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Initiation of *in Vitro* Lipid Peroxidation by *N*-Hydroxynorcocaine and Norcocaine Nitroxide

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SUMMARY

Norcocaine nitroxide and *N*-hydroxynorcocaine were found to stimulate hepatic microsomal lipid peroxidation *in vitro*, as measured by spin-trapping techniques using the spin trap α-[4-pyridyl-1-oxide]-*N*-*tert*-butylnitrone. It was determined that either norcocaine nitroxide or *N*-hydroxynorcocaine markedly enhanced the rate of spin trapping of lipid peroxyl radicals when added to hepatic microsomal preparations. Glutathione, in the presence of dialyzed cytosol, inhibited the formation of lipid peroxyl spin-trapped adducts. This finding suggests that cytosolic glutathione-dependent enzymes perhaps including glutathione peroxidase play an important role in the prevention of norcocaine nitroxide- or *N*-hydroxynorcocaine-mediated lipid peroxidation.

Cocaine has been found to cause hepatotoxicity in both induced and noninduced mice (1, 2). Extensive studies have determined that cocaine must be biotransformed before it can initiate hepatotoxicity (3–5). Recent findings have demonstrated that the toxic intermediate is most likely norcocaine nitroxide (6–8); however, the mechanism by which this nitroxyl free radical initiates liver damage remains unclear. In two recent abstracts, Evans (6) and Evans and Johnson (7) reported that *in vivo* binding of cocaine to hepatic protein correlated well with the degree of hepatotoxicity observed. On the basis of this finding and the fact that norcocaine nitroxide appears to be the species responsible for cocaine-mediated hepatotoxicity, these investigators (6, 7) suggested that convalent binding of norcocaine nitroxide to cellular macromolecules was the mechanism by which hepatocellular damage was mediated.

Contrary to this hypothesis, we have demonstrated that if norcocaine nitroxide binds to cellular proteins, as suggested by Evans (6, 7), it is not by a direct covalent reaction of the nitroxide free radical with cellular macromolecules (8). Furthermore, we have shown that norcocaine nitroxide uncouples hepatic microsomal enzymes such as FAD-containing monooxygenase and, in so doing, produces superoxide (8). With the generation of superoxide and the potential formation of hydrogen peroxide from the oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide (9) as well as from the dismutation of superoxide (10), it is possible that lipid peroxidation may be an important factor responsible for cocaine-induced hepatotoxicity.

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Because of the uncertainty as to the mechanism responsible for cocaine-mediated hepatotoxicity, we decided to investigate whether or not lipid peroxidation may be a viable mechanism to account for such cellular damage. Our results demonstrate that *in vitro* lipid peroxidation is greatly stimulated by the cocaine metabolites *N*-hydroxynorcocaine and norcocaine nitroxide.

Unless otherwise stated, chemical reagents were purchased from Sigma Chemical Company (St. Louis, Mo.). The following chemicals or reagents were obtained from various sources: NADPH (Boehringer Mannhein Biochemicals, Indianapolis, Ind.); Chelex-100 ion exchange resin, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.), and 4-POBN[2] (Aldrich Chemical Company, Milwaukee, Wisc.). Norcocaine nitroxide was synthesized according to the procedures of Baldwin *et al.* (11) and Rauckman *et al.* (8). *N*-Hydroxynorcocaine was prepared prior to use by catalytic hydrogenation (platinum and ethanol, 1 atmos) of norcocaine nitroxide (20 mg) for 1 hr at 20°. This reaction is known to produce hydroxylamines from nitroxides (12). Under these conditions, *N*-hydroxynorcocaine is stable for several hours.

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.

Washed hepatic microsomes from rat, hamster, and mouse were prepared by differential centrifugation of the liver homogenates produced with a Polyton homogenizer

[2] The abbreviation used is: 4-POBN, α-[4-pyridyl-1-oxide]-*N*-*tert*-butylnitrone.

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(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 1.15% KCl. The washed microsomal pellets were resuspended to a protein concentration of 10 mg/ml as measured by the dye binding assay developed by Bradford (13).

Spin trapping of free radicals during norcocaine nitroxide-induced lipid peroxidation was undertaken using the water-soluble spin trap 4-POBN. In a typical experiment, the reaction medium contained 0.1 M 4-POBN; 250 μM NADPH; 0.1 ml of liver microsomal suspension (2 mg/ml final volume, from rat, hamster, or mouse); 10 μM *N*-hydroxynorcocaine; and sufficient buffer [0.1 M sodium phosphate, which had been passed through a Chelex-100 ion exchange column as described by Poyer and McCay (14) to remove divalent metal ion impurities, and 0.1 mM diethylenetriaminepentaacetic acid, pH 7.4] to bring the final volume to 0.5 ml. When these experiments were conducted in the absence of *N*-hydroxynorcocaine, the rate of spin-trapping free radicals was 10 times slower than in the presence of this hydroxylamine. The following necessary control experiments were performed, and no discernible EPR signals were detected: 4-POBN was incubated with microsomes in the presence and absence of *N*-hydroxynorcocaine without NADPH, and 4-POBN was incubated with NADPH without hepatic microsomes in the absence of *N*-hydroxynorcocaine. When the latter experiment was conducted in the presence of *N*-hydroxynorcocaine, it was possible to detect a very small quantity (at maximal gain on the EPR spectrometer) of 4-POBN-OOH which rapidly decomposed into an EPR-invisible species (15). This observation suggests that a small quantity of norcocaine nitroxide was not reduced to *N*-hydroxynorcocaine. It is also possible that oxygen might have oxidized *N*-hydroxynorcocaine to norcocaine nitroxide. Once this free radical is produced, it will uncouple NADPH to generate superoxide, which apparently is spin-trapped by 4-POBN (8).

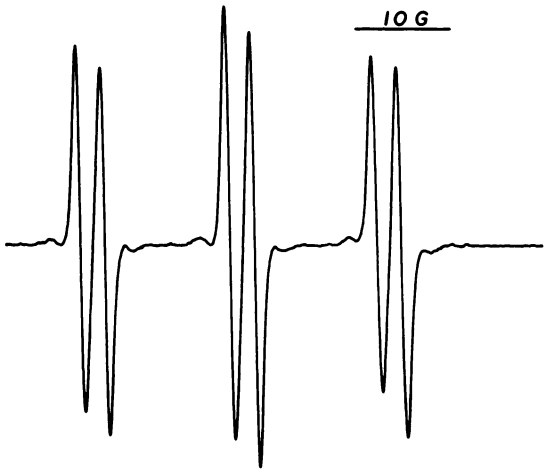

FIG. 1. *EPR of the spin-trapped adduct*

The EPR spectrum of 4-POBN-OOL (the spin-trapped adduct resulting from the reaction of 4-POBN with lipid peroxyl radicals) was observed when *N*-hydroxynorcocaine was incubated with mouse hepatic microsomes and NADPH in the presence of 4-POBN, A_N = 15.8 G and A_H = 2.6 G. The microwave power was 20 mW and the modulation frequency was 100 kHz with an amplitude of 0.63 G. The sweep time was 12.5 G/min and the response time was 0.3 sec.

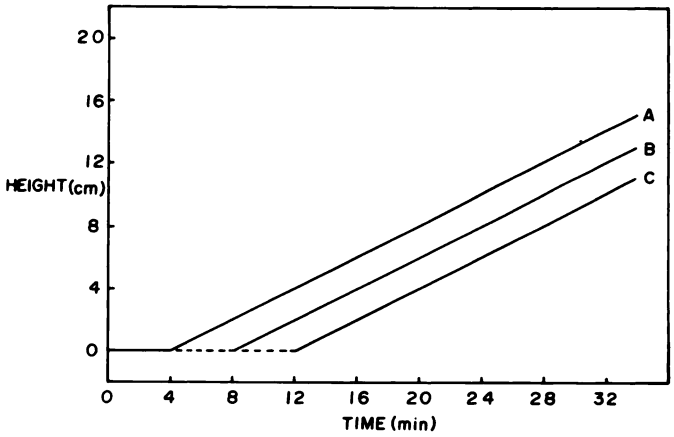

FIG. 2. *Rate of formation of the spin-trapped lipid peroxyl radical as a function of time*

Measurement of the rate of spin-trapped lipid peroxyl radical formation as a function of time when *N*-hydroxynorcocaine was incubated with mouse hepatic microsomes and NADPH in the presence of 4-POBN (*A*), with the addition of mouse hepatic cytosol (*B*), and with the addition of mouse hepatic cytosol and glutathione (0.1 mM) (*C*). The EPR spectrometer was set at the top of the middle peak of the nitroxide triplet (field set was 3383 G). The microwave power was 10 mW and the modulation frequency was 100 kHz with an amplitude of 1.0 G. The receiver gain was 1 × 10^4. The sweep time was 6.25 G/min and the response time was 1.0 sec.

