkylation activities in rat liver microsomes were also determined by using inhibitory monoclonal (mAb) or polyclonal antibodies (pAb). The substrate specificity and carbon cleavage selectivity of these cytochrome P450 isoforms in the metabolism of long chain nitrosamines were also investigated.

Materials and methods

Chemicals

NDPA (99% pure), NBDA (99% pure), NBPA (99% pure), NADP⁺ (Tris salt), glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (Type IX) and semicarbazide hydrochloride were obtained from Sigma Chemical Co. (St Louis, MO). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade from commercial sources.

Preparation of liver microsomes

Male Fischer 344 rats (90–100 g) were purchased from Harlan Sprague Dawley Co. (Indianapolis, IN) and divided into seven groups containing six rats each. Treated groups received phenobarbital (PB), pyridine (PYR), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), β-naphthoflavone (BNF) or clofibrate (CLO). Rats in the control group did not receive any treatment. The PB-pretreated rats were given 0.1% PB in their drinking water for 10 days; PYR was administered i.p. at a daily dose of 200 mg/kg body wt for 3 days; the CLO-pretreated group was administered a Purina laboratory diet containing 0.3% CLO for 10 days; the BHA- and BHT-pretreated groups were administered an AIN-76-A diet containing either 0.45% BHA or BHT for 7 days following a 7 day regimen on an AIN-76-A diet alone; the BNF-pretreated group was administered BNF (80 mg/kg) in corn oil i.p. three times 24 h apart. The rats were fasted overnight before killing. Microsomes were prepared from liver homogenates by differential centrifugation and washed once with a solution containing 100 mM tetrasodium pyrophosphate, pH 7.4, 1 mM EDTA (27). The washed microsomal pellets were resuspended in buffer containing 100 mM potassium phosphate, pH 7.4, 20% glycerol, 1 mM EDTA. Microsomal protein concentrations were determined using the method of Lowry et al. (28) with serum albumin as the standard. The cytochrome P450 content was determined spectrophotometrically as described by Omura and Sato (29). The microsomes were stored frozen in small aliquots at −80°C prior to use.

Dealkylation assays using the liver microsomal enzyme system

The metabolism of NDPA, NBDA and NBPA by the different liver microsomal preparations was determined by measuring the formation of formaldehyde (demethylation), acetaldehyde (deacetylation), propionaldehyde (depropylation) or butyraldehyde (debutylation) using the HPLC method of Farrelly et al. (30), as modified by Yoo et al. (31). Since inhibition was observed when NDPA was dissolved in ethanol, a substrate for some cytochrome P450s, NDPA and NBPA were dissolved in 100% acetone, which is not a substrate for cytochrome P450s and has no effect on the activity of individual cytochrome P450 isoforms. NDPA was dissolved in 50% acetone/50% H2O. The acetonitrile concentration in all reaction mixtures was 0.05%, under which no inhibition was observed. Various concentrations of NDPA, NBDA or NBPA were preincubated with liver microsomes (0.5 mg protein) in 50 mM Tris buffer, pH 7.4, containing 150 mM KC1 and 10 mM MgCl2 for 3 min in 16×100 mm culture tubes sealed with air-tight rubber stoppers to prevent evaporation of the volatile aldehyde products. The reactions were initiated by addition of a NADPH-generating mixture containing 3.8 mM glucose-6-phosphate dehydrogenase (G6PD) and 0.4 mM NADP⁺ (Tris salt, adjusted to pH 7.4 prior to use). For control reactions either the NADPH-generating system or the nitrosamine was omitted. Under these conditions product formation for all reactions was linear with time for at least 40 min. After incubation for 20 min at 37°C the reactions were quenched by addition of 0.1 ml of a 1:1 mixture of 50% acetic acid and 0.1 M semicarbazide, which trapped the aldehydes as their semicarbazone derivatives. The test tubes were mixed rapidly by vortexing and chilled on ice for 20 min. Saturated barium hydroxide solution (0.1 ml) was added and the proteins precipitated by centrifugation. The supernatant (0.4 ml) was extracted using a two phase medium containing 1.0 ml water and 0.1 ml 2,4-dinitrophenylhydrazine (DNPH) solution (0.25% in N HCl) plus 1.5 ml hexane and mixed for 1 h to convert the carbonyl compounds to 2,4-dinitrophenylhydrazine derivatives (32). The hexane layer was removed and 1.0 ml was mixed with 0.35 ml acetonitrile by vortexing. The acetonitrile was removed and 0.05 ml of the acetonitrile layer was analyzed on a Vanpak model 5020 HPLC. The metabolites were separated on a Rad-Pak C18 reverse phase (10 μm) column filled in a Waters RCM-100 Module and eluted isocratically with 65:35% acetonitrile/water. The peaks corresponding to the DNPH derivatives of the various aldehydes were monitored using a Model 116 Gilson UV detector at a wavelength of 340 nm (31). For the standards known quantities of aldehydes were run in parallel to the sample assays. Butyraldehyde was added as an internal standard for the NDPA assay and valeraldehyde was added as the internal standard for the NBDA and NBPA assays. All assays were run in duplicate and the differences between the duplicates were usually <5%.

Metabolism studies using purified forms of cytochrome P450 in the reconstituted system

Purified cytochrome P450 2B1 (13.3 nmol/mg protein), IA1 (9.3 nmol/mg protein) and NADPH-cytochrome P450 reductase (11.1 nmol/mg protein) were prepared from rat liver microsomes pretreated with PB or BNF using standard procedures (33–35). Rabbit cytochrome P450 2E1 (17.0 nmol/mg protein) was obtained from Dr Minor J.Coon as a gift. Purified cytochrome P450 450 (0.1 nmol), reductase (0.2 nmol) and dilauroylphosphatidylcholine (DLPC) (25 μg, sonicated briefly prior to use) were reconstituted at 4°C for at least 30 min prior to initiating reactions. The incubation mixtures contained the reconstituted proteins, 50 mM Tris buffer, pH 7.4, and substrate (NDPA, NBDA or NBPA) at the concentration indicated in a total volume of 1.0 ml. The reactions were initiated by addition of the NADPH-generating system. After incubation for 20 min at 37°C, the reactions were terminated by addition of 0.1 ml of a saturated solution containing 50% zinc sulfate and 0.1 M semicarbazide (1:1). The samples were analyzed for aldehyde formation as previously described for microsomal metabolism.

Immunoinhibition study

Monoclonal antibodies (mAb) I-91-3 (mAb-2E I) and 2-66-3 (mAb-2B I/2) were generous gifts from Dr Harry Gelboin (National Institute of Health, Bethesda, MD). Rabbit anti- rat 3AI/2 pAb (pAb-3AI/2) was a generous gift from Dr Frederick P.Ouwerkerk (Vanderbilt University, Nashville, TN). For analysis of antibody inhibition of enzyme activity of cytochrome P450 the monoclonal antibodies were added to reaction mixtures using a ratio of 5:1 ascites fluid protein to microbial protein. It was reported that this ratio produced maximal inhibition of the specific cytochrome P450 activities (36). The reaction mixtures containing microbial protein and mAb or pAb were kept at room temperature for 15 min before initiation of the reaction. The reactions were started by addition of the NADPH-generating system, as described above, in a total volume of 1
Cytochrome P450-dependent nitrosamine metabolism

Fig. 1. HPLC separation of 2,4-dinitrophenylhydrazone derivatives of NBPA and NDBA metabolites. The reaction mixtures contained microsomes from PYR-pretreated rats (0.5 mg protein) and 2 mM NBPA in the absence (a) or presence (b) of a NADPH-regenerating system or 2 mM NDBA and microsomes (0.5 mg protein) from either control (c) or PB-pretreated (d) rats in the presence of the NADPH-regenerating system. Incubations, sample treatments and HPLC separations were performed as described in Materials and methods. The peaks and elution times are: formaldehyde, 8.90 min; acetaldehyde, 11.05 min; propionaldehyde, 13.70 min; butyraldehyde, 17.60 min. Valeraldehyde (23-30 min) was added as the internal standard. The peak eluting at 6.58 min is DNPH.

The incubations and HPLC separations of metabolites were as for the dealkylation assays using the microsomal system.

Results

Metabolism of NDPA, NDBA and NBPA by rat liver microsomes

HPLC analysis of reaction mixtures containing NDPA, NDBA or NBPA demonstrated that these long chain nitrosodialkylamines were metabolized by rat liver microsomes. Figure 1 shows representative chromatograms for separation of the 2,4-dinitrophenylhydrazone derivatives of the aldehyde metabolites formed as a result of a 20 min incubation of NDPA, NDBA or NBPA with rat liver microsomes. Under the experimental conditions used for these studies aldehyde metabolites were not detected when the NADPH-generating system was omitted from the reaction mixtures, except for a small background amount of formaldehyde which was observed when microsomes prepared from PYR-pretreated rats were used in the incubations (Figure 1a). The background formaldehyde was generated from glycerol, which was used to stabilize the protein against denaturation or conformational changes, by cytochrome P450 (37). An additional control in which substrate was omitted in the presence of the NADPH-generating system also gave no detectable aldehyde products, except when NADP⁺ (monosodium salt) was used in the NADPH-generating system. In that case a large amount of acetone, a contaminant from the NADP⁺, was observed in the elution pattern, with an elution time close to that of propionaldehyde (data not shown). Therefore, the Tris salt of NADP⁺, which is devoid of acetone, was used for these studies. With the complete microsomal incubation system NBPA was metabolized by microsomes from PYR-pretreated rats to give formaldehyde, propionaldehyde and butyraldehyde (Figure 1b). Incubation of NDBA with hepatic microsomes from untreated rats gave acetaldehyde and butyraldehyde in very low yields (Figure 1c). However, with microsomes from PB-pretreated rats NDBA metabolism was greatly increased (Figure 1d).

Effects of inducers of cytochrome P450 on metabolism of NDPA, NDBA and NBPA

Metabolism of these three nitrosamine carcinogens by liver microsomes from male F344 rats pretreated with various inducers was investigated. The metabolic profiles for metabolism of NDPA (2 mM) by liver microsomes from rats pretreated with various inducers are shown in Figure 2. With liver microsomes from control rats the major metabolite of NDPA was propionaldehyde, with essentially no formaldehyde formation. Pretreatment of rats with PB or PYR substantially increased the rate of formation of propionaldehyde from NDPA, up to 6- and 4-fold respectively, but a similar enhancement was not observed with microsomes from BHT- or BHA-pretreated rats. Although microsomes from both PB- and PYR-pretreated rats catalyzed
formation of formaldehyde from NDBA, the catalytic activity of acetaldehyde formation with PYR induction was 3-fold higher than that observed with PB induction. However, even in this case formaldehyde was a minor product of the overall metabolism of NDPA. Compared with microsomes from control rats microsomes from BNF-, BHA-, BHT, and CLO-pretreated rats exhibited relatively little change in metabolism of NDPA.

The effects of pretreating rats with these inducers on NDPA metabolism are presented in Figure 3. The metabolism of NDPA by liver microsomes from control rats followed a pattern similar to that of NDPA, in that the product of \( \alpha \)-carbon hydroxylation (in this case butyraldehyde) was the predominant product formed, with relatively little formaldehyde formation. With microsomes from PB-pretreated rats the rate of dealkylation was ~6-fold higher than in control animals. A small amount of formaldehyde was detected only in microsomes from PB-pretreated rats. In addition, butyraldehyde formation by microsomes from PYR-pretreated rats occurred at a rate approximately three times higher than that with control microsomes. No significant difference was found between control microsomes and microsomes from CLO-pretreated rats in the overall level of dealkylation of NDPA. NDPA dealkylation was slightly increased by BHT pretreatment, but was slightly decreased by BHA pretreatment. Acetaldehyde was a minor metabolite produced from NDPA during metabolism by microsomes from untreated, PB- and CLO-pretreated rats.

As shown in Figure 4, induction with PB led to the formation of significant quantities of both propionaldehyde and butyraldehyde from NBPA, as well as a very small amount of formaldehyde. In addition to the depropylation and dealkylation activities, microsomes from PYR-pretreated rats also catalyzed formaldehyde formation from NBPA. Treatment of rats with BNF or CLO had no significant effect on metabolism of NBPA as compared with controls. Pretreatment with either BHT or BHA resulted in increased metabolism of NBPA via \( N \)-dealkylation to give both propionaldehyde and butyraldehyde. Under our experimental conditions acetaldehyde formation from NDPA or NBPA could not be detected.

### Table I. Apparent kinetic parameters for the demethylation of NDPA, NDBA and NBPA by microsomes from PB- and PYR-pretreated rats\(^a\)

| Inducer | NDPA | | | NDBA | | | NBPA |
|---------|------|-------------------------------|-----------------|------|-------------------------------|-------------------------------|
|         | \( K_m \) (mM) \( b \) | \( V_{\text{max}} \) | \( K_m \) (mM) | \( V_{\text{max}} \) | \( K_m \) (mM) | \( V_{\text{max}} \) |
| PB      | 0.77±0.05 | 0.62±0.04 | ND\(^c\) | 0.45±0.04 | 0.62±0.05 |
| PYR     | 0.25±0.04\(^d\) | 1.56±0.18\(^d\) | 0.24±0.02 | 0.65±0.07 | 1.52±0.08\(^d\) |

\(^a\)Animal treatments, incubation conditions and HPLC assays for the formaldehyde derivative are described in Materials and methods.

\(^b\)The values of \( K_m \) (mM) and \( V_{\text{max}} \) (nmol/min/mg protein) were derived from double reciprocal plots of aldehyde formation versus substrate concentration.

\(^c\)Metabolite not detectable.

\(^d\) \( n = 6 \), Significantly different from microsomes from PB-pretreated rats (\( P < 0.01 \) by Student’s \( t \) test).

### Table II. Apparent kinetic parameters for the depropylation of NDPA and NBPA by microsomes from rats pretreated with different inducers\(^a\)

<table>
<thead>
<tr>
<th>Inducer</th>
<th>NDPA</th>
<th></th>
<th></th>
<th>NBPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>( V_{\text{max}} )</td>
<td>( K_m ) (mM)</td>
<td>( V_{\text{max}} )</td>
</tr>
<tr>
<td>None</td>
<td>0.38±0.03</td>
<td>2.75±0.08</td>
<td>0.19±0.02</td>
<td>1.40±0.12</td>
</tr>
<tr>
<td>PB</td>
<td>0.37±0.02</td>
<td>19.1±3.15(^b)</td>
<td>0.49±0.01(^b)</td>
<td>9.71±2.08(^b)</td>
</tr>
<tr>
<td>PYR</td>
<td>0.30±0.01(^b)</td>
<td>13.8±2.7(^b)</td>
<td>0.32±0.02(^b)</td>
<td>6.43±1.76(^b)</td>
</tr>
<tr>
<td>BNF</td>
<td>0.28±0.05(^b)</td>
<td>1.98±0.29</td>
<td>0.32±0.02(^b)</td>
<td>3.15±0.35(^b)</td>
</tr>
<tr>
<td>BHT</td>
<td>0.23±0.01(^b)</td>
<td>3.23±0.25</td>
<td>0.19±0.03</td>
<td>2.94±0.11(^b)</td>
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<tr>
<td>BHA</td>
<td>0.20±0.02(^b)</td>
<td>2.63±0.19</td>
<td>0.32±0.03(^b)</td>
<td>2.28±0.24(^b)</td>
</tr>
<tr>
<td>CLO</td>
<td>0.29±0.02(^b)</td>
<td>1.75±0.08(^b)</td>
<td>0.29±0.02(^b)</td>
<td>1.48±0.14</td>
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</table>

\(^a\)The incubations and HPLC assays for the butyraldehyde derivatives are described in Materials and methods. Values represent the mean ± SD of three separate experiments performed in duplicate.

\(^b\)Significantly different from untreated microsomes (\( P < 0.01 \) by Student’s \( t \) test).

\(^c\)Significantly different from untreated microsomes (\( P < 0.05 \) by Student’s \( t \) test).

### Table III. Apparent kinetic parameters for the dealkylation of NDBA and NBPA by microsomes from PB- and PYR-pretreated rats\(^a\)

<table>
<thead>
<tr>
<th>Inducer</th>
<th>NDBA</th>
<th></th>
<th></th>
<th>NBPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>( V_{\text{max}} )</td>
<td>( K_m ) (mM)</td>
<td>( V_{\text{max}} )</td>
</tr>
<tr>
<td>None</td>
<td>0.26±0.03</td>
<td>3.17±0.33</td>
<td>1.21±0.07</td>
<td>2.50±0.68</td>
</tr>
<tr>
<td>PB</td>
<td>0.20±0.01</td>
<td>21.2±5.46(^b)</td>
<td>0.41±0.04(^b)</td>
<td>9.19±2.51(^b)</td>
</tr>
<tr>
<td>PYR</td>
<td>0.26±0.01</td>
<td>9.57±2.22(^b)</td>
<td>0.36±0.08(^b)</td>
<td>5.63±2.04(^b)</td>
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<tr>
<td>BNF</td>
<td>0.28±0.02</td>
<td>1.90±1.42</td>
<td>0.69±0.05(^b)</td>
<td>1.65±0.07</td>
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<tr>
<td>BHT</td>
<td>0.24±0.02</td>
<td>5.61±2.69</td>
<td>0.29±0.01</td>
<td>2.64±0.11</td>
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<tr>
<td>BHA</td>
<td>0.22±0.02</td>
<td>1.92±0.08(^b)</td>
<td>0.31±0.01(^b)</td>
<td>2.28±0.23</td>
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<tr>
<td>CLO</td>
<td>0.21±0.03</td>
<td>2.68±0.35</td>
<td>0.46±0.03(^b)</td>
<td>2.13±0.25</td>
</tr>
</tbody>
</table>

\(^a\)The incubations and HPLC assays for the butyraldehyde derivatives are described in Materials and methods. Values represent the mean ± SD of three separate experiments performed in duplicate.

\(^b\)Significantly different from untreated microsomes (\( P < 0.01 \) by Student’s \( t \) test).
Kinetic studies of metabolism of NDPA, NDBA and NBPA by rat liver microsomes

A series of increasing concentrations of NDPA, NDBA and NBPA ranging from 0.25 to 4 mM were selected to determine the apparent kinetic constants $K_m$ and $V_{max}$ for metabolism of these three long chain nitrosodialkylamines by rat liver microsomes. As shown in Figure 5, formation of all metabolites from these three nitrosamine carcinogens was concentration dependent. Apparent Michaelis–Menten kinetics were observed for metabolism of NDPA, NDBA and NBPA by all of the various types of microsomes used for these studies. Formaldehyde was produced from NDPA (Figure 5a) and NBPA (Figure 5c) by microsomes from both PB- and PYR-pretreated rats. However, the rates of formation were very different. Formation of formaldehyde from NBPA was only catalyzed by microsomes from PYR-pretreated rats at a very low yield (Figure 5b). Although all of the liver microsomal preparations tested were capable of metabolizing NDPA (Figure 5d) and NBPA to propionaldehyde (Figure 5e) and generating butyraldehyde from NDBA (Figure 5f) and NBPA (Figure 5g), the specific activities of the microsomes from PB- and PYR-treated rats differed significantly from the others.

The apparent $K_m$ and $V_{max}$ values were determined from the data in Figure 5 as indicators of the enzyme specificity and substrate selectivity of the different microsomal preparations for metabolism of NDPA, NDBA and NBPA. The overall kinetic parameters for NDPA, NDBA and NBPA, calculated by linear regression analysis, are summarized in Tables I–III. Liver microsomes from PB-induced rats exhibited the lower apparent $K_m$(0.25 mM) for demethylation of NDPA (Table I). Although formaldehyde was formed upon incubation of NDBA with liver microsomes from PB-induced rats with a similar apparent $K_m$ value, the corresponding $V_{max}$ for demethylation of NDBA decreased from 1.56 to 0.65 nmol/min/mg protein. Liver microsomes from PYR-pretreated rats exhibited the larger apparent $K_m$ and the smaller $V_{max}$ for NDPA demethylation (Table I). For depropylation of NDPA and NBPA catalyzed by liver microsomes from PB-induced rats the apparent $K_m$ values were 0.37 and 0.49 mM respectively, with corresponding $V_{max}$ values of 19.04 and 9.71 nmol/min/mg protein (Table II). Microsomes from rats pretreated with PYR also exhibited significant induction of catalytic activity, with $V_{max}$ values of 13.8 and 6.4 and apparent $K_m$ values of 0.30 and 0.32 for NDPA and NDBA respectively. The apparent $K_m$ value for debutylation of NDBA by liver microsomes from PB-induced rats was lower than that for depropylation of NDPA, but the $V_{max}$ values were similar (Table III). The next highest $V_{max}$ value was observed for debutylation of NDBA catalyzed by liver microsomes from PYR-induced rats. The lowest $V_{max}$ value for debutylation of NDBA and NBPA was seen with liver microsomes from BNF-pretreated rats (Table III).

Metabolism of NDPA, NDBA and NBPA by purified cytochrome P450s in the reconstituted system

Purified cytochrome P450 2E1 in the reconstituted system effectively catalyzed formation of formaldehyde from NDPA and NBPA, but was much less active in catalyzing formaldehyde formation from NDBA (Figure 6a). Although cytochrome P450 2E1 did metabolize NDPA and NBPA to propionaldehyde, it did not catalyze formation of acetaldehyde from the three nitrosamines. Our data also show that cytochrome P450 2E1 had no catalytic activity for debutylation of NDBA or NBPA (Figure 6a). In contrast, purified cytochrome P450 2B1 catalyzed not
only depropylation of both NDPA and NBPA, but also depropylation of both NDPA and NBPA with a relatively high specific activity (Figure 6b). However, purified cytochrome P450 2B1 exhibited no catalytic activity for formation of formaldehyde or acetaldehyde from NDPA, NDBA, and NBPA. We did not detect the production of any metabolites of NDPA, NDBA, and NBPA in the presence of purified rat liver cytochrome P450 1A1 in the reconstituted system (data not shown), suggesting that these three forms of cytochrome P450 have very different substrate specificities with respect to these three nitrosamines.

Inhibition of rat liver microsomal metabolism of NDPA and NDBA by monoclonal or polyclonal antibodies

To determine the role of cytochromes P450 2B1 and 2E1 in microsomal metabolism of these long chain nitrosodialkylamines, we investigated the effects of inhibitory antibodies specific for cytochromes P450 2B1 and 2E1 on depropylation of NDPA and depropylation of NDPA, mAb 2-66-3, an inhibitory antibody specific for cytochrome P450s 2B1 and 2B2 (mAb-2B1/2), inhibited 65% of propionaldehyde formation from NDPA (Table IV) and 62% of butyraldehyde formation from NDBA (Table IV) and 62% of butyraldehyde formation from NDBA. mAb 1-91-3, an inhibitory antibody for cytochrome P450 2E1 (mAb-2E1), inhibited 28% of pyruvaldehyde formation from PYR-pretreated rats by 28%, whereas mAb-2B1 inhibited this reaction by 41% (Table IV). Rabbit anti-rat 3A1/2 pAb was used to examine the possible role of cytochrome P450 3A1/2 in metabolism of NDBA. However, this pAb had no inhibitory effect on dealkylation activities in microsomes from either untreated rats or those pretreated with PB or BHT (Table V).

Discussion

A large number of different forms of cytochrome P450 differing in their amino acid sequences, regulation of expression, substrate specificities and catalytic activities have been identified (38,39). Changes in the cytochrome P450 composition of liver microsomes due to induction of various cytochrome P450 isoenzymes by different inducers significantly influences metabolism of nitrosodialkylamines in vivo and in vitro and consequently may alter their biological end points (8,18,19,31). Our results demonstrate that metabolism of NDPA, NDBA and NBPA by rat liver microsomes is significantly affected by changes in the activities of specific cytochrome P450 isoforms due to induction by inducers such as PB and PYR.

For all three nitrosamines the overall yield of metabolites with microsomes from untreated rats was low, particularly for NDBA. The relatively low metabolic rates observed with control microsomes indicates they lack or have a relatively low level of the specific cytochrome P450 isoforms involved in metabolism of these long chain nitrosamines. However, pretreatment of rats with PB, an effective inducer of cytochrome P450 2B1 (40,41), dramatically increased microsomal activity for dealkylation of NDPA, NDBA and NBPA. The catalytic activity enhanced by PB pretreatment is specific for hydroxylation of α-carbons, regardless of the length of the alkyl group. This high N-dealkylation activity was also observed in microsomes from PYR-pretreated rats. It is known that PYR, a component of tobacco and tobacco smoke, induces cytochrome P450 2E1 (42,43). However, besides inducing cytochrome P450 2E1, higher doses of PYR (200 mg/kg body wt) result in coordinated induction of cytochrome P450 2B1 (44). Co-induction of both cytochromes P450 2B1 and 2E1 in rat liver by PYR apparently shows a synergistic effect on formation of N-dealkylation products from NDPA, NDBA and NBPA.

The N-dealkylation reaction, as well as the other dealkylation activities of microsomes, was not increased by pretreating rats with BNF or CLO in all cases. These results suggest that cytochromes P450 1A1/1A2 and 4A1/4A2, which are highly inductible by BNF (33,45) and CLO (46,47), respectively, are not involved in metabolism of NDPA, NDBA and NBPA.

BHT and BHA are two phenolic antioxidants which are widely used as food additives for both humans and livestock. BHT was reported to produce a 'PB-type' induction of hepatic cytochromes P450 in rats (48). However, BHT pretreatment did

Table IV. Inhibition of propionaldehyde formation from NDPA by mAbs in microsomes from rats pretreated with different inducers

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Specific activity (propionaldehyde formed, nmol/mg protein)</th>
<th>% Inhibition</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2.83±0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11</td>
<td>2.23±0.76</td>
</tr>
<tr>
<td>PB-treated</td>
<td>17.5±2.48</td>
<td>65</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PYR-treated</td>
<td>10.5±1.97</td>
<td>41</td>
<td>7.21±2.71</td>
</tr>
<tr>
<td>BHT-treated</td>
<td>2.98±0.77</td>
<td>57</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The samples, incubations and HPLC assays for the propionaldehyde derivatives are described in Materials and methods. 
<sup>b</sup>n = 4.
<sup>d</sup>ND, not determined.
<sup>**</sup>Significantly different from control (P < 0.01 by Student's t test).

Fig. 6. Metabolism of NDPA, NDBA and NBPA by purified cytochrome P450 2E1 (a) and 2B1 (b) in the reconstituted system. The samples, incubations and assays were performed as described in Materials and methods. The products formed were: formaldehyde (■), propionaldehyde (□) and butyraldehyde (▲). Data are taken from three separate experiments with each point determined in duplicate. Bars, SEM.
not significantly increases the activities of rat liver microsomes for N-dealkylation of NDBA and NBPA when compared with PB and PYR pretreatment and had essentially no effect on metabolism of NDPA. The low N-dealkylation activity observed in microsomes from BHT-pretreated rats is presumably due to a low level induction of hepatic cytochrome P450 2B1, because BHT primarily induces hepatic cytochrome P450 3A1/2 in rats (48). High level expression of cytochrome P450 3A1/2 in rat liver microsomes following pretreatment with BHT did not increase dealkylation activity of rat liver microsomes for these nitrosodialkylamines, suggesting that NDPA, NDBA and NBPA are not substrates for cytochrome P450 3A1/2. Since, in addition to inducing cytochrome P450 2B1, both PB and BHT induce hepatic cytochrome P450 3A1/2 in rats (41,48), we examined the possible role of cytochrome P450 3A1/2 in metabolism of NDPA with an inhibitory pAb. However, we could not detect any statistically significant inhibition of NDPA metabolism by this antibody. This result indicates that cytochrome P450 2B1, rather than 3A1/2, is the critical isozyme for metabolism of NDPA in liver microsomes from both PB- and BHT-pretreated rats.

Pretreatment of rats with BHA has been reported to inhibit esophageal carcinogenesis due to NDPA, but to significantly stimulate hepato- and urinary bladder carcinogenesis due to NDPA (49). This alteration in the organotropy of NDPA by BHA may result from changes in the catalytic activities of certain cytochrome P450 isoforms upon BHA treatment. BHA pretreatment decreased formation of butyraldehyde from NDPA by 50% when compared with control microsomes. However, it did not have any significant effect on N-dealkylation of NDPA. It is possible that BHA inhibits the activity or suppresses expression of cytochrome P450 isoforms, such as cytochrome P450 2B1, which are involved in metabolism of NDPA and NDPA.

Generally, the cytochrome P450 isoforms which display low \( K_m \) values and high turnover numbers for a given nitrosamine are considered to be the ones that are important physiologically for metabolism of these indirect carcinogens (22). Although microsomes from animals pretreated with PB or PYR catalyzed metabolism of NDPA and NDPA to give both formaldehyde and propionaldehyde/butyraldehyde, the \( V_{max} \) values were much higher for \( \alpha \)-carbon hydroxylation, determined as depropylation or debutylation, than for formation of formaldehyde, indicating that the preferred position for oxidative metabolism of these nitrosamines is the \( \alpha \)-position.

Studies by Mirvish, Yang and co-workers (19) on liver microsomal metabolism of \( N \)-nitrosomethyl-\( n \)-amylamine have also demonstrated specific effects of a variety of inducing agents on metabolism of the pentyl chain. While control microsomes exhibited primarily 4-hydroxylation activity, induction by PB increased hydroxylation at all positions of the pentyl chain, with a 4- to 5-fold induction in depentylation as a result of \( \alpha \)-hydroxylation. 3-Methylcholanthrene specifically induced 3-hydroxylation, whereas aroclor-1254 strongly induced both 2- and 3-hydroxylation (19). Studies using purified cytochrome P450 2B1 in the reconstituted system gave metabolite ratios similar to those in PB-induced microsomes, with depentylation being the predominant metabolic pathway (19).

The substrate specificity and regioselectivity of several purified isoforms of cytochrome P450 were further confirmed using the reconstituted system. The results of these studies demonstrate that purified cytochrome P450 2B1 is specific for catalyzing \( \alpha \)-hydroxylation of long chain nitrosamines such as NDPA, NDBA and NBPA and exhibits a relatively high specific activity for these substrates. The specificity of cytochrome P450 2B1 for catalyzing \( \alpha \)-hydroxylation suggests that it may catalyze metabolism of a wide spectrum of \( N \)-nitrosodialkylamines at the \( \alpha \)-position. Previous studies from our laboratory have implicated cytochrome P450 2B1 as having a significant role in \( \alpha \)-hydroxylation of NDEA (13). In contrast, cytochrome P450 2E1 appeared to be unable to cleave an alkyl group with more than three carbons from either NDPA or NBPA. Cytochrome P450 2E1 catalyzed a small amount of formaldehyde formation from both NDPA and NBPA, but it was unable to produce a statistically significant amount of formaldehyde from NDPA. Cytochrome P450 1A1 in the reconstituted system did not catalyze metabolism of any of these nitrosamines to any significant extent. This lack of activity with purified cytochrome P450 1A1 in the reconstituted system further demonstrates the substrate specificity of the individual isoforms of cytochrome P450.

Kawanishi and co-workers have also investigated dealkylation of a series of \( N \)-nitrosodialkylamines by several purified cytochrome P450s in the reconstituted system (21). Cytochrome P450 2B1 exhibited a very high activity for NDPA depentylation and NDBA debutylation. Compared with cytochrome P450 2B1, cytochrome P450 1A2 exhibited 65% of the specific catalytic activity for depentylation of NDPA but had only 13% of the debutylation activity for NDPA, whereas cytochrome P450 2B2 and cytochrome P450 1A1 were less active for metabolism of these compounds (21).

In view of our data using microsomes from rats pretreated with a variety of different inducers, the reconstituted system containing purified isoforms of cytochrome P450 (2B1, 2E1 and 1A1/2) and inhibitory antibodies to 2B1/2, 3A1/2, and 2E1, our results clearly indicate that cytochromes P450 2B1 and 2E1 appear to play an important role in metabolism of NDPA, NDBA and NBPA, whereas cytochrome P450 forms 1A1/1A2, 3A1 and 4A1 do not appear to play any significant role in metabolism of these long chain nitrosamines in rat liver.

These studies demonstrate that \( \alpha \)-hydroxylation is the predominant pathway for hepatic metabolism of \( C_3 \) and \( C_4 \) nitrosodialkylamines. Cytochrome P450 2B1 appears to play an
important role in metabolism of NDPA, NDBA and NBPA by rat liver microsomes via \( \alpha \)-hydroxylation. Cytochrome P450 2E1 also appears to be involved in propionaldehyde formation from NDPA by directly catalyzing formation of propionaldehyde from NDPA via \( \alpha \)-hydroxylation. However, it is possible that cytochrome P450 2E1 may also selectively hydroxylate NDPA at the \( \beta \)-carbon position to initiate another important bioactivation pathway (Figure 7). It is known that NDPA can undergo \( \beta \)-oxidation to produce propionaldehyde and a methylating agent (11). Krüger (50) has suggested that NDPA may be metabolically degraded via \( \beta \)-oxidation to the nitrosomethylpropylamine, which may then release either propionaldehyde or formaldehyde via \( \alpha \)-hydroxylation. However, it is also possible that the formaldehyde produced from NDPA by cytochrome P450 2E1 was directly generated by cleavage of the terminal carbon of NDPA, as shown in Figure 7.

Of all possible cytochrome P450-dependent oxidations of NDBA (i.e. \( \alpha \), \( \beta \), \( \omega \)-1 and \( \omega \)) \( \alpha \)- and \( \omega \)-1-hydroxylation have been shown to be the two principal pathways for NDBA metabolism by microsomal preparations (51). Formation of butylaldehyde...
Cytochrome P450-dependent nitrosamine metabolism

Fig. 9. Proposed pathways for metabolism of NBPA by rat liver microsomes.

dehyde, a convenient probe for α-hydroxylation (Figure 8), substantially increased upon PB pretreatment. The significant increase in formation of butyraldehyde from NDBA following induction of cytochrome P450 2B1 by PB pretreatment indicates that α-hydroxylation is an inducible and cytochrome P450 2B1-dependent pathway. Pretreatment of rats with PB not only significantly increased biotransformation of NDBA via α-hydroxylation, but also enhanced metabolism of NDBA via ω-1-hydroxylation in vitro (51). The metabolic fate of ω-1-hydroxy-NDBA has not yet been established. Whether it can be converted into acetaldehyde in vitro by chain shortening remains to be investigated. ω-Hydroxylation has previously been reported to be a major pathway for bioactivation of NDBA (6). Since non-microsomal enzymes have been shown to be involved in the further metabolism of ω-hydroxy derivatives of NDBA (17,18), liver microsomes would, therefore, probably not be predictive for formation of aldehydes from NDBA via ω-hydroxylation. However, contamination of the microsomal preparation with ADH, ALDH or mitochondrial enzymes during preparation could be responsible for formation of certain aldehydes, e.g. formaldehyde or acetaldehyde, from NDBA following ω-hydroxylation.

The metabolic fate of NBPA in rat liver microsomes appears to be highly dependent on the activities of cytochrome P450 2B1 and 2E1. Like NDBA and NDBA, α-hydroxylation is a major metabolic pathway for NBPA in vitro (Figure 9). The small amount of formaldehyde observed may be formed from the propyl chain of NBPA via chain shortening and this could be catalyzed by cytochrome P450. However, it is also possible that non-microsomal enzymes may be involved in the further metabolism of hydroxy derivatives of NBPA formed via other pathways, as indicated in Figure 9.

Although the sequence of steps leading to formation of formaldehyde is not clear, it has previously been demonstrated that nitrosodipropylamine methylates DNA (11). Later it was shown that this is also true for N-nitrosobis(2-hydroxypropyl)amine, N-nitrosodi-n-propylamine, 2-(2-oxopropyl)-2-oxopropanoic acid and N-nitrosobis(2-oxopropyl)amine, three known pancreatic carcinogens metabolically derived from N-nitrosodipropylamine (52,53). Experimental evidence supports the hypothesis that methyloxopropynitrosamine and methylhydroxypropynitrosamines are intermediates in this process (50,54).

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L. Shu and P. F. Hollenberg


