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Reexamination of the Microsomal Transformation of *N*-Hydroxynorcocaine to Norcocaine Nitroxide

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SUMMARY

Cocaine is known to be associated with hepatotoxicity in laboratory animals, and there is recent evidence that it also induces liver damage in humans. In both cases an *N*-oxidative pathway is responsible. Cocaine (NCN) is first *N*-demethylated to norcocaine, followed by oxidation to *N*-hydroxynorcocaine (NCNOH) and norcocaine nitroxide (NCNO•). On the basis of ESR studies of NCNOH with rat liver microsomes, it has been proposed that NCNO• induces hepatotoxicity by futile redox cycling between NCNO• and NCNOH at the expense of NADPH. The reaction is reported to be accompanied by formation of superoxide and lipid peroxyl radicals. It has also been reported that the same toxic sequence occurs with rat brain microsomes, leading to the formation of reactive free radicals in the brain. We have reexamined the microsomal metabolism of NCNOH to investigate the mechanism more thoroughly. Spin traps [5,5-dimethyl-1-pyrroline *N*-oxide and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone] were used to investigate the formation of reactive free radicals, including superoxide, in liver and brain microsomal incubations. In agreement with the literature, we detected a six-line spectrum of a radical adduct of α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone from liver microsome incubations. In contrast, our results showed that brain microsomes were completely inactive, contrary to the literature. In addition, we did not find any NCNO•- or NCNOH-dependent formation of superoxide with either brain or liver microsomes.

Cocaine is known to be associated with hepatotoxicity in laboratory animals (1), and there is recent evidence that it also induces liver damage in humans (2). It is extensively metabolized in the human body by several major pathways, principally spontaneous or enzymatic ester hydrolysis (2, 3). The quantitatively less important *N*-oxidative pathway, primarily taking place through liver oxidases, leads to the metabolites believed to be most closely associated with hepatotoxicity in animal models, and recent work suggests that the same mechanism operates in humans (4). The initial step in cocaine oxidation is *N*-demethylation to NCN, followed sequentially by oxidation to NCNOH (5) and NCNO• (6, 7). Evans and Johnson (6, 7) used ESR spectroscopy to show that mouse microsomal enzymes produced a nitroxide free radical from NCNOH. NCNO• is a member of a class of nitroxides in which the nitroxide nitrogen is joined to two bridgehead carbon atoms of a bicyclic structure (8). Radicals of this type containing α-hydrogens are protected from the normal disproportion, which yields a hydroxylamine and a nitrone, because the resulting nitrone double bond would violate Bredt's rule, which states that in small, bridged, bicyclic compounds double bonds at the bridgehead are impossible.

In a series of papers Rosen and co-workers (9–13) compared independently synthesized NCNO• with the nitroxide produced by microsomal metabolism of NCNOH to clearly identify the latter as NCNO•. They suggested that, even though nitroxides are generally considered to be relatively stable and unreactive free radicals, NCNO• precipitated cocaine hepatotoxicity by futile redox cycling between NCNOH and NCNO• at the expense of NADPH and eventually depleted reduced glutathione (8, 9). The proposed mechanism of hepatotoxicity includes the formation of the superoxide radical during NCNO•/NCNOH redox cycling. On the basis of spin-trapping experiments with the spin trap 4-POBN, they reported that NCNO•/NCNOH mediated lipid peroxyl radical formation. They proposed that microsomal lipid peroxidation was stimulated by NCNOH and played an important role in hepatic toxicity (12). However, a number of recent studies have disputed this proposal (2, 5, 14).

In contrast to the proposed toxicity of NCNO•/NCNOH, many stable nitroxides that undergo redox cycling, such as 2,2,6,6-tetramethylpiperidineoxyl (15), are not hepatotoxic, even at high dose levels (1, 16–18). Charkoudian and Shuster (19) believe that NCNO• is not the final reactive metabolite and that it must be converted to a more toxic product. They showed that NCNO• can easily be oxidized to the highly reactive NCN nitrosonium ion, which reacts readily with glutathi-

ABBREVIATIONS: NCN, norcocaine; NCNO•, norcocaine nitroxide; NCNOH, *N*-hydroxynorcocaine; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; RLM, rat liver microsomes; RBM, rat brain microsomes.

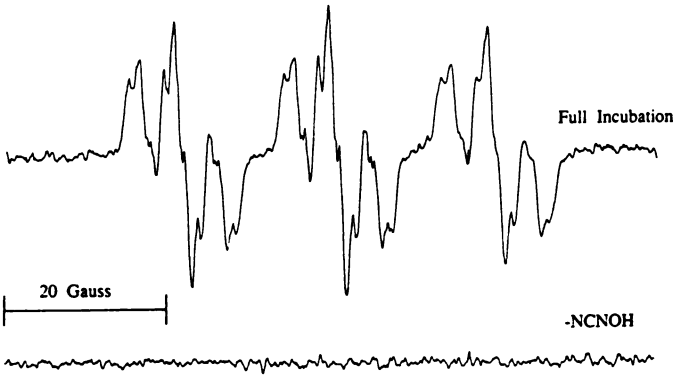

Fig. 1. Microsomal formation of NCNO•. *Full Incubation*, ESR spectrum obtained with the complete incubation as described in Materials and Methods. *–NCNOH*, same incubation minus NCNOH, showing that the spectrum requires NCNOH. Instrument settings (on a Varian E-109 spectrometer) for all experiments, except as indicated, were as follows: scan range, 80 G; scan time, 8 min; time constant, 1 sec; modulation amplitude, 0.33 G; microwave frequency, 9.335 GHz; microwave power, 20 mW; receiver gain, 4×10^4 (typical).

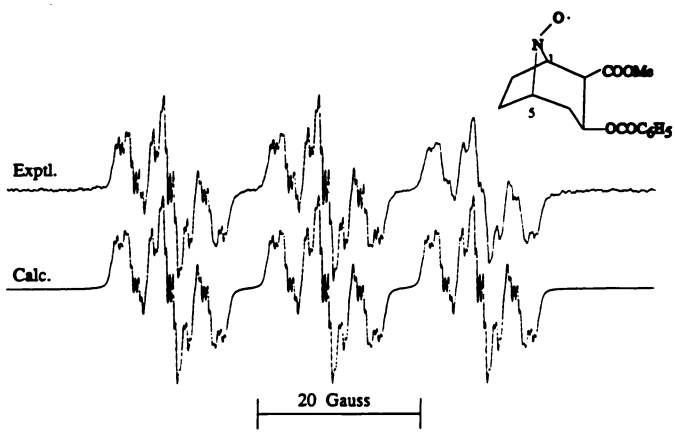

Fig. 2. ESR spectrum of NCNO•. *Exptl.*, spectrum obtained for pure NCNO• in distilled water, showing the nitrogen triplet and hydrogen hyperfine couplings. *Calc.*, simulation based on the hyperfine coupling constants listed in Table 1.

one. Depletion of glutathione could then contribute to cocaine hepatotoxicity. In fact, nitroxides actually inhibit free radical-mediated toxicity such as the hydrogen peroxide-induced damage to *Escherichia coli* (20), V79 Chinese hamster cells (21), and beating cultured cardiomyocytes (16). In addition, the hypoxic cytotoxicity of mitomycin C (22) and the aerobic radiation damage to V79 cells (23) are inhibited by nitroxides.

Finally, because NCN has been shown to be a cocaine metabolite in rat brain (24), Kloss *et al.* (11) attempted to demonstrate that RBM are also effective in producing NCNO• from NCNOH. Although the authors stated that RBM had far lower specific activity than did RLM (only 10% as much), so that lower concentrations of free radical metabolites per mg of protein would be expected, the ESR evidence presented implies that the activities of RBM and RLM are essentially equal (11, 12).

In this work we attempted to resolve the inconsistencies in the literature regarding the reported brain microsomal activity. Also, we wished to investigate more thoroughly the roles of superoxide and lipid peroxide radicals in the microsomal transformation of NCNOH.

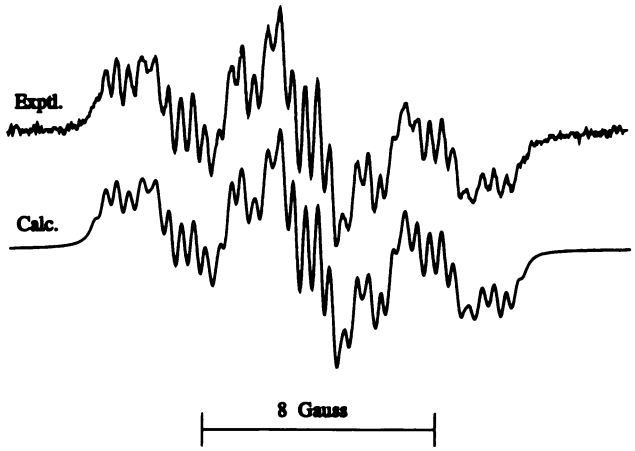

Fig. 3. Low-field peak-expanded ESR spectrum of NCNO•. *Exptl.*, ESR spectrum of the low-field peak of the nitrogen triplet, run under high-resolution conditions. *Calc.*, simulation based on the hyperfine coupling constants listed in Table 1, with the nitrogen coupling omitted. Instrument settings: scan range, 25 G; scan time, 8 min; time constant, 0.5 sec; modulation amplitude, 0.033 G; microwave power, 2 mW; receiver gain, 1.6×10^4.

TABLE 1

Hyperfine coupling constants (HFCC) for NCNO•

Nucleus	HFCC	
	This work	Literature[a]
	G	
N	19.14	18.0
H-β (2)[b]	4.265	4.25
H	1.813	
H	1.227	
H	0.990	
H	0.478	
H	0.427	
H	0.385	
H	0.364	
H	0.265	

[a] Ref. 28.
[b] The number in parentheses indicates the number of equivalent protons.

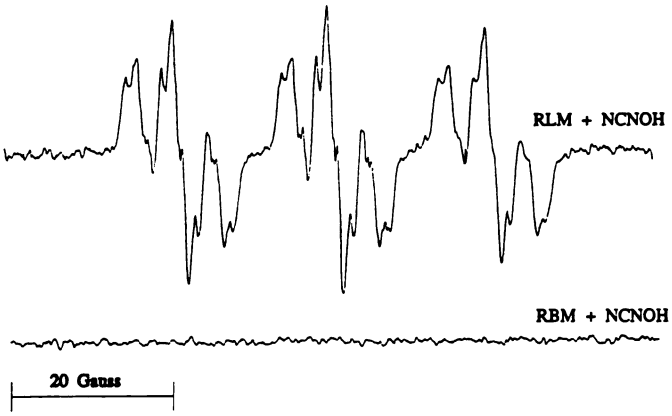

Fig. 4. Comparison of RLM and RBM. *RLM + NCNOH* (repeat Fig. 1), EPR spectrum obtained with the standard incubation prepared as described in the text, using RLM. *RBM + NCNOH*, spectrum obtained with the standard incubation but prepared with RBM instead of RLM. The conditions, including the concentration of microsomal protein, were the same for both RLM and RBM incubations.

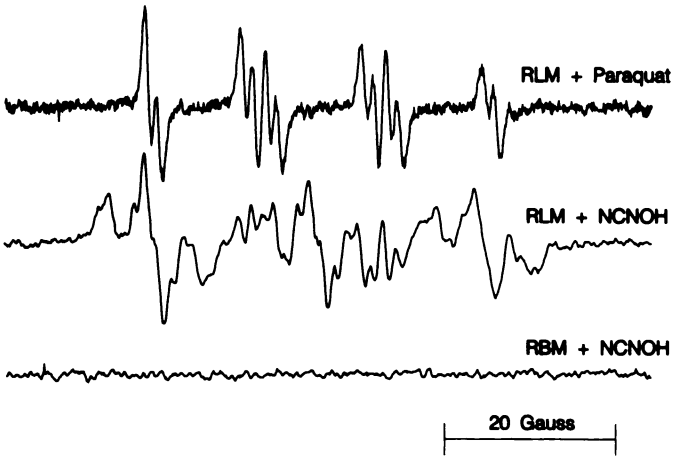

Fig. 5. ESR spectra of incubations with DMPO and possible superoxide-generating systems. *RLM + Paraquat*, standard incubation prepared with RLM, except containing 250 μM paraquat instead of NCNOH, with 100 mM DMPO. *RLM + NCNOH*, standard incubation prepared with RLM, including NCNOH, with 100 mM DMPO. *RBM + NCNOH*, standard incubation prepared with RBM instead of RLM, including NCNOH, with 100 mM DMPO.

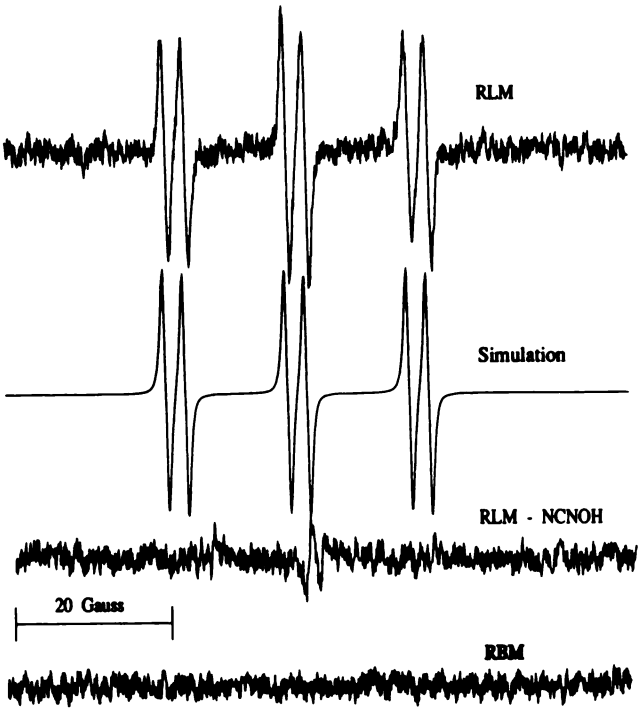

Fig. 6. ESR spectrum of NCNOH/NCNO•-dependent 4-POBN-radical adduct. *RLM*, standard incubation prepared with RLM, with 100 mM 4-POBN added. *Simulation*, simulation of the spectrum above, with a^N = 15.58 G and a^H_β = 2.50 G. *RLM − NCNOH*, standard incubation prepared with RLM, with 100 mM 4-POBN added but with the NCNOH substrate omitted. *RBM*, standard incubation prepared with RBM instead of RLM, with 100 mM 4-POBN added.

Materials and Methods

NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and deferoxamine mesylate (desferal) were obtained from Sigma. The spin traps DMPO and 4-POBN were also from Sigma. The DMPO was purified by vacuum distillation at room temperature and stored at −70° under a nitrogen atmosphere. Superoxide dismutase and catalase were from Boehringer Mannheim. The tricine buffer (100 mM, pH 7.4) used as the stock solution was stirred overnight with Chelex 100 ion exchange resin (Bio-Rad) before use.

NCNOH was synthesized from NCN (provided by the National Institute on Drug Abuse) according to the method of Beckett *et al.* (25), as modified by Thompson *et al.* (26). NCNO• was prepared by oxidation of NCNOH with *m*-chloroperbenzoic acid, according to the method of Rauckman *et al* (9). In some cases, the nitroxide was isolated by preparative thin layer chromatography as described by those authors. In others, it was stored at −20° as a red solution in a few hundred microliters of methylene chloride.

RLM were prepared from phenobarbital-treated male Sprague-Dawley rats according to published methods (27), and the final pellets were vortexed in tricine buffer before use. RBM were prepared from 17-day-old female Sprague-Dawley rats as described by Kloss *et al.* (11). Microsomes were analyzed for protein content by the Lowry assay. Solutions were made up with tricine buffer except for NCNOH, which was prepared as a 10 mg/ml solution in ethanol. Standard incubations contained microsomes (2 mg of protein/ml), 0.4 mg/ml NCNOH substrate (1.3 mM), 3 mM $MgCl_2$, and an NADPH-generating system consisting of 3 mM glucose-6-phosphate, 3 units/ml glucose-6-phosphate dehydrogenase, and 0.1 mM NADPH in tricine buffer (100 mM, pH 7.4), to make a total volume of 0.50 ml.

Immediately after mixing, incubation mixtures were pipetted into a 17-mm quartz flat cell, which was then placed in a TM_{110} microwave cavity. EPR spectra were obtained at room temperature with a Varian E-109 X-band spectrometer, interfaced to a personal computer for data acquisition.

Results

With RLM in standard incubations, a strong EPR signal corresponding to NCNO• was obtained (Fig. 1). There was no signal in the absence of the NCNOH substrate. The spectrum was identical to that obtained for the synthetic nitroxide in distilled water (Figs. 2 and 3) and to the previously reported spectrum (9). Simulations were done with a locally written, least-squares fitting program, and the hyperfine coupling constants obtained are listed in Table 1. Coupling was observed between the unpaired electron and all protons, and the best simulation (highest correlation coefficient) was obtained with two large and equivalent proton couplings, in agreement with the work of Rozantsev and Sholle (28). The three remaining protons with larger couplings are likely on the three methylene carbons (carbons 4, 6, and 7; see Fig. 2 for numbering scheme), but specific assignments would be highly speculative. In experiments in which up to 500 units/ml superoxide dismutase or up to 13,000 units/ml catalase was added to the standard incubation, there was no significant change in the intensity of the NCNO• signal. The addition of 50 μM desferal to chelate adventitious iron also had no effect on NCNO• concentration. In spite of numerous attempts, we were unable to reproduce the literature claim (11) of nitroxide formation from NCNOH when RBM were used in place of RLM in an identical incubation with an equal concentration of protein (Fig. 4).

We investigated the possible formation of superoxide in the microsome-NCNOH incubations with the spin trap DMPO. Addition of paraquat, which is known to produce superoxide in rat liver microsomal incubations containing NADPH (29), provided a positive control for superoxide formation. As can be seen in Fig. 5, an excellent DMPO/•OOH spectrum similar to that reported for RBM incubations containing either NCNO• or NCNOH was detected (11). A microsomal incubation containing RLM, an NADPH-generating system, and DMPO (100 mM) gave a weak composite DMPO/•OOH and DMPO/•OH

spectrum commonly seen in microsomal systems (30), dominated by the expected NCNO· spectrum. A control experiment without the NCNOH substrate gave a relatively weak spectrum of the same DMPO adducts (data not shown). An experiment repeated with the identical concentration of protein from RBM showed no ESR signal whatsoever, again contrary to the literature report (11).

In a second spin-trapping experiment, in which 4-POBN (100 mM) was added to the incubation, RBM and RLM again behaved differently (Fig. 6). For consistency with the work of Kloss *et al.* (11), a 100 mM phosphate buffer treated with Chelex and containing 100 μM diethylenetriaminepentaacetic acid was used in place of tricine buffer, and the concentration of NCNOH was only 10 μM instead of 1.3 mM. RLM gave the six-line 4-POBN-radical adduct spectrum reported previously (13), but in our hands RBM again did not show any activity. The NCNO· spectrum was not observed, presumably because of the much lower substrate concentration.

The identity of the NCNOH-dependent 4-POBN-radical adduct detected in RLM incubations is also in dispute (31). Based on the similarity of the hyperfine coupling constants reported for this radical adduct to those reported for radical adducts detected during lipoxygenase-catalyzed peroxidation of linoleic acid and in other peroxidation systems, the trapping of lipid peroxyl radicals was reported (13). Subsequent investigations have shown that this 4-POBN-radical adduct is most probably due to the trapping of one or more carbon-centered lipid-derived free radicals (32–36).

Discussion

On the basis of experiments with RBM, Rosen and coworkers (11, 12) proposed a mechanism for NCNO· formation and cocaine-mediated hepatotoxicity that included the generation of superoxide and lipid peroxyl radicals by RLM and RBM. We have been unable to confirm any of their ESR results with RBM and, thus, could not corroborate their mechanism. We found no indication of free radical formation and no evidence for any RBM activity under conditions in which RLM activity was readily detectable. The hypothesis that the known presence of NCN in the brain necessarily leads to enhanced free radical production is not supported.

Because we could not detect an increase in superoxide generation in the presence of NCNOH, we do not believe that redox cycling of NCNOH/NCNO· leads to superoxide formation in either RLM or RBM systems.

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