Incubation of *N*-hydroxynorcocaine with mouse hepatic microsomes and the spin trap 4-POBN in the presence of NADPH led to the formation of the spin-trapped adduct whose EPR spectrum is shown in Fig. 1. Similar results were found when either rat or hamster hepatic microsomes were substituted for mouse hepatic microsomes in the above experiment. Identification of the free radical spin-trapped by 4-POBN was made using the model system of Fe^{3+}-linoleic acid and 4-POBN as well as our previous experience with spin-trapping lipid peroxyl radicals in hepatic microsomes (16, 17). When NADPH was omitted from the reaction mixture, no EPR signal was detected. When *N*-hydroxynorcocaine was omitted from the reaction mixture, an EPR spectrum was observed which was identical with that depicted in Fig. 1; however, the rate of spin trapping was only 10% of that observed in the presence of *N*-hydroxynorcocaine. On the basis of these findings, we conclude that lipid peroxyl radicals were generated during the oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide (8) and spin-trapped by the acyclic nitrone 4-POBN.

Substituting norcocaine nitroxide for *N*-hydroxynorcocaine in the above-described experiments presented one minor problem. Since the EPR signal from norcocaine nitroxide overlapped that of the lipid peroxyl spin-trapped adduct, the appearance of this latter nitroxide was possible only when the concentration of norcocaine nitroxide was significantly diminished. For this reason, it was impossible to determine the initial rate of spin-trapping lipid peroxyl radicals in the presence of norcocaine nitroxide. Nevertheless, these observations clearly demonstrate that lipid peroxyl radicals can be spin-trapped

during either the microsomal oxidation of *N*-hydroxynorcocaine to norcocaine nitroxide (8) or the reduction of norcocaine nitroxide to *N*-hydroxynorcocaine (8). Since norcocaine nitroxide is reduced to *N*-hydroxynorcocaine and this hydroxylamine is then oxidized back to norcocaine nitroxide, it is impossible to determine under the present experimental conditions whether lipid peroxyl radical formation is the result of the oxidative or reductive step, or both.

The ability of glutathione peroxidase to prevent the spin trapping of lipid peroxyl radicals was investigated since this enzyme is known to protect cells against the toxic effects of lipid hydroperoxides (18). When mouse hepatic microsomes, *N*-hydroxynorcocaine, and 4-POBN were incubated with dialyzed mouse hepatic cytosol in the presence of NADPH, the rate of spin-trapping lipid peroxyl radicals demonstrated a several-minute lag before the appearance of the nitroxide became apparent. Once the spin-trapped adduct became EPR-visible, the rate of formation was no different from that observed in the absence of dialyzed cytosol (Fig. 2). To the above reaction mixture was added reduced glutathione (0.1 mM), and the following observations were noted. Although lipid peroxyl radicals were spin-trapped, the appearance of this nitroxide free radical was significantly delayed as compared with either control or control in the presence of dialyzed cytosol (Fig. 2). However, once spin trapping had commenced, the rate was no different from that observed under control conditions. Finally, reduced glutathione (0.1 mM) was incubated with mouse hepatic microsomes, *N*-hydroxynorcocaine, 4-POBN, and NADPH and it was determined that the presence of reduced glutathione did not retard the rate of lipid peroxyl radical spin trapping as compared with control.

These observations suggest that, during the *in vitro* cycling of *N*-hydroxynorcocaine to norcocaine nitroxide, lipid peroxidation occurs as measured by the spin trapping of lipid peroxyl radicals by 4-POBN. This process can be retarded by glutathione-dependent cytosolic enzymes, which may include glutathione peroxidase acting on lipid hydroperoxides.Whether lipid peroxidation occurs *in vivo* and is the mechanism responsible for cocaine-mediated hepatotoxicity remains to be determined; however, our findings clearly demonstrated that, during the metabolic cycling of *N*-hydroxynorcocaine to norcocaine nitroxide and back again, free radicals are generated which lead to *in vitro* lipid peroxidation.

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