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Norcocaine Nitroxide

A Potential Hepatotoxic Metabolite of Cocaine

ELMER J. RAUCKMAN, GERALD M. ROSEN, AND JOY CAVAGNARO

Departments of Surgery and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

Norcocaine nitroxide was found to be produced via the one-electron oxidation of *N*-hydroxynorcocaine by hepatic microsomal enzymes from induced and noninduced rats, hamsters, and mice in the presence of an NADPH-generating system. This reaction was demonstrated to be mediated by cytochrome P-450 as suggested by induction experiments using phenobarbital, which markedly enhanced the production of this nitroxide, and by the inhibition of this monooxygenase by metyrapone, which depressed the formation of this free radical. Unlike other nitroxides, norcocaine nitroxide was rapidly reduced by flavoproteins such as cytochrome P-450 reductase and FAD-monooxygenase, but not cytochrome P-450. We believe that since NADPH is consumed during the futile cycling of *N*-hydroxynorcocaine/norcocaine nitroxide and since NADPH is an essential cofactor of the glutathione reductase system, diminished reduced nucleotide may lead to depressed levels of cellular glutathione. In this manner, we theorize that cocaine initiates hepatotoxicity.

INTRODUCTION

The local anesthetic and central nervous system-stimulating properties of cocaine have been well recognized (1). However, in recent years, this pharmacological agent has also been shown to mediate hepatotoxicity induced in mice (2, 3). It is believed that a metabolite of cocaine and not cocaine itself is responsible for such hepatic necrosis. Evans *et al.* (4) originally proposed that the toxic intermediate was cocaine *N*-oxide. However, studies by Misra *et al.* (5) have demonstrated that the hepatotoxicity of cocaine *N*-oxide is probably due to its metabolism to cocaine, norcocaine, or some other metabolite. This latter hypothesis has been confirmed by Thompson *et al.* (6), who have presented data indicating that *N*-hydroxynorcocaine participates in cocaine-induced liver necrosis. Finally, Nayak *et al.* (7) have proposed that norcocaine nitroxide, a one-electron oxidation product of *N*-hydroxynorcocaine, may play an important role in initiating hepatic toxicity after cocaine treatment. However, it should be pointed out that Nayak *et al.* (7) presented no scientific data to support their suggestion that norcocaine nitroxide is produced during the bioactivation of cocaine to its hepatotoxic metabolite. Evans (8) and Evans and Johnson (9) have recently reported that mouse hepatic microsomes biotransform *N*-hydroxynorcocaine to its nitroxide and suggested that this free radical covalently binds to proteins (8).

The scientific literature, to date, points to the fact that norcocaine nitroxide is the metabolite responsible for cocaine-induced hepatotoxicity; however, nitroxides are known to be quite stable and relatively unreactive toward electrophilic and nucleophilic moieties (10). Furthermore, there is no evidence to support the theory that microsomal enzymes directly oxidize hydroxylamines to nitroxides. However, we have demonstrated that microsomal enzymes indirectly oxidize certain hydroxylamines to nitroxides via the intermediate superoxide (11). The purposes of this investigation were to examine the putative microsome-catalyzed oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide and to study the reactivity of this nitroxide.

Our results indicate that *N*-hydroxynorcocaine is oxidized to norcocaine nitroxide by control and phenobarbital-induced rat, mouse, and hamster hepatic microsomal cytochrome P-450 in the presence of NADPH and oxygen. Like other nitroxides, norcocaine nitroxide is reduced by hepatic microsomes and NADPH; however, contrary to our previous observations (12), this reaction is not mediated by cytochrome P-450 but by microsomal flavoproteins, including cytochrome P-450 reductase. In addition, this nitroxide was found to be unreactive with microsomal proteins in the absence of NADPH. Finally, we determined that both *N*-hydroxynorcocaine and norcocaine nitroxide stimulate microsomal NADPH oxidation.

MATERIALS AND METHODS

General comments. Unless otherwise stated, chemical reagents were purchased from Sigma Chemical Company (St. Louis, Mo.). The following chemicals or reagents

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were obtained from various sources: NADPH (Boehringer Mannhein Biochemicals, Indianapolis, Ind.); cocaine-HCl (Mallinckrodt, St. Louis, Mo.); Chelex-100 ion exchange resin, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.); metyrapone and 2,2,6,6-tetramethylpiperidine (Aldrich Chemical Company, Milwaukee, Wisc.). The nitroxide TEMPO[1] was synthesized by the oxidation of 2,2,6,6-tetramethylpiperidine with hydrogen peroxide (13). EPR spectra were recorded using a Varian Associates Model E-9 spectrometer.

Animals. Male Fisher-344 rats (125–200 g) and male LVG hamsters (90–110 g) were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Male DBA/2Ha mice (15–20 g) were obtained from Health Research, Inc. (Buffalo, N. Y.). All animals were maintained on corncob bedding for at least 10 days prior to use in rooms where soft wood bedding was not used. Animals had free access to Purina laboratory chow (No. 5001) and tap water. Phenobarbital-induced animals received 0.1% sodium phenobarbital in their drinking water for 4 days prior to sacrifice.

Microsomes. Washed microsomes were prepared from fresh livers by differential centrifugation of the liver homogenates produced with a Polytron homogenizer (Brinkmann Instrument Company, Westbury, N. Y.) in sucrose/EDTA buffer and were washed twice (resuspended and centrifuged at 100,000 × *g* for 40 min) with 1.25% KCl. The washed microsomal pellets were resuspended to a protein concentration of 2–30 mg/ml.

Preparation of compounds. Norcocaine was prepared from cocaine by the method of Baldwin *et al.* (14). Norcocaine nitroxide was synthesized from norcocaine by oxidizing norcocaine (100 mg, 346 μmoles) using *m*-chloroperbenzoic acid (250 mg, 1.44 mmoles) in 5 ml of methylene chloride for 10 min. This mixture was washed twice with 5% K_2CO_3, filtered, and evaporated to a volume of about 0.2 ml. This red solution was streaked on a 10 × 20 cm, 1000-μm silica gel GF preparative thin-layer chromatography plate (Analtech, Inc. Newark, Del.) and eluted with ethyl acetate. The red norcocaine nitroxide band (R_F = 0.65; the R_F for norcocaine is 0.50) was scraped off the plate and extracted from the silica gel with 95% ethanol. Generally, this ethanol solution was concentrated to 5 ml and used in reactions. Complete evaporation of this ethanol solution yields 35 mg of pure norcocaine nitroxide (15).

N-hydroxynorcocaine was prepared prior to its use by catalytic hydrogenation (platinum and EtOH, 1 atmos) of norcocaine nitroxide (20 mg) for 1 hr at 20°. This reaction is known to produce hydroxylamines from nitroxides (16).

EPR experiments. All of the incubation experiments concerning the microsomal oxidation of *N*-hydroxnorcocaine were conducted with an NADPH-generating system consisting of 3 mM glucose 6-phosphate, glucose 6-phosphate dehydrogenase (3 units/ml), and 3 mM magnesium chloride in 100 mM tricine buffer (pH 7.4). Unless otherwise indicated, *N*-hydroxynorcocaine was added as an ethanolic solution (10 mg/ml) to a final concentration of 0.4 mg/ml.

Experiments in which the reduction rates of nitroxides were determined, unless otherwise specified, were conducted with an NADPH concentration of 0.1 mg/ml and a microsomal protein concentration of 0.5–2.0 mg/ml in 100 mM phosphate buffer (pH 7.4). The K_m determinations for NADPH were conducted by varying the NADPH concentration between 4 μM and 20 μM and measuring the rate of disappearance of EPR signal from TEMPO (10 μM) or norcocaine nitroxide (35 μM).

The rate of reduction of norcocaine nitroxide by either purified FAD-containing monooxygenase (a gift from Dr. Daniel M. Ziegler, University of Texas, Austin, Tex.) or purified cytochrome P-450 reductase (a gift from Dr. Bettie Sue Masters, University of Texas, Dallas, Tex.) was determined by measuring the decrease in the height of the central peak of the nitroxide triplet as a function of time. In a typical experiment, the reaction medium contained 35 μM norcocaine nitroxide, NADPH (0.1 mg/ml, purified FAD-containing monooxygenase (0.05 mg/ml), or purified cytochrome P-450 reductase and sufficient buffer (0.1 M chelexed potassium phosphate) to bring the final volume to 0.5 ml.

RESULTS

Formation of norcocaine nitroxide. The addition of *N*-hydroxynorcocaine to microsomes obtained from non-induced (animals maintained on corncob bedding) mice, rats, or hamsters in the presence of an NADPH-generating system resulted in the formation of norcocaine nitroxide (Fig. 1). Norcocaine nitroxide was identified in the reaction mixture by virtue of its EPR spectrum, which was identical with that of norcocaine nitroxide prepared by the oxidation of norcocaine with *m*-chloroperbenzoic acid (Fig. 1). This nitroxide exhibits the characteristic hyperfine splitting pattern for a nitroxide with two β-hydrogen atoms (17). The norcocaine nitroxide signal appeared immediately[2] after the reaction was initiated by the addition of *N*-hydroxynorcocaine to the microsomes and an NADPH-generating system. The nitroxide signal increased in intensity for approximately 10 min and then slowly disappeared. If either the microsomes or the NADPH-generating system were deleted, no EPR signal was observable.

Microsomes obtained from phenobarbital-induced animals as compared with control animals produced a significantly greater norcocaine nitroxide EPR signal when incubated at equivalent microsomal protein concentrations. Since induced animals have markedly enhanced levels of cytochrome P-450 as compared with control animals, this observation suggests that cytochrome P-450 may be responsible for the one-electron oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide. To substantiate this possibility, metyrapone (0.1 mM) was added to the reaction mixture and found to inhibit formation of the nitroxide by 50% (0.4 mM inhibited 95%). Other inhibitors of this monooxygenase, e.g., carbon monoxide and butylated hydroxytoluene (18), were found to inhibit

[1] The abbreviations used are: TEMPO, 2,2,6,6-tetramethylpiperidinoxyl; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate; DETAPAC, *N,N*-bis[2-[bis(carboxymethyl)amino]ethyl]glycine.

[2] There is approximately a 30-sec time lag between commencement of the reaction and recording of the EPR signal.

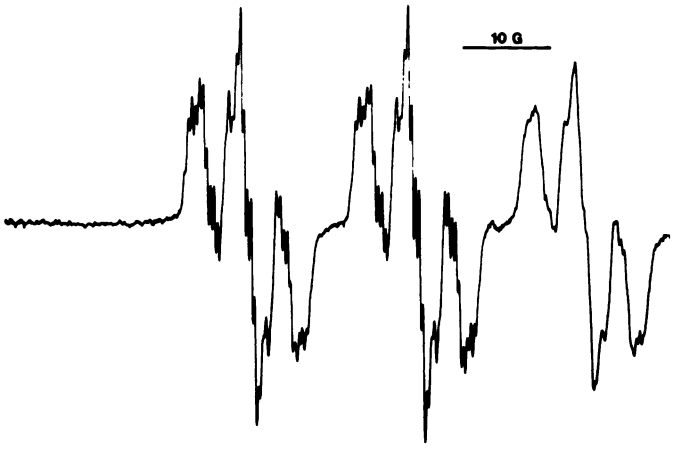

FIG. 1. *EPR spectrum of norcocaine nitroxide*

The spectrum was obtained from a dilute solution of synthetic norcocaine nitroxide in water at ambient temperature. The instrumental parameters were as follows: gain, 4×10^3; microwave power, 10 mW; modulation amplitude, 0.32 G; time constant, 0.3 sec; scan time, 8 min; scan range, 100 G; field set, 3385 G.

the oxidation of *N*-hydroxynorcocaine. In support of these observations, Evans (8) has recently reported that SKF 525-A prevented the formation of norcocaine nitroxide.

Oxygen was found to be required for the appearance of norcocaine nitroxide in these reaction mixtures. It was determined that *N*-hydroxynorcocaine exhibits a typical Type I binding spectrum with phenobarbital-induced rat microsomes (Fig. 2). All of these experimental results are consistent with the fact that cytochrome P-450 is the enzyme responsible for conversion of *N*-hydroxynorcocaine to norcocaine nitroxide; however, it is possible that either superoxide or hydrogen peroxide, produced as a by-product of microsomal enzymatic oxidation of NADPH, is the actual oxidizing species. Superoxide dismutase (0.2 mg/ml) or catalase (0.2 mg/ml) was added to the reaction mixture and found not to inhibit the formation or norcocaine nitroxide.

