Liver Toxicity from Norcocaine Nitroxide, an N-Oxidative Metabolite of Cocaine

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ABSTRACT

The oxidative metabolism of cocaine to norcocaine nitroxide has been postulated to be essential for cocaine hepatotoxicity. The hepatic effects of norcocaine nitroxide have never been evaluated in vivo, however. In this study mice were administered norcocaine nitroxide i.p., and hepatotoxicity was assessed using serum alanine aminotransferase activities and microscopic examination of liver tissue. Hepatotoxicity of norcocaine nitroxide was dose-related; significant injury was detectable at doses of 20 to 30 mg/kg i.p., and severe hepatic necrosis was observed at doses of 40 and 50 mg/kg. Elevated serum alanine aminotransferase activities peaked between 12 and 18 hr after norcocaine nitroxide treatment. Electron microscopy revealed the presence of pronounced changes in cell morphology as early as 30 min after the norcocaine nitroxide dose. Pretreatment of mice with phenobarbital had no effect on the magnitude of hepatic injury but shifted the intralobular site of necrosis from the midzonal to the perportal region. Pretreatment with diazoxon, an esterase inhibitor, increased norcocaine nitroxide-induced liver damage, whereas each of the P450 inhibitors SKF 525A, cimetidine, trenalol and mycin, ketoconazole and chloramphenicol significantly diminished norcocaine nitroxide hepatotoxicity. The results indicate that norcocaine nitroxide is hepatotoxic and suggest the involvement of P450 enzymes.

Cocaine has been found to produce hepatic necrosis in mice (Shuster et al., 1977; Evans and Harbison, 1978; Freeman and Harbison, 1981), rats (Watanabe et al., 1987) and humans (Perino et al., 1987; Wanless et al., 1990; Kanel et al., 1990; Silva et al., 1991; Radin, 1992). Cocaine hepatotoxicity has been studied most extensively in mice, where it appears that cytochrome P450-mediated oxidation of cocaine is required for toxicity to occur (see Kloss et al., 1984; Boelsterli and Goldlin, 1991; and Roberts et al., 1992 for reviews). Through the oxidative pathway, cocaine is N-demethylated to norcocaine (Kloss et al., 1983a), which is further metabolized to N-hydroxynorcocaine (Kloss et al., 1982). N-Hydroxynorcocaine is then oxidized to norcocaine nitroxide (Misra et al., 1979; Kloss et al., 1984). P450 enzymes have been shown to participate in each of these steps of oxidative cocaine metabolism, and there is evidence that flavin-containing monoxygenase may also partially mediate the metabolism of cocaine to norcocaine (Rauckman et al., 1982a; Kloss et al., 1983a). There has been speculation that norcocaine nitroxide, which is a stable radical, undergoes a further, one-electron oxidation to produce a highly reactive nitrosonium species (Charkoudian and Shuster, 1985).

Evidence for the role of oxidative cocaine metabolism in its hepatotoxicity comes principally from experiments that examined the effects of enzyme inducers and inhibitors on cocaine-induced liver injury. In general, pretreatments that increase P450 enzyme activity or decrease the activity of competing esteratic metabolism increase cocaine hepatotoxicity, whereas P450 inhibitors block or diminish liver injury from cocaine (Roberts et al., 1992a). The intermediate metabolites norcocaine and N-hydroxynorcocaine are hepatotoxic when administered directly to mice (Thompson et al., 1979), with potency greater than or equal to that of cocaine. The toxicity of both can be blocked by pretreatment with the P450 inhibitor SKF 525A (Thompson et al., 1979), which suggests that at least one additional oxidative step beyond N-hydroxynorcocaine formation is required to produce the toxic species.

Although evidence clearly points to the nitroxide metabolite as critical in the hepatotoxic effects of cocaine, there has been little study of the effects of this metabolite on the liver. Incubation of norcocaine nitroxide with mouse hepatic microsomal suspensions has been shown to stimulate lipid peroxidation (Rosen et al., 1982; Kloss et al., 1983b), but there has not yet been any direct evaluation of the toxicity of norcocaine nitroxide in vivo. In order to gain a better understanding of the potential role of the nitroxide metabolite of cocaine

ABBREVIATIONS: ALT, alanine aminotransferase; ESR, electron spin resonance; TEM, transmission electron microscopy; TLC, thin-layer chromatography.
in its hepatotoxicity, we synthesized norcocaine nitroxide and administered it to mice. To facilitate comparisons with cocaine, a mouse strain (ICR) used previously in studies of cocaine hepatotoxicity in this laboratory was utilized for these experiments. We report here the results of studies examining both temporal aspects and dose-response relationships for toxicity, as well as the importance of both P450 and esteratic metabolism in the hepatic effects of norcocaine nitroxide.

Materials and Methods

Chemicals and reagents. Cocaine hydrochloride, treoleandomycin, ