Biochemical Pharmacology, Vol. 30, No. 10, pp. 1115–1119, 1981.
Printed in Great Britain.

0006-2952/81/101115-05 $02.00/0
Pergamon Press Ltd.

OXIDATION OF *N*-HYDROXYPHENTERMINE TO 2-METHYL-2-NITRO-1-PHENYLPROPANE BY LIVER MICROSOMES

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(*Received* 14 *August* 1980; *accepted* 10 *November* 1980)

Abstract—The role of cytochrome(s) P-450 in the oxidation of *N*-hydroxyphentermine to 2-methyl-2-nitro-1-phenylpropane by liver microsomes was examined using inhibitors of this heme system. The reaction resembled the *N*-hydroxylation of phentermine and the *N*-demethylation of benzphetamine in its sensitivity to carbon monoxide, metyrapone, and 2,4-dichloro-6-phenylphenoxy-ethylamine, but it differed from these reactions in its inhibition by superoxide dismutase. Superoxide, generated by xanthine oxidase, was also capable of oxidizing *N*-hydroxyphentermine, but glucose oxidase generated hydrogen peroxide was not. Superoxide dismutase completely blocked nitro compound formation when substantial levels of hydrogen peroxide were present in the incubation mixture. These observations suggest that this hydroxylamine-nitro oxidation is mediated by microsome-generated superoxide.

Previous reports from this laboratory have described the *in vitro* *N*-hydroxylation of phentermine by rabbit [1] and rat liver microsome preparations. The reaction was sensitive to carbon monoxide and inducible with phenobarbital [2] indicating the participation of cytochrome P-450 in the oxidation. The subsequent metabolism of phentermine, i.e. oxidation of *N*-hydroxyphentermine (NOHP)† to2-methyl-2-nitro-1-phenylpropane (MNPP), wasdescribed in studies with rat liver microsomes and differed from the *N*-hydroxylation reaction in its inhibition by catalase and liver cytosol [3]. Thus, it is possible for an arylalkylamine to be oxidized at the nitrogen function to the nitro state *in vitro*.

Nitro compounds related to phentermine have also been reported as *in vivo* excretion products of *p*-chlorophentermine [4] and phentermine [5], so their origin and mechanism of formation are of interest in understanding the metabolic fate of aliphatic amines. This report describes results of a detailed study investigating the formation of MNPP from NOHP in rat and rabbit liver microsomes. Of particular interest was the sensitivity of the reaction to catalase since it suggested the participation of H_2O_2 in the reaction. This possibility, as well as the role of other active oxygen species, was examined, and the results indicate that superoxide, generated by P-450, is responsible for the conversion.

MATERIALS AND METHODS

Livers from male Sprague–Dawley rats (180–230 g) or male white New Zealand rabbits (2.2–2.5 kg) were used. Livers from two rats or one rabbit were homogenized (25%, w/v, in 1.15% KCl) and then centrifuged at 10,000 *g* for 20 min. The microsomal fraction was prepared by gel filtration on a Sepharose 2B column [6]. One milliliter of rat microsome suspension contained 3.85 ± 0.30 mg (mean ± S.E.) protein and 2.38 ± 0.14 nmoles cytochrome P-450. For normal rabbit liver, 1 ml of microsome suspension contained 3.12 ± 0.25 mg protein and 3.25 ± 0.20 nmoles cytochrome P-450.

One milliliter of microsome suspension was incubated with 4 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 2.0 μmoles NADP, 25 μmoles glucose-6-phosphate, 75 μmoles $MgCl_2$, 2 units glucose-6-phosphate dehydrogenase, and varying amounts of substrate. The NADPH-generating system was omitted in experiments using NADPH. All incubations were conducted at 37° in 25-ml Erlenmeyer flasks with shaking for 10 min in air unless noted differently. The carbon monoxide inhibition studies were conducted by bubbling CO/O_2 (2:1) through the reaction vessel for 5–7 min and then sealing the vessel. The reaction was terminated by the addition of 5 ml of cold CH_2Cl_2, followed by shaking in an ice–water bath for 5 min.

The procedures used for isolation and quantitation of NOHP and MNPP were identical to those described earlier [3].

Experiments conducted with enzyme systems other than microsomes were performed in 5 ml of 0.2 M potassium phosphate buffer, pH 7.5. Xanthine oxidase (0.05 units) was incubated with 1 mM xanthine, and glucose oxidase (0.04 units) was incubated with 0.2 M glucose. All incubations were conducted at 37° for 10 min and were terminated by extraction with cold CH_2Cl_2.

H_2O_2 was assayed spectrophotometrically by the formation of $Fe(SCN)_3$ [7], and O_2^- was determined by the oxidation of epinephrine to adrenochrome [8]. Measurement of microsomal cytochrome P-450

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† Abbreviations: NOHP, *N*-hydroxyphentermine; MNPP, 2-methyl-2-nitro-1-phenylpropane; SOD, superoxide dismutase; and DPEA, 2,4-dichloro-6-phenylphenoxy-ethylamine.

used the method of Omura and Sato [9], and protein was determined by the method of Lowry *et al.* [10] and/or the Bio-Rad Protein Assay with crystalline bovine serum albumin as the standard.

Chemicals. Phentermine hydrochloride was a gift of the Pennwalt Corp. (Rochester, NY). NOHP was synthesized from phentermine [11] and MNPP and 2-methyl-2-nitroso-1-phenylpropane by procedures described by Lindeke *et al.* [12]. Sodium azide was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and mannitol from the J. T. Baker Co. (Pillipsburg, NJ). The following chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO): $NADP^+$, NADPH, glucose-6-phosphate dehydrogenase, xanthine oxidase (21 units/ml), glucose oxidase (1385 units/ml), albumin, xanthine, catalase (2400 units/mg), and superoxide dismutase (2500 units/mg). 2,4-Dichloro-6-phenylphenoxyethylamine (DPEA) was a gift of Dr. R. E. McMahon of Lilly Research Laboratories (Indianapolis, IN).

RESULTS

The formation of MNPP from NOHP was linear with time for at least 20 min over the concentration range examined, was protein-dependent, and displayed saturation kinetics with a half-maximum concentration at approximately 0.2 mM (Fig. 1). The substrate and MNPP levels at the end of the incubation period accounted for at least 95 per cent of the original substrate level in both rat and rabbit, so that MNPP was the major metabolite under the conditions described. Some of the characteristics of NOHP oxidation to MNPP by rat and rabbit liver microsomes are shown in Table 1. For comparison, the oxidation of phentermine to NOHP was examined under the same conditions. NOHP formation has been shown [1, 2] to be P-450 mediated and displays the expected sensitivity to CO, DPEA and metyrapone. MNPP formation is also affected by these agents but differs in that it is inhibited by superoxide dismutase (SOD) and catalase and is enhanced by azide. Phentermine *N*-hydroxylation is increased by, and benzophetamine is insensitive to, these chemicals. The increase noted in the *N*-hydroxylation of phentermine reflects the inhibition of the oxidation of NOHP to MNPP [3]. The effects of these reagents on MNPP formation were about the same in both rat and rabbit tissue. In experiments not shown, 0.1 mM EDTA reduced MNPP formation in rat tissue by 25 per cent.

The inhibitory effect of catalase on MNPP formation had been observed previously [3], but the effects of SOD had not and, therefore, a more complete concentration effect curve was determined (Fig. 2). The reaction was essentially blocked at a SOD concentration of 12.5 units/ml, whereas a catalase concentration sufficient to scavenge all H_2O_2 had no effect on MNPP levels. A concentration of 20,000 units of catalase per ml was necessary for complete suppression of MNPP formation. Thus, SOD appears to have been a much better inhibitor of this oxidation reaction.