The norcocaine nitroxide EPR spectrum could not be obtained if NADPH (0.5 mM) was used instead of an NADPH-generating system (maintaining NADPH concentration at about 5 μM). Norcocaine nitroxide was also not observed if as little as 5 μl (final protein concentration

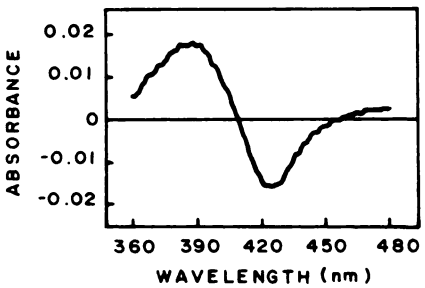

FIG. 2. *Difference spectrum of N-hydroxynorcocaine with phenobarbital-induced rat liver microsomes*

The spectrum was obtained according to the method of Jeffcoate (20). Microsomal protein concentration was 1.5 mg/ml in 100 mM phosphate buffer (pH 7.4). *N*-Hydroxynorcocaine was added to a final concentration of 0.70 mM.

1.0 mg/ml) of autologous dialyzed cytosol was added to the reaction mixture.

Reactivity of norcocaine nitroxide. The instability of the norcocaine nitroxide signal is easily understood if the reactivity of norcocaine nitroxide with components of the reaction mixture is investigated. Norcocaine nitroxide is relatively stable, and a solution of it in phosphate buffer (pH 7.4) decomposes less than 1% within 1 hr. However, norcocaine nitroxide is rapidly reduced by hepatic microsomes in the presence of NADPH or NADH, and the rate of the signal loss is proportional to the quantity of microsomes added. Without either pyridine nucleotide, there is a neglible reduction of norcocaine nitroxide by microsomes; however, NADPH or NADH was found to react, at a significant rate, directly with norcocaine nitroxide (Table 1). Dialyzed hepatic cytosol was likewise found to be unreactive with norcocaine nitroxide unless NADPH was added to the reaction mixture, at which point the norcocaine nitroxide EPR signal disappeared rapidly.

Michaelis constants for the reduction of norcocaine nitroxide by microsomes in the presence of reducing equivalents were determined. The K_m for norcocaine nitroxide with rat microsomes at constant NADPH at a constant norcocaine nitroxide concentration was determined to be 3.8 μM. The K_m for NADPH, if TEMPO is used as a substrate, was determined to be 1.8 μM. This represents the K_m for cytochrome P-450 reductase, since this is where NADPH binds. These data further support the theory that different enzymes may be involved in the microsomal reduction of norcocaine nitroxide and TEMPO.

Purified enzyme studies were conducted to determine what enzymes may be responsible for the microsomal reduction of norcocaine nitroxide. Table 2 shows that both of the purified flavoproteins, cytochrome P-450 reductase (rat liver) and FAD-containing monooxygenase (porcine liver) catalyzed the reduction of this nitroxide in the presence of either NADPH or NADH. The turnover number for cytochrome P-450 reductase was considerably larger than that for FAD-containing monooxygenase (Table 2). However, one cannot predict which enzyme has the more important role in the microsomal reduction since cytochrome P-450 reductase is coupled

TABLE 1

Reduction of norcocaine nitroxide and TEMPO by non-induced rat hepatic microsomes

Mixture	Rate of substrate reduction[a]	
	TEMPO	Norcocaine nitroxide
	μmoles/min/mg protein	
NADPH	0.0	2.2
NADPH + microsomes	2.9	17.0
NADPH + microsomes + metyrapone	1.0	16.8
NADH	0.0	1.2
NADH + microsomes	0.7	8.9
NADH + microsomes + metyrapone	0.4	8.8

[a] Rates were determined by following the loss of EPR signal intensity with time. The NADPH and NADH rates were divided by the protein values so they are comparable directly to the enzymatic rates.

TABLE 2

Reaction of norcocaine nitroxide with NADPH, NADH, and purified enzymes

Mixture[a]	NCN reduction[b]	Superoxide production[c]
	nM/min	*nM/min*
NADPH + NCN	580	160
NADH + NCN	640	158
NADPH + FADM	—	26
NADPH + FADM + NCN	940	391
NADH + FADM	—	12
NADH + FADM + NCN	900	400
NADPH + RED	—	618
NADPH + RED + NCN	4320	395
NADH + RED + NCN	1000	—

[a] FADM, FAD-containing monooxygenase; RED, cytochrome P-450 reductase; NCN, norcocaine nitroxide.

[b] Norcocaine nitroxide reduction was measured by loss of EPR signal intensity. NCN = 3 μM; NAD(P)H = 55 μM; FADM = 170 nM; RED = 44 nM in tricine buffer [*N*-tris(hydroxymethyl)methyl glycine (Sigma Chemical Company)] (pH 8.3) containing 1 mM DETAPAC.

[c] Superoxide production was measured as the superoxide dismutase (10 μg/ml)-inhibitable cytochrome *c* reduction. The cytochrome P-450 reductase superoxide production was measured using succinylated cytochrome *c*. Conditions were the same as in footnote *b* except that the norcocaine nitroxide concentration used was 30 μM.

to cytochrome P-450 whereas FAD-containing monooxygenase is the terminal oxidase in its system.

The reduction of norcocaine nitroxide to *N*-hydroxynorcocaine is a one-electron process. If the agent or enzyme reducing norcocaine nitroxide is a nonradical, it must undergo a one-electron oxidation to a free radical species. In the case of NAD(P)H this would result in the formation of a pyridinium nucleotide free radical, which is a species known to react with oxygen to produce superoxide (19). The production of superoxide during the reduction of norcocaine nitroxide to *N*-hydroxynorcocaine by NAD(P)H, cytochrome P-450 reductase, and FAD-containing monooxygenase was determined and the results are presented in Table 2. NADPH and NADH were found to generate about equal quantities of superoxide when reducing norcocaine nitroxide. This nitroxide was determined to stimulate greatly the production of superoxide from FAD-containing monoxoygenase; however, norcocaine nitroxide inhibited the formation of superoxide by cytochrome P-450 reductase. This observation supports the proposition that this flavoprotein is able to transfer electrons, one at a time, to one-electron acceptors, e.g., cytochrome P-450.

The reactivity of norcocaine nitroxide with low molecular weight sulfhydryl compounds was investigated on the basis of the hypothesis that glutathione protects liver against cocaine-induced hepatotoxicity (8, 9). Neither reduced glutathione (1 mM) nor cysteine (1 mM) was found to react with norcocaine nitroxide in the presence of chelexed phosphate buffer (0.1 M, pH 7.4) or phosphate buffer (0.1 M, pH 7.4) containing 0.1 mM DETAPAC. However, in the absence of the chelator (DETAPAC) or in nonchelexed buffer, glutathione reduced norcocaine nitroxide. The sulfhydryl reagents dithiothreitol and β-mercaptoethanol were found to react almost instantaneously with norcocaine nitroxide, resulting in the total loss of the nitroxide EPR signal.

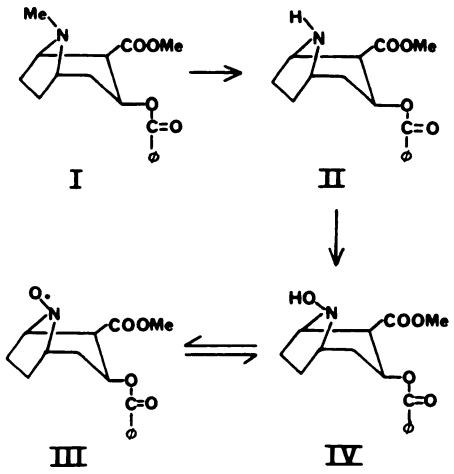

FIG. 3. *Structure of compounds*

I, Cocaine; *II*, norcocaine; *III*, *N*-hydroxynorcocaine; *IV*, norcocaine nitroxide.

DISCUSSION

Previous studies indicated that neither rat nor hamster microsomal enzymes could initiate the conversion of *N*-hydroxynorcocaine to norcocaine nitroxide (9). However, our results clearly demonstrate that rat, mouse, and hamster microsomal enzymes are capable of carrying out this oxidation. Our observation that cytosol plus NADPH rapidly converts norcocaine nitroxide to an EPR-invisible species and that the addition of small amounts of cytosol to the *N*-hydroxynorcocaine-microsomal NADPH-generating system mixture prevents the appearance of the norcocaine nitroxide spectrum indicates that contamination of microsomes with cytosol could be a major factor in the inability to observe this microsomal conversion by EPR spectrometry.

The oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide is a one-electron oxidation. We have previously reported that the microsomal one-electron oxidation of hydroxylamines to nitroxides is a result of microsomal superoxide production and not direct enzymatic conversion (11). *N*-Hydroxynorcocaine seems to be an exception to this observation in that the data indicate that cytochrome P-450 directly mediates this oxidation. The microsomal oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide is enhanced by phenobarbital induction; the microsomal conversion is inhibited by metyrapone and SKF 525-A (8, 9); and a Type I binding spectrum is observed for *N*-hydroxynorcocaine with phenobarbital-induced microsomes. Compounds which exhibit a Type I binding spectrum are almost invariably substrates for cytochrome P-450 (20).

Cytochrome P-450-mediated one-electron oxidations are unusual. Stier and Reitz (21) initially proposed that cytochrome P-450 catalyzed the oxidation of *N*-hydroxy-2,2,6,6-tetramethylpiperidine to its nitroxide; however, we have not been able to reproduce these results and have found that the superoxide radical generated in microsomes is responsible for this oxidation (11). Floyd *et al.* (22) have observed that nitroxides are formed from *N*-acetyl-*N*-hydroxy-2-aminofluorene by the action of lipid hydroperoxides and hemoproteins. However, the evidence for a direct oxidation of this compound by

cytochrome P-450 using NADPH and oxygen as cofactors is weak. Thus, as far as we know, the data presented here for the oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide represent the best-documented case of a one-electron-mediated cytochrome P-450 reaction using NADPH and oxygen as cofactors.

The observation that near steady-state levels of norcocaine nitroxide are maintained for at least 10 min in these incubation mixtures and the observation that norcocaine nitroxide is rapidly reduced by microsomes and NADPH indicate that microsomal oxidation of *N*-hydroxynorcocaine and microsomal reduction of the resulting norcocaine nitroxide occur at the same rate. After about 10 min, the reductive phase appears to predominate, since the EPR signal slowly disappears. Initially, we thought that this was caused by the reaction mixture's becoming anaerobic; thus, since oxygen is required for the oxidative reaction, the reductive reaction would predominate. This was found not to be the case. When the reaction mixture allowed to incubate for 20 min in the EPR cell (at which time the norcocaine nitroxide signal had diminished to about one-third of its maximal size) was removed, bubbled with oxygen for 20 sec, and reinserted into the instrument, the EPR signal did not increase. One possibility is that *N*-hydroxynorcocaine is depleted during the incubation period; however, when the concentration of microsomes added to the incubation mixture was decreased 5-fold, the time course for the disappearance of the norcocaine nitroxide was not changed. Another explanation, and the one we favor, is that a subpopulation of cytochrome P-450 responsible for the oxidation of *N*-hydroxynorcocaine to its nitroxide is destroyed during the incubation period by lipid peroxidation. The addition of 0.4% vitamin E (in ethanol) to the reaction mixture was found to maintain the level of norcocaine nitroxide at a steady state for at least 25 min. This observation is consistent with the second hypothesis.

Norcocaine nitroxide can be considered to be a relatively stable nitroxide, since in phosphate buffer it decomposes about 1% within 1 hr. Nitroxides which are fully substituted at the α position are generally stable for long periods of time (10). The chemical reactivity of nitroxide radicals is relatively low; in fact, a wide variety of chemical reactions may be conducted on compounds containing the nitroxide moiety without affecting it (10). However, nitroxides may participate in oxidation-reduction reactions and generally are reduced with a half-wave potential around 300 mV (23). Ascorbate and ferrous ions, for example, are known to reduce nitroxides (10). It is often stated, based on a report by Morrisett and Drott (24), that sulfhydryls reduce nitroxides; however, we have found that this is not correct if divalent cation impurities are removed from the buffers or if chelators (e.g., DETAPAC) are added.[3] Even powerful sulfhydryl reductants such as dithiolthreitol do not appreciably reduce typical nitroxides such as TEMPO. However, norcocaine nitroxide is rapidly reduced by dithiolthreitol and β-mercaptoethanol, but not by glutathione or cysteine. This suggests that norcocaine nitroxide has a greater reactivity toward one-electron reductions than do other nitroxides. NADPH and NADH were found to react directly with norcocaine nitroxide, causing loss of its EPR signal. More common nitroxides do not react directly with NADPH or NADH under these conditions.

