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Role of Superoxide in the N-Oxidation of *N*-(2-Methyl-1-phenyl-2-propyl)hydroxylamine by the Rat Liver Cytochrome P-450 System†

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ABSTRACT: The N-oxidation of *N*-(2-methyl-1-phenyl-2-propyl)hydroxylamine (*N*-hydroxyphentermine, MPPNHOH) and the N-hydroxylation of 2-methyl-1-phenyl-2-propylamine (phentermine) by reconstituted systems that contained cytochromes P-450 purified from rat liver microsomes were demonstrated. The oxidation of MPPNHOH, but not of phentermine, could also be mediated by a superoxide and hydrogen peroxide generating system that contained xanthine and xanthine oxidase. Superoxide dismutase completely inhibited the oxidation of MPPNHOH by the xanthine/xanthine oxidase system and inhibited by 70% the oxidation mediated by a reconstituted cytochrome P-450 oxidase system. The majority of the microsomal oxidation was inhibited by an antibody raised against the major isozyme of cytochrome P-450 purified from livers of phenobarbital-pretreated rats. 2-Methyl-2-nitroso-1-phenylpropane (MPPNO) was found to be an intermediate in the overall oxidation of MPPNHOH to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$). Superoxide dismutase appeared to inhibit the first step, the conversion of MPPNHOH to MPPNO. These observations are accounted for by a sequence of two mechanistically distinct P-450-mediated oxidations. In the first reaction, N-hydroxylation of phentermine occurs by a normal cytochrome P-450 pathway. The formed hydroxylamine then uncouples the cytochrome P-450 system to generate superoxide and hydrogen peroxide. The superoxide oxidizes MPPNHOH to MPPNO which is then oxidized to $MPPNO_2$, the ultimate product. This superoxide-mediated oxidation represents another pathway for N-oxidation by cytochrome P-450.

Previous studies in this laboratory have shown that phentermine (2-methyl-1-phenyl-2-propylamine; $MPPNH_2$)[1] is oxidized to *N*-hydroxyphentermine (MPPNHOH) and that MPPNHOH is further oxidized to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$) (Sum & Cho, 1979; Maynard & Cho, 1981) by rat liver microsomal preparations. The N-hydroxylation of $MPPNH_2$ appeared to be a normal cytochrome P-450 catalyzed reaction in that it is a two-electron oxidation and was inhibited by carbon monoxide and DPEA (Sum & Cho, 1977). The oxidation of MPPNHOH to $MPPNO_2$ was also inhibited by carbon monoxide and DPEA (Sum & Cho, 1979, Maynard & Cho, 1981) but differed from the first oxidation in its sensitivity to superoxide dismutase. Further studies with rat and rabbit liver microsomes (Maynard & Cho, 1981; Cho et al., 1982) indicated that MPPNHOH caused a NADPH-dependent increase in H_2O_2 and that superoxide was responsible for the oxidation of MPPNHOH to $MPPNO_2$ by liver microsomes. Thus, MPPNHOH appeared to uncouple cytochrome P-450 to generate the superoxide that subsequently oxidized MPPNHOH.

The present investigation was initiated to obtain direct evidence for cytochrome P-450 involvement in the reaction by incubating MPPNHOH with reconstituted systems containing the major cytochrome P-450 isozyme from either pheno-

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[1] Abbreviations: $MPPNH_2$, 2-methyl-1-phenyl-2-propylamine; MPPNHOH, *N*-(2-methyl-1-phenyl-2-propyl)hydroxylamine; MPPNO, 2-methyl-2-nitroso-1-phenylpropane; $MPPNO_2$, 2-methyl-2-nitro-1-phenylpropane; PB, phenobarbital; 3MC, 3-methylcholanthrene; PC, dilauroylphosphatidylcholine; DPEA, *N*-(2,4-dichloro-6-phenylphenoxy)ethylamine; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography.

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barbital- (PB-) or 3-methylcholanthrene- (3 MC-) pretreated rats. The oxidation of $MPPNH_2$ to MPPNHOH and of MPPNHOH to $MPPNO_2$ by a superoxide-generating system which consisted of xanthine and xanthine oxidase was also investigated. The results indicate that the oxidation of MPPNHOH but not $MPPNH_2$ is mediated by superoxide anion generated by either xanthine oxidase or cytochrome P-450.

Experimental Procedures

Materials

Phentermine hydrochloride was a gift of Pennwalt Corp. (Rochester, NY). MPPNHOH was synthesized from $MPPNH_2$ (Beckett et al., 1975). 2-Methyl-2-nitroso-1-phenylpropane (MPPNO) and $MPPNO_2$ were synthesized from $MPPNH_2$ according to the methods of Lindeke et al. (1975). NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, bovine blood superoxide dismutase (2750 units/mg), dilauroylphosphatidylcholine (PC), xanthine, and xanthine oxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Catalase (65 000 units/mg) was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). DEAE Affi-Gel Blue was obtained from Bio-Rad Laboratories (Richmond, CA) and Sephadex G-100 from Pharmacia (Piscataway, NJ).

Methods

Purification of Cytochrome P-450 and NADPH–Cytochrome P-450 Reductase. The major PB-inducible isozyme of cytochrome P-450 was purified from the livers of male Long-Evans rats to a specific content of 14 nmol/mg of protein or greater as described by West et al. (1979). Cytochrome P-448 was a gift from Regina Wang and Dr. Anthony Lu (Merck Sharp & Dohme, Rahway, NJ) and had been purified to a specific content of 13 nmol/mg of protein from the livers of rats that had previously been injected with 3MC. Cytochrome P-450 concentrations were determined by the method of Omura & Sato (1964) using an extinction coefficient of 91 M^{-1} cm^{-1}.

NADPH-dependent cytochrome P-450 reductase was purified from hepatic microsomes of PB-pretreated, male, New Zealand white rabbits by DEAE-Sephadex A-25 chromatography as described by Dignam & Strobel (1977) and affinity chromatography on 2′,5′-ADP–agarose as described by Yasukochi & Masters (1976) with some modifications. The final reductase preparation contained 11.5 nmol/mg of protein and gave one major band upon SDS–polyacrylamide gel electrophoresis. Reductase activity was determined by the method of Phillips & Langdon (1962). Concentrations of reductase were calculated with the assumption of a maximal specific activity of 3160 nmol of cytochrome *c* reduced min^{-1} (nmol of reductase)$^{-1}$ at 22 °C (Miwa et al., 1979).

Preparation of Antibodies. Immune sera from untreated and immunized, female, New Zealand white rabbits treated with the cytochrome P-450 purified from PB-pretreated rats were gifts from Dr. Cecil Pickett of Merck Sharp & Dohme (Rahway, NJ) (Thomas et al., 1976). The IgG was purified by chromatography on DEAE Affi-Gel by procedures provided by the manufacturer. Briefly, the serum preparation (30–35 mL) was dialyzed against two changes of 2 L of 0.02 M Tris-HCl (pH 8.0), 0.028 M NaCl, and 0.02% NaN_3 and then applied to a column of DEAE Affi-Gel Blue (2.6 × 35 cm) equilibrated buffer, and the fractions containing IgG were combined. The pool was concentrated to a small volume by ultrafiltration (Amicon XM-50 membrane) and applied to a Sephadex G-100 column to remove hemoglobin which remained in the preparation after the DEAE Affi-Gel Blue step. The purified IgG was concentrated over Amicon XM-50 membranes, and the final protein concentration was 40 mg/mL. Control antibody was purified by an identical procedure from sera obtained from nonimmunized rabbits. The final protein concentration of this preparation was 32 mg/mL. Each antibody preparation was heated to 56 °C for 60 min before use as described by Thomas et al. (1977), who had found this treatment to inactivate an inhibitory factor present in some of their preparations of control antibody without affecting the inhibitory activity of antibodies prepared against cytochrome P-450.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Incubations with $MPPNH_2$ and MPPNHOH. Four different enzyme systems were used for this investigation These were two different reconstituted cytochrome P-450 oxidase systems, a superoxide-generating system, which contained xanthine and xanthine oxidase, and rat liver microsomes. For incubations with the reconstituted systems, 1.0 nmol of cytochrome P-450 or cytochrome P-448, 1.0 nmol of reductase, and 60 μg of PC [1 mg/mL in 0.1 M $KPHO_4$ (pH 7.7) sonciated immediately before] were combined and allowed to equilibrate at room temperature for 5 min, and then the amount of distilled water calculated to give a final incubation volume of 3 mL was added followed by an NADPH-regenerating system [1.3 μmol of NADP, 15 μmol of glucose 6-phosphate, 6 units of glucose-6-phosphate dehydrogenase, 6 μmol of $MgCl_2$, and 240 μmol of potassium phosphate (pH 7.4) in 0.5 mL total volume]. Superoxide dismutase was then added to some of the incubation mixtures. After a 5-min preincubation at 37 °C with shaking, the reaction was started by the addition of either 15 μmol of $MPPNH_2$ or 1.5 μmol of MPPNHOH. The reaction was run for 10 min and was terminated by pouring the incubation mixture into 10 mL of ice-cold dichloromethane that had previously been bubbled with N_2 for 20 min and that contained an internal standard for analysis by HPLC or gas chromatography (see below).

Incubation mixtures for the xanthine/xanthine oxidase system contained 0.75 μmol of xanthine, 500 μmol of potassium phosphate (pH 7.4), 0.05 unit of xanthine oxidase, and either 15 μmol of $MPPNH_2$ or 1.5 μmol of MPPNHOH in a final volume of 3 mL. Superoxide dismutase, catalase, mannitol, or Me_2SO were also included in some incubation mixtures. After a 5-min preincubation at 28 °C with shaking, the reaction was started by the addition of xanthine oxidase. The production of $MPPNO_2$ was linear under these conditions for at least 15 min. The reaction was stopped, and products were analyzed as described above.

For microsomal incubations, washed hepatic microsomes were prepared as previously described (Sum & Cho, 1977) from male Sprague-Dawley rats which had been pretreated with PB. The incubation mixture contained microsomes (equivalent to 1 nmol of cytochrome P-450), the NADPH-regenerating system described above, and 1.5 μmol of MPPNHOH in a total volume of 3 mL. After a 5-min preincubation at 37 °C the reaction was started by the addition of substrate, allowed to proceed for 10 min, and stopped as described above.

Isolation and Quantitation of Products. The dichloromethane–aqueous mixture from above was shaken and centrifuged, and 8 mL of the dichloromethane layer was transferred to a 15-mL conical tube and concentraed to a small volume under a stream of dry N_2. One milliliter of methanol

Table I: Oxidation of MPPNHOH by Reconstituted Cytochrome P-450 Oxidase Systems[a]

components	nmol of $MPPNO_2$
PB P-450, reductase, PC	0
PB P-450, PC, NADPH	0
3 MC P-450, reductase, PC	0
3 MC P-450, PC, NADPH	0
reductase, PC, NADPH	14 ± 10
reductase, PC	0
PB P-450, reductase, PC, NADPH	70 ± 7
3 MC P-450, reductase, PC, NADPH	35 ± 3

[a] Incubations were performed for 10 min as described under Methods, except that only the indicated components were included in the incubation mixture. When the NADPH-regenerating system was omitted, it was replaced with buffer that contained 240 μmol of potassium phosphate (pH 7.4) and 6 μmol of $MgCl_2$. $MPPNO_2$ was determined by either HPLC or gas chromatography. Each value is an average ± standard deviation of at least four determinations.

was then added, and the mixture was concentrated down to 0.5 mL. An additional 1-mL portion of methanol was added and the mixture concentrated to 0.2 mL. The tube was capped and placed on ice, and the products were analyzed by HPLC on an Altex RP-18 column. The eluant was a 75:25 mixture of methanol and potassium phosphate buffer (50 mM, pH 2.8). Alternatively, if only MPPNHOH and $MPPNO_2$ were to be determined, methanol was not added to the concentrated dichloromethane extract and instead trifluoroacetyl derivatives were formed and the products analyzed by gas chromatography as previously described (Sum & Cho, 1977; 1979). The internal standards were 100 nmol of 1-phenyl-2-hexanone for analysis by HPLC or 1-phenyl-2-butanone for analysis by gas chromatography. The metabolites were quantitiated by comparison of the peak area ratio (metabolite/internal standard) to the appropriate standard curve (peak area ratio vs. nanomoles of metbolite) which was run in each experiment. The amounts of $MPPNO_2$ determined by HPLC or gas chromatography from the extracts of identical incubation mixtures were very similar. The HPLC assay was validated by comparing the chromatographic behavior of incubation mixture extracts with authentic samples of $MPPNH_2$, MPPNHOH, MPPNO, and $MPPNO_2$ on different columns (RP-C_8, RP-C_{18}) and solvents (methanol buffer in varying proportions). In each case, retention times of authentic compound and its corresponding peak in the extract were changed identically. The GC assay was validated by comparison of the mass spectra of enzymatic reaction products with authentic compounds (Sum & Cho, 1979).

MPPNO has properties that made direct experiments difficult. Aliphatic nitroso compounds exist as an equilibrium mixture of monomer and dimer, with the dimer favored in polar or aqueous media and the monomer favored in nonpolar or organic media. When MPPNO is dissolved in benzene, it is rapidly oxidized by air to $MPPNO_2$, but the conversion is reduced 6-fold in methanol. Oxygen solubility in the two solvents is comparable, so the only chemical difference in these solutions was the existence of MPPNO as a dimer in methanol. These data indicate that only the monomer is oxidized so that in polar media the reaction depends on the dissociation of dimer.

Determination of the Effects of Antibodies on the Microsomal Oxidation of MPPNHOH. The indicated amount of either control antibody or antibody raised against the major PB-inducible isozyme of cytochrome P-450 from rat liver was added directly to the microsomes in the incubation flask and the mixture allowed to equilibrate for 5 min at room temperature. The remaining components of the incubation mixture were added and the incubation performed in the usual fashion. The control incubation mixture (no antibody) received a corresponding volume of the antibody buffer.

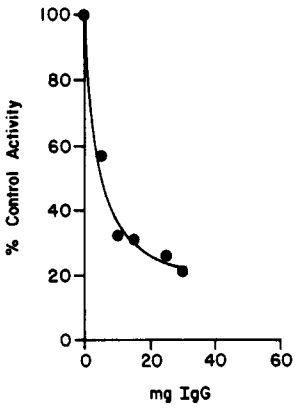

FIGURE 1: Effects of anti-cytochrome P-450 IgG on the microsomal oxidation of MPPNHOH. Microsomes (1 nmol of cytochrome P-450) were preincubated with the indicated amount of either control or anti-cytochrome P-450 IgG before incubation with MPPNHOH as described under Methods. Products were determined by HPLC. Activity is expressed as percent control $MPPNO_2$ formation (100% times the nanomoles of $MPPNO_2$ formed in the presence of anti-cytochrome P-450 IgG divided by nanomoles of $MPPNO_2$ formed in the presence of control IgG). The activity in the absence of antibody was 62 nmol of $MPPNO_2$ formed in 10 min. Each point is an average of two determinations.

Results

Oxidation of NOHP by Reconstituted Cytochrome P-450 Oxidase Systems. Table I shows results from incubations of MPPNHOH with the two reconstituted cytochrome P-450 oxidase systems. The major product was $MPPNO_2$, and no products were detected in the absence of either NADPH or cytochrome P-450 reductase. A low but variable level of $MPPNO_2$ formation was detectable in the absence of cytochrome P-450. However, even this formation of $MPPNO_2$ was NADPH dependent. Twice as much $MPPNO_2$ was formed in the reconstituted system which contained cytochrome P-450 purified from PB-pretreated animals than was formed in the system which contained cytochrome P-448 from 3MC-pretreated animals. However, if the activity observed in the absence of cytochrome is taken into account, it would appear that the isozyme from the PB-pretreated rats is considerably more than twice as active as the isozyme from 3MC-pretreated rats for N-oxidation of MPPNHOH. Although the reductase used in these studies was obtained from rabbits, reconstitution with reductase isolated from PB-pretreated rats oxidized MPPNHOH to $MPPNO_2$ at rates similar to the complete systems shown in Table I.

MPPNO was detected in highest levels in incubation mixtures that contained cytochrome P-450 from PB-pretreated rats, probably because of the greater rate of MPPNHOH oxidation. The levels were variable and low, less than 6 nmol in 10 min, indicating that MPPNO did not accumulate to appreciable levels in incubation of MPPNHOH with the reconstituted systems.

Effects of Anti-Cytochrome P-450 IgG on the Microsomal Oxidation of NOHP. In studies examining the inhibition of MPPNHOH oxidation by anti-cytochrome P-450 IgG, MPPNHOH was incubated with microsomes in the presence of increasing amounts of IgG that had been isolated from sera of either control rabbits or rabbits that had been inoculated with cytochrome P-450 purified from PB-pretreated rats as described under Methods. $MPPNO_2$ was the major product, and MPPNO was not detected in significant quantities. Approximately 80% of the $MPPNO_2$ formation was inhibited

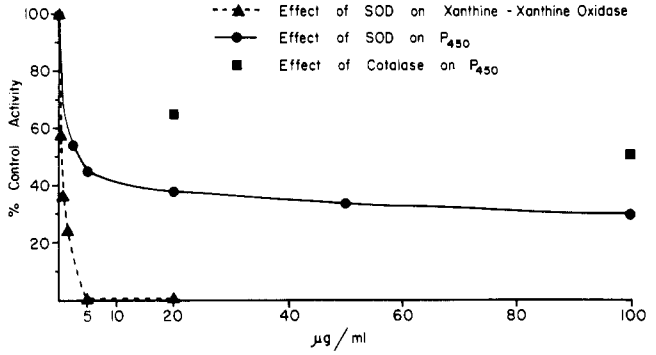

FIGURE 2: Effects of superoxide dismutase and catalase on the oxidation of MPPNHOH. Cytochrome P-450 isolated from PB-pretreated rats or xanthine oxidase was used for these incubations. The indicated concentration of superoxide dismutase (●) or catalase (■) was present in the incubation mixture. Incubations were for 10 min and were performed as described under Methods. Both HPLC and gas chromatography were used for product determination. Activity is expressed as percent control. The control activity from the reconstituted P-450 system was 70 nmol, and from the xanthine oxidase/xanthine mixture was 25 nmol of $MPPNO_2$ formed in 10 min. Each point is an average of at least two determinations.

at the highest concentration of the anti-cytochrome P-450 IgG (Figure 1), indicating that at least 80% of the microsomal oxidation of MPPNHOH was catalyzed by the cytochrome P-450 system. The residual oxidation might have been catalyzed by isozymes of cytochrome P-450 which did not cross-react with the antibody, NADPH-dependent cytochrome P-450 reductase, or other enzymes besides those involved in the cytochrome P-450 system. This antibody also inhibited MPPNHOH oxidation and benzphetamine N-demethylation in reconstituted cytochrome P-450 oxidase systems which contained cytochrome P-450 isolated from PB-pretreated rats (data not shown).

Effects of Superoxide Dismutase and Catalase on the Oxidation of MPPNHOH by a Reconstituted Cytochrome P-450 Oxidase System and Xanthine/Xanthine Oxidase System. Figure 2 shows the effects of increasing concentrations of superoxide dismutase on the oxidation of MPPNHOH to $MPPNO_2$ by a reconstituted system that contained cytochrome P-450 purified from PB-pretreated animals. A 50% inhibition of the oxidation occurred at a concentration of superoxide dismutase between 5 and 10 μg/mL (14–28 units/mL). In reconstituted oxidase systems in which the reductase had been isolated from PB-pretreated rats, the oxidation of MPPNHOH was also inhibited by superoxide dismutase. Therefore, the reaction does not appear to be affected by the source of the reductase, in agreement with studies by Guengerich et al. (1981). The NADPH-dependent oxidation of MPPNHOH to $MPPNO_2$ by cytochrome P-450 reductase was also inhibited by superoxide dismutase (data not shown). Superoxide dismutase which had been boiled did not inhibit the oxidation of MPPNHOH. Low, but variable, amounts of MPPNO were also detected and appeared to decrease as the concentration of superoxide dismutase in the incubation mixture was increased.

High levels of catalase, at concentrations of 20 (1300 units/mL) and 100 μg/mL (6500 units/mL), inhibited the oxidation by 35% and 50%, respectively. It is unlikely that this inhibition was due to contamination of the catalase with superoxide dismutase since the reduction of cytochrome *c* by superoxide generated via a xanthine/xanthine oxidase system was inhibited by less than 10% by 100 μg/mL catalase while 1 μg/ml superoxide dismutase resulted in 80% inhibition. The oxidation of MPPNHOH to $MPPNO_2$ in the presence of a xanthine/xanthine oxidase system was reported earlier (Maynard & Cho, 1981). This system, which generates both superoxide and hydrogen peroxide, was used in this study to further investigate the role of superoxide in the oxidation. Figure 2 shows the effects of increasing concentrations of superoxide dismutase on the oxidation of MPPNHOH to $MPPNO_2$ by the xanthine/xanthine oxidase system. The formation of $MPPNO_2$ was inhibited more than 50% at a concentration of 0.5 μg/mL superoxide dismutase. MPPNO was also detected in these incubation mixtures at levels slightly higher than those for $MPPNO_2$. Overall, the levels of MPPNO appeared to decrease as the concentration of superoxide dismutase in the incubation mixture increased. Inhibition of $MPPNO_2$ formation by catalase was variable but was always less than 30% up to 80 μg/mL.

Thus, in both the reconstituted cytochrome P-450 oxidase system and the xanthine/xanthine oxidase system, most of the oxidation of MPPNHOH appears to be mediated by superoxide anion. The oxidation that occurs with reductase in the absence of cytochrome P-450 also appears to be mediated by superoxide. It is also possible, however, that hydrogen peroxide is involved in part of the cytochrome P-450 mediated oxidation of MPPNHOH since catalase was a better inhibitor and superoxide dismutase was a poorer inhibitor for oxidation by the reconstituted cytochrome P-450 oxidase system than for oxidation by the xanthine/xanthine oxidase system.

Hydroxyl radicals produced either via a Haber–Weiss reaction or a Fenton-type reaction could also participate in the oxidation process. To test this possibility, incubations of MPPNHOH with the xanthine/xanthine oxidase system were performed in the presence of mannitol and Me_2SO which have been found to be effective as hydroxyl radical scavengers (Cederbaum et al., 1978). Concentrations of mannitol between 0 and 80 mM were tested and had no effect on the oxidation of MPPNHOH to $MPPNO_2$. Me_2SO at a concentration of 80 mM increased the conversion of MPPNHOH to $MPPNO_2$ by 25%. These scavengers had no effect on the microsomal oxidation of MPPNHOH (Maynard & Cho, 1981). At the concentrations used in the experiments, neither mannitol nor Me_2SO affected the level of xanthine oxidase generated superoxide as measured by cytochrome *c* reduction. Thus, it appears unlikely that the oxidation of MPPNHOH to $MPPNO_2$ is mediated by hydroxyl radicals.

As already mentioned, MMPNO could be identified as a product from incubations of MPPNHOH with the reconstituted cytochrome P-450 oxidase systems and the xanthine/xanthine oxidase system. The amount of MPPNO that was recoverd from similar incubation mixtures was often quite variable, presumably due to air/O_2 oxidation during workup procedures, unless the dichloromethane used for extraction of the incubation mixture was bubbled with N_2 and the extracts kept on ice until the analysis by HPLC. This was an unwieldy procedure in a multitube assay so that the levels of the nitro compound were usually followed as a measure of oxidation.

To determine the role of MPPNO as an intermediate, the effect of superoxide dismutase on the oxidation of MPPNHOH to MPPNO and $MPPNO_2$ was investigated. MPPNHOH was incubated with a xanthine/xanthine oxidase system in the absence or presence of superoxide dismutase (20 μg/mL) or superoxide dismutase (20 μg/mL) plus catalase (20 μg/mL). The results (Table II) show that superoxide dismutase or superoxide dismutase plus catalase inhibited the formation of MPPNO and $MPPNO_2$ to similar extents. This is good evidence that superoxide is involved in the oxidation of MPPNHOH to MPPNO which is then further oxidized to

Table II: Effects of Superoxide Dismutase and Catalase on the Formation of MPPNO and $MPPNO_2$ from MPPNHOH by a Xanthine/Xanthine Oxidase System[a]

superoxide dismutase	catalase	MPPNO		$MPPNO_2$	
		expt 1	expt 2	expt 1	expt 2
–	–	100 (46.7)	100 (32.5)	100 (39.6)	100 (39.4)
+	+	21	17	2.6	1.4
+	+	4.5	0	1	0

[a] Incubations were performed for 15 min and products identified by HPLC as described under Methods. Superoxide dismutase (60 μg) and catalase (60 μg) were added as indicated. The values represent percent control activity. Parentheses indicate nanomoles of product formed in the control incubations and represent the 100% values. Each value is an average from four determinations.

Table III: $MPPNH_2$ *N*-Hydroxide by Reconstituted Cytochrome P-450 Oxidase System[a]

cytochrome P-450	superoxide dismutase	nmol of MPPNHOH	nmol of $MPPNO_2$
PB	–	15 ± 1	3.3 ± 0.6
PB	+	17.7 ± 0.6	0.7 ± 1
3MC	–	3	0
3MC	+	3	0

[a] Incubations were performed for 10 min in cytochrome P-450 reconstituted systems as described under Methods. Cytochrome P-450 was the major isozyme isolated from either PB- or 3MC-pretreated rats. Superoxide dismutase (60 μg) was added where indicated. Each value for the system with the PB isozyme is an average of three determinations, and each value for the 3MC system is an average of two determinations.

$MPPNO_2$ by oxygen. If superoxide oxidized MPPNHOH to $MPPNO_2$ by a pathway that did not involve MPPNO, then inhibition of the oxidation of MPPNHOH to MPPNO by superoxide dismutase would not have been expected.

N-Hydroxylation of $MPPNH_2$ by Reconstituted Cytochrome P-450 Oxidase Systems. The N-hydroxylation of $MPPNH_2$ by reconstituted systems that contained cytochrome P-450 from either PB- or 3MC-pretreated rats is shown in Table III. There was no detectable formation of MPPNHOH when either NADPH or reductase was omitted from an otherwise complete incubation mixture. The rates were much lower than those for the N-oxidation of MPPNHOH. The system that contained cytochrome P-450 from PB-pretreated rats was 6 times as active as the system that contained cytochrome P-448 from 3MC-pretreated rats. The low levels of MPPNHOH formed with 3MC precluded detection of $MPPNO_2$. Superoxide dismutase, as expected, substantially reduced $MPPNO_2$ formed during the incubation. MPPNO was not determined since it did not accumulate to significant levels when MPPNHOH was incubated with the reconstituted systems. In agreement with the finding that the oxidation of $MPPNH_2$ to MPPNHOH was not affected by superoxide dismutase, MPPNHOH could not be detected as a product when $MPPNH_2$ was incubated with a xanthine/xanthine oxidase system. Thus, the oxidation of $MPPNH_2$ to MPPNHOH appears to be mediated by the normal cytochrome P-450 pathway and is independent of superoxide.

Discussion

These data indicate that cytochrome P-450 can oxidize a hydroxylamine by a mechanistically distinct pathway involving superoxide that is generated in this case from the action of MPPNHOH as an uncoupler. An uncoupler is a compound that binds to cytochrome P-450 and causes an increase in the rate of NADPH utilization and oxygen consumption without undergoing oxidation itself. The oxygen can be reduced to superoxide (Kuthan et al., 1978), hydrogen peroxide (Nordblom & Coon, 1977), or water (Staudt et al., 1974). MPPNHOH had been shown previously to stimulate H_2O_2 (Cho et al., 1982) and superoxide (Maynard & Cho, 1981) production in microsomes by an NADPH-dependent mechanism.

However, an alternative mechanism for the generation of superoxide by the cytochrome P-450 system should be considered. Misra & Fridovich (1976) found that superoxide is produced and then serves as an oxidant in the oxidation of phenylhydrazine by iron(II) oxyhemoglobin. The process can be summarized as follows:

$$HbFe^{2+}\text{–}O_2 + R\text{–}NH\text{–}NH_2 \xrightarrow{H^+} HbFe^{3+} + R\text{–}NH\text{–}\dot{N}H + H_2O_2 \quad (1)$$

$$RNH\text{–}\dot{N}H + O_2 \rightarrow R\text{–}N{=}NH + O_2^- + H^+ \quad (2)$$

$$R\text{–}NH\text{–}NH_2 + O_2^- \xrightarrow{H^+} R\text{–}NH\text{–}\dot{N}H + H_2O_2 \quad (3)$$

In this sequence a radical is produced that can then reduce molecular oxygen to superoxide. According to this mechanism, the production of one phenylhydrazyl radical (step 1, initiation) can lead to the oxidation of many molecules of phenylhydrazine in steps 2 and 3 (propagation). If an analogous reaction sequence was the primary mechanism for the production of superoxide in incubations of the cytochrome P-450 system with MPPNHOH, then one molecule of NADPH should serve to initiate the oxidation of many molecules of MPPNHOH. However, in an earlier study (Cho et al., 1982), it was found that the rate of NADPH utilization was 6 times that of $MPPNO_2$ formation in incubations with microsomes from PB-pretreated rats. Such a result would be expected if MPPNHOH was acting as an uncoupler.

Flavoproteins such as xanthine oxidase (Fridovich, 1970), the mixed-function flavin amine oxidase (Rauckman et al., 1979), and NADPH-dependent cytochrome P-450 reductase (Fong et al., 1973) will also reduce oxygen to superoxide. Xanthine oxidase generates both superoxide and hydrogen peroxide during its oxidation of xanthine to uric acid. At pH 7, the ratio of superoxide to hdyrogen peroxide generation is about 1:4 (Fridovich, 1970). The oxidation of MPPNHOH to $MPPNO_2$ by the xanthine/xanthine oxidase system could be completely inhibited by the addition of superoxide dismutase but only slightly by the addition of catalase to the incubation mixture, indicating that superoxide, but neither hydrogen peroxide nor hydroxyl radicals, serves as the primary oxidant of MPPNHOH in this system. Further evidence against the involvement of hydroxyl radicals was the inability of hydroxyl radical scavengers to decrease oxidation of MPPNHOH to $MPPNO_2$ by the xanthine/xanthine oxidase system. Hydroxyl radicals also do not appear to be involved in the oxidation of MPPNHOH by potassium superoxide (Fukuto et al., 1985) or the oxidation of hindered secondary amines to nitroxides by a xanthine/xanthine oxidase system (Rauckman et al., 1979).

The data also indicate that superoxide serves as the primary oxidant for the oxidation of MPPNHOH by the cytochrome P-450 system since addition of superoxide dismutase to the

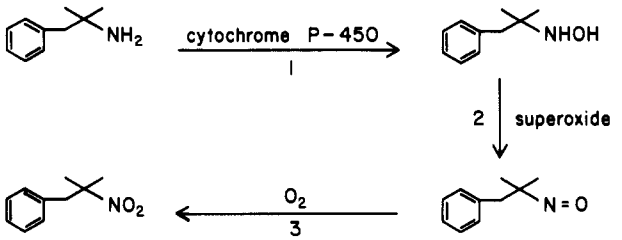

FIGURE 3: Proposed pathway for the N-oxidation of $MPPNH_2$ by the cytochrome P-450 system. Step 1 is mediated by a normal cytochrome P-450 reaction. Step 2 is mediated by a direct oxidation by superoxide generated from cytochrome P-450 via uncoupling and possibly also by a peroxidatic pathway involving cytochrome P-450 and hydrogen peroxide generated via uncoupling. Step 3 is a chemical oxidation, most likely by oxygen.

incubation mixture decreased the overall formation of $MPPNO_2$ to about 30% of control levels. That this reaction is chemically feasible is shown in the accompanying paper (Fukuto et al., 1985) in which superoxide is shown to oxidize MPPNHOH in both aqueous and organic media. In the case of the cytochrome P-450 system, O_2^- is generated as a result of the uncoupling interaction with MPPNHOH (Cho et al., 1982). The generated O_2^- might then oxidize both MPPNHOH bound to the active site of cytochrome P-450 and MPPNHOH that is free in solution. Thus, the O_2^- might need to diffuse only a short distance before it could oxidize substrate which is bound to cytochrome P-450. If this site were not accessible to superoxide dismutase, the reaction would not be sensitive to the enzyme. In this manner, the cytochrome P-450 mediated reaction would differ in its inhibition by superoxide dismutase from that mediated by xanthine and xanthine oxidase in which the O_2^- and MPPNHOH react in solution.

Addition of catalase to incubation mixtures which contained the reconstituted system inhibited the oxidation about 50% at the highest concentration tested. The oxidation of MPPNHOH can be mediated directly by hydrogen peroxide (0.05–0.1 M) in aqueous solutions near neutral pH (Fukuto et al., 1985). It is known that the cytochrome P-450 system can oxidize some substrates via a peroxidatic pathway (Nordblom et al., 1976), and evidence for some oxidation of MPPNHOH by such a pathway has been found (Maynard, 1982). The addition of either hydrogen peroxide or cumene hydroperoxide to partially purified cytochrome P-450 from rat liver supported the formation of N-oxidation products from MPPNHOH (Maynard, 1982). It is also possible that some oxidation of MPPNHOH occurs by a normal cytochrome P-450 reaction, independently of superoxide and hydrogen peroxide.

At the highest concentration tested, the antibody raised against the major isozyme of cytochrome P-450 from PB-pretreated rats inhibited 80% of the oxidation of MPPNHOH by rat liver microsomes. Therefore, it is likely that the majority of the oxidation of MPPNHOH by rat liver microsomes was mediated by the major PB-inducible isozyme of cytochrome P-450 which others have estimated represents about 80% of the total cytochrome P-450 in microsomes from PB-induced rats (Pickett et al., 1981; Ryan et al., 1982). The remainder of the activity might have been mediated by other cytochrome P-450 isozymes, NADPH-dependent cytochrome P-450 reductase, or the flavin amine oxidase. The major isozyme induced by 3MC appeared to be much less active in reconstituted oxidase systems than the isozyme isolated from PB-pretreated rats but might contribute slightly to the total microsomal oxidation.

MPPNO could be identified as a product from incubations of MPPNHOH with the reconstituted cytochrome P-450 system or the xanthine/xanthine oxidase system. However, substantially higher levels of MPPNO were recovered from incubations with the xanthine/xanthine oxidase system than with the reconstituted system. The results in Table III indicate that superoxide mediates the oxidation of MPPNHOH to MPPNO. This is in agreement with the chemical study in which it was demonstrated that potassium superoxide oxidizes MPPNHOH to MPPNO but does not oxidize MPPNO to $MPPNO_2$. MPPNO can be oxidized to $MPPNO_2$ by either oxygen or hyroperoxide anion (Fukuto et al., 1985). However, the much lower levels of MPPNO in incubation mixture with the reconstituted system and microsomes than in the xanthine/xanthine oxidase system indicate that MPPNO might be further metabolized to $MPPNO_2$ directly by the cytochrome P-450 system. It is also possible that the presence of lipid in the reconstituted system and in microsomes might facilitate the air oxidation of MPPNO. MPPNO exists predominantly as the dimer in aqueous solutions and as the monomer in lipophilic solvents. Partitioning into lipid phase would increase the total amount of monomer which could then be oxidized by air. Although a direct determination of the metabolic fate of MPPNO would clarify this point, such a study is complicated by the physical properties of MPPNO. MPPNO exists predominantly as a dimer in aqueous solution whereas the monomer is likely to be the actual substrate and appears to be the species oxidized by oxygen.

The overall pathway for the N-oxidation of phentermine by the cytochrome P-450 system involves three distinct steps which are outlined in Figure 3. $MPPNH_2$ is oxidized by a normal cytochrome P-450 dependent process to the hydroxylamine which acts as an uncoupler of the cytochrome P-450 system generating superoxide and hydrogen peroxide. Most of the oxidation of MPPNHOH to MPPNO is mediated directly by superoxide and appears to involve a radical mechanism in which a one-electron abstraction from MPPNHOH leads to the formation of a nitroxide (Fukuto et al., 1985). This nitroxide then disproportionates to MPPNO and MPPNHOH. Oxidation of MPPNHOH to MPPNO by hydrogen peroxide might also either occur directly or occur in conjunction with cytochrome P-450 via a peroxidatic pathway. Some oxidation of MPPNHOH to MPPNO by a normal cytochrome P-450 pathway cannot be excluded. MPPNO is oxidized directly by oxygen to $MPPNO_2$ with possibly some enzyme oxidation also occurring.

Several compounds are known that uncouple the cytochrome P-450 system to produce superoxide or hydrogen peroxide. MPPNHOH is unique in that it is an uncoupler and can also react with and consume superoxide. As a consequence, it serves as a substrate for a novel pathway of cytochrome P-450 oxidation. While it is possible that oxidation of hydroxylamines by superoxide might be a general reaction, the structural requirements for uncouplers appears to be stringent. For example, the structurally similar compound *N*-hydroxyamphetamine is not an uncoupler and instead leads to the formation of an inhibitory complex with cytochrome P-450 (Cho et al., 1982).

Registry No. MPPNHOH, 38473-30-2; MPPNO, 52497-67-3; $MPPNO_2$, 34405-43-1; $MPPNH_2$, 122-09-8; cytochrome P-450, 9035-51-2; NADPH-cytochrome P-450 reductase, 9039-06-9; superoxide, 11062-77-4; monooxygenase, 9038-14-6.

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Mechanism of Oxidation of *N*-Hydroxyphentermine by Superoxide†

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ABSTRACT: Cytochrome P-450 oxidizes *N*-hydroxyphentermine (MPPNHOH) by an indirect pathway involving superoxide. The chemical details of this oxidation, in which *N*-hydroxyphentermine is converted to 2-methyl-2-nitro-1-phenylpropane ($MPPNO_2$), have been elucidated by examining the interaction of MPPNHOH with superoxide in aqueous and organic solvents. The role of peroxide, hydroperoxy radicals, and oxygen in the reaction was also examined. The results indicate that superoxide itself is oxidizing MPPNHOH to a nitroxide that disproportionates to MPPNHOH and 2-methyl-2-nitroso-1-phenylpropane (MPPNO). MPPNO is then oxidized to $MPPNO_2$ by O_2 or hydroperoxide. Two possible mechanisms for the superoxide oxidation were considered, a proton abstraction and a hydrogen atom abstraction. Stoichiometric and oxygen evolution studies favor the hydrogen abstraction pathway.

Superoxide radical anion, $O_2^{-}\cdot$, is of interest in oxidations catalyzed by cytochrome P-450 because it is a potential by-product of some reactions. As it is both a radical and an anion, it has the potential for dual reactivity. As an anion, it can react as either a base or a nucleophile. Its reactivity is frequently dominated by its ability to act as a base. The conjugate acid of superoxide, HOO·, disproportionates via a bimolecular process to yield hydrogen peroxide and molecular oxygen, and the thermodynamics of this disproportionation has been proposed (Fee & Valentine, 1977) to be the basis for the effective pK_a of about 24 although the actual value is 4.69 in water (Sawyer & Valentine, 1981). In aprotic solvents, many of the oxidations attributed to superoxide can be accounted for by initial proton abstraction from the substrate to yield a substrate anion that is then oxidized by either O_2 or H_2O_2, the products of proton-induced disproportionation of superoxide (Nanni et al., 1980). The nucleophilic reactions of superoxide are usually seen only in aprotic, non-hydrogen-bonding solvents.

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