The effects of catalase and azide suggested that H_2O_2 was involved in the reaction, and those of SOD suggested that O_2^- could be responsible for this reaction. To determine the relative efficiencies of

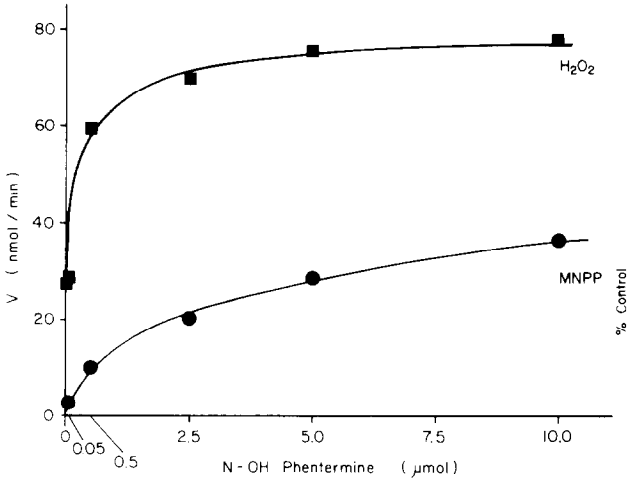

Fig. 1. Substrate concentration and MNPP, H_2O_2 formation in rat liver microsomes. NOHP at the indicated concentrations was incubated for 10 min with rat liver microsomes. The reaction was stopped, and H_2O_2 and MNPP were assayed in the incubate as described in Materials and Methods. The H_2O_2 values have had the substrate-free H_2O_2 levels subtracted and thus represent the substrate-dependent values. Each value shown is the mean of two experiments with each determination done in triplicate. The maximum rates of formation of H_2O_2 and MNPP are 45.9 nmoles/min and 28.2 nmoles/min respectively. The standard errors of the means are contained within the symbols of the data points.

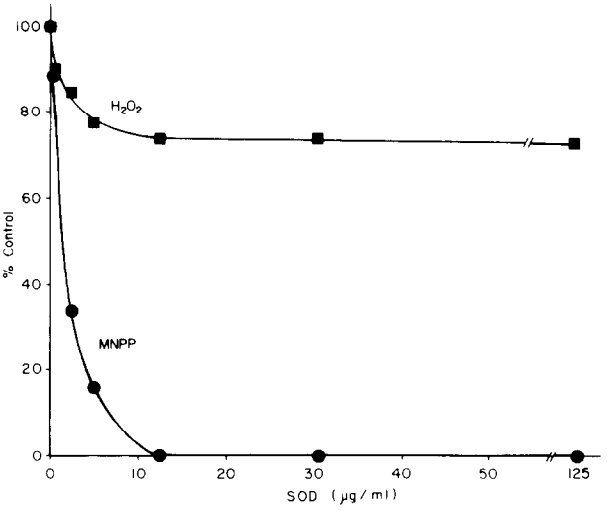

Fig. 2. Effects of superoxide dismutase (SOD) on H_2O_2 and MNPP levels. Superoxide dismutase at the indicated levels together with 1.0 mM NOHP and 0.5 mM azide were incubated with microsomes for 10 min, and the levels of H_2O_2 and MNPP were determined as described in Materials and Methods. Data are expressed as a percentage of the values of products formed in the absence of SOD, and represent the mean values of two experiments done in duplicate. The standard errors of the means are contained within the symbols of the data points.

Table 1. NOHP and MNPP formation in liver microsomes*

Product conditions	% NOHP		% MNPP		% HCHO
	Rat	Rabbit	Rat	Rabbit	Rat
Control	100	100	100	100	100
Minus cofactors	2	3	9	2	3
CO/O_2 (2:1)	46	15	22	17	—
DPEA (1 mM)	0	0	1	35	—
Metyrapone (1 mM)	36	59	52	75	—
SOD	123	108	34	23	98
Catalase	118	106	30	56	101
Azide (0.5 mM)	93	81	270	166	99

* The effects of different inhibitors on the formation of *N*-hydroxyphentermine (NOHP) from phentermine (4 mM), 2-methyl-2-nitro-1-phenylpropane (MNPP) from *N*-hydroxyphentermine (1 mM), and formaldehyde (HCHO) from benzphetamine (1 mM) were measured after incubation at 37° for 10 min in the presence of microsomes from the indicated species. Data are the means of at least two separate experiments with each determination in triplicate and with the following control values expressed as nmoles formed in 10 min ± S.E.: NOHP (rat, 20 + 1; rabbit, 55 + 2), MNPP (rat, 220 + 3; rabbit, 54 + 1), and HCHO (rat, 175 ± 3). Superoxide dismutase (SOD) levels were 2 μg/ml for NOHP incubations and 25 μg/ml for phentermine incubations. Catalase levels were 100 μg/ml for NOHP and 1 mg/ml for phentermine incubations.

Table 2. NOHP and MNPP formation in oxidase systems*

Conditions	MNPP (nmoles)
Glucose oxidase (0.04 units)/glucose (0.2 mM)	39.3 ± 3.6
−Glucose	30.2 ± 2.4
+Catalase	29.7 ± 1.9
Xanthine oxidase (0.05 units)/xanthine (1 mM)	219.6 ± 6.2
+SOD (2 μg/ml)	42.2 ± 3.0
+SOD (2 μg/ml) and catalase (100 μg/ml)	21.3 ± 0.9

* The formation of MNPP from NOHP (0.2 mM) was measured after a 10-min incubation at 37°. Each value is the mean ± S.E. of at least three determinations. Under these conditions, the glucose oxidase system generated 1.5 μmoles of H_2O_2 and the xanthine oxidase generated 0.6 μmole of O_2^-. When phentermine (4 mM) was incubated under these conditions, the levels of NOHP in the incubate were not detectable.

Table 3. H_2O_2 generation by liver microsomes*

Conditions	H_2O_2 (nmoles)	
	Rat	Rabbit
Control	272.5 ± 12.2	196.8 ± 10.4
−NADPH-generating system	29.1 ± 1.8	30.8 ± 2.0
+NOHP (4 mM)	872.0 ± 18.3	612.1 ± 16.3
+Benzphentermine (4 mM)	934.2 ± 20.2	679.4 ± 17.0
+Phentermine (4 mM)	470.5 ± 15.8	407.5 ± 14.9

* H_2O_2 levels were determined after a 10-min incubation of microsomes with cofactors and the indicated substances. Control refers to H_2O_2 generated in the absence of exogenously added substrates. Each value is the mean ± S.E. of at least six determinations. MNPP at 0.4 mM and 1-methyl-2-nitroso-1-phenylpropane at 0.1 mM reduced H_2O_2 levels slightly (approximately 20 per cent).

these reduced oxygen species, enzyme-generated O_2^- and H_2O_2 were compared for their abilities to oxidize phentermine and NOHP (Table 2). The activities of the two enzymes were such that the number of moles of H_2O_2 generated by glucose oxidase was about twice that of the moles of O_2^- generated by xanthine oxidase. In spite of the large H_2O_2 excess, the yield of MNPP from O_2^- (xanthine oxidase) oxidation was five times greater, indicating that suproxide is much more efficient in this conversion. Furthermore, the conversion with glucose oxidase was independent of glucose and insensitive to catalase, so that H_2O_2 may not be important for this effect. Neither of these enzyme systems was able to oxidize phentermine. The possible role of hydroxyl radicals was examined with ethanol, mannitol, and DMSO [13], but none of these compounds inhibited MNPP in rat tissue, and DMSO caused an increase in MNPP levels.

As another attempt to investigate the role of O_2^- or H_2O_2 in the reaction, levels of H_2O_2 formed during the oxidation were measured. Superoxide is formed by microsomes [14, 15] and dismutates to H_2O_2 so that the H_2O_2 levels measured reflect that which is formed directly [7, 16] and that formed by O_2^- dismutation. Table 3 shows the NADPH-dependent formation of H_2O_2 and the effects of several substrates. Phentermine, NOHP, and benzphetamine all caused increases in H_2O_2 levels in both animal species, with NOHP and benzphetamine being substantially higher. The NOHP concentration effect on H_2O_2 levels is shown in Fig. 1. Saturation is observed with a half-maximal NOHP concentration of 0.034 mM. In a control experiment, MNPP at 0.4 mM and 2-methyl-2-nitroso-1-phenylpropane reduced H_2O_2 levels slightly, so that these species were not responsible for the increase in H_2O_2 seen with NOHP.

This substrate-dependent H_2O_2 generation was sensitive to the P-450 reagents CO, DPEA and metyrapone (Table 4). In contrast to MNPP formation, however, H_2O_2 formation was partially inhibited (30 per cent) by superoxide dismutase at concentrations that completely prevented MNPP formation (Fig. 2).

DISCUSSION

Although the reaction sequence—phentermine to NOHP to MNPP—occurs in liver microsomes and appears to be P-450 dependent, there are major differences in the character of the first and second reactions. Chemically, the *N*-hydroxylation of phentermine is a two-electron oxidation that is the usual P-450-type oxidation, whereas the oxidation of NOHP to MNPP is a four-electron change. The two-electron intermediate, the nitroso state, has been described by Beckett and Belanger [17], but it could not be detected under the conditions described here using their assay procedure or by high pressure liquid chromatography (procedure to be reported). Therefore, if it is formed, it must be rapidly oxidized to MNPP. The nitroso state is formed as the major product under autoxidation conditions [18], and subsequent oxidation or reaction results in formation of styrene by elimination of the nitrogen function and only small quantities of MNPP. The nitroso compound also readily forms a dimer that is unstable in aqueous media [18]. Autoxidation is not likely to occur in the presence of microsomal protein [19], but other oxidants such as lead tetracetate [20] and *t*-butylhydroperoxide [21] also yield nitroso compounds upon reaction with aliphatic hydroxylamines. The nitro state is thus not a readily formed chemical oxidation product of this group of hydroxylamines.

The other distinguishing characteristic of MNPP formation is its sensitivity to scavengers for O_2^- and H_2O_2. Neither *N*-hydroxylation nor *N*-demethylation reactions displayed this sensitivity, which suggests that if O_2^- or H_2O_2 is involved in the reaction, the mechanism is not typical for P-450. OH· is unlikely as the oxidizing species because of the lack of inhibition by the family of OH· scavengers. The minimal effect of high (0.1 mM) concentrations of EDTA also eliminates possible Fe^{2+} catalyzed oxidation [22]. The formation of MNPP by O_2^- is the active species. The greater sensitivity of the reaction of SOD is also consistent with this interpretation, particularly since the commercially available catalase has been shown to have traces of SOD contamination

Table 4. NOHP-dependent H_2O_2 generation in liver microsomes

Conditions	H_2O_2 generated* (%)	
	Rat	Rabbit
Control	100	100
−NOHP	32	31
−NADPH-generating system	6	6
CO/O_2 (2:1)	38	31
DPEA (1 mM)	32	43
Metyrapone (1 mM)	44	45

* Controls (100 per cent) represent the amount of H_2O_2 generated from normal liver microsomes in the presence of an NADPH-generating system and NOHP (1 mM). Control values were: rat, 562 ± 15 nmoles H_2O_2 generated in 10 min; and rabbit, 483 ± 10 nmoles H_2O_2 generated in 10 min. The H_2O_2 formed by liver microsomes with cofactors and 1 mM NOHP was measured after incubation at 37° for 10 min under the additional conditions indicated.

[23]. A comparison of the effects of SOD on MNPP and H_2O_2 formation also supports the notion that H_2O_2 is an unlikely reactant since, at SOD concentrations that completely suppress MNPP formation, H_2O_2 levels remain high and are depressed by only 30 per cent (Fig. 2).

Superoxide is thought to be the initial product of the uncoupling of the P-450 oxidase system whereby molecular oxygen is reduced by P-450 without the corresponding oxidation of substrate [26]. The superoxide formed then dismutates to H_2O_2 which is measured [14, 25]. The uncoupling occurs when a substrate binds to the enzyme system but, because of a lack of appropriate chemical reactivity (e.g. absence of α-hydrogen), does not undergo reaction [24]. Uncoupling apparently does not have to be complete, and some P-450 substrates such as benzphetamine undergo reaction and generate H_2O_2 as well [26].

The data presented point out another possible route of drug metabolism in which a drug or its metabolite uncouples the P-450 system to generate an active oxygen species that can then chemically oxidize substrate to the isolated metabolite.

Acknowledgement—This research was supported by USPHS Grants GM26024 and CA9030.

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