The one-electron reduction of norcocaine nitroxide by cytochrome P-450 reductase is unusual in that we have not previously observed such a reaction with this enzyme; however, the primary function of this reductase is one-electron transfer (to cytochrome P-450), and the one-electron reduction of other xenobiotics by this enzyme is well known (25). On the other hand, reaction of norcocaine nitroxide with FAD-containing monooxygenase is unprecedented since, other than superoxide production (11), this enzyme has never been demonstrated to undergo one-electron reactions.

It is known that cocaine administration to mice results in depletion of glutathione and that depressed levels of this thiol are somehow linked to hepatic necrosis (6). The lack of direct reactivity of norcocaine nitroxide with glutathione eliminates direct conjugation or reduction of the nitroxide as a possibility to account for glutathione depletion. The lack of norcocaine nitroxide reduction when reduced glutathione and dialyzed cytosol were incubated with the nitroxide indicates that cytosolic glutathione peroxidase does not participate in the reduction of norcocaine nitroxide. On the basis of these observations, we suggest that cocaine-induced glutathione loss is more a result of NADPH depletion with the concomitant buildup of excessive oxidized glutathione which is actively excreted by the hepatocyte to maintain a normal GSH:GSSG ratio. The determination that NADPH is consumed both in the conversion of *N*-hydroxynorcocaine to norcocaine nitroxide and in its subsequent reduction back to *N*-hydroxynorcocaine suggests that a futile cycle is created resulting in eventual loss of cellular NADPH. Since NADPH is also the cofactor necessary for the conversion of oxidized to reduced glutathione via glutathione reductase, a depletion of NADPH theoretically can result in an accumulation of oxidized glutathione, which can lead to its efflux from liver (26). This mechanism is also consistent with the established relationship of exogenous hydroperoxide administration to oxidized glutathione efflux. Sies *et al.* (27) have demonstrated that oxidized glutathione is released from isolated perfused liver in the presence of exogenously administered hydroperoxide with a concomitant decrease in the NADPH:$NADP^+$ ratio as observed by surface fluorescence. With this in mind, Sies *et al.* (28) have proposed that, when levels of NADPH are diminished beyond a critical level, the cell's ability to reduce oxidized glutathione to glutathione is compromised.

In conclusion, we have demonstrated that norcocaine nitroxide is an unusual nitroxide which we believe participates in the hepatotoxicity of cocaine by virtue of its one-electron oxidation-reduction cycling between *N*-hydroxynorcocaine and norcocaine nitroxide. The cytochrome P-450-mediated conversion of *N*-hydroxynorcocaine to norcocaine nitroxide utilizes NADPH, as do the flavoprotein reductions of the nitroxide to the hydroxylamine. The direct reaction of norcocaine nitroxide with NADPH may also result in the oxidation of a significant amount of NADPH. These reactions may result in a

[3] E. J. Rauckman, G. M. Rosen, and J. Cavagnaro, unpublished observations.

depletion of NADPH with a concomitant decrease in the GSH:GSSG ratio. These factors, combined with other (as yet unidentified) elements, result in cocaine-induced hepatotoxicity. There is no evidence that norcocaine nitroxide binds covalently with cellular proteins by direct reaction of the nitroxide with nucleophilic substituents on the protein. The ability of microsomal preparations from rats, mice, and hamsters to convert *N*-hydroxynorcocaine to norcocaine nitroxide correlates well with the occurrence of hepatotoxicity in these species. Our data clearly demonstrate that mouse, rat, and hamster hepatic microsomes can oxidize *N*-hydroxynorcocaine to norcocaine nitroxide with approximately equal facility; however, only mice are susceptible to this hepatotoxicity (8). This suggests that factors other than just the ability of cells to produce norcocaine nitroxide are involved in the hepatotoxicity of cocaine. It is not known whether or not rat and hamster liver cells actually convert *N*-hydroxynorcocaine to norcocaine nitroxide *in vivo*. Perhaps these species have the ability to hydrolyze and excrete cocaine at a rate which does not allow a dangerous level of *N*-hydroxynorcocaine to accumulate. Other possibilities are that rats and hamsters have a greater capacity to produce NADPH or that these species have another system with which to deal with norcocaine nitroxide. Establishing why mice and not rats or hamsters demonstrate cocaine-induced hepatotoxicity will add to our understanding of the role of norcocaine nitroxide in cocaine-induced hepatotoxicity.

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Send reprint requests to: Dr. Gerald M. Rosen, Department of Pharmacology, Duke University Medical Center, Durham, N. C. 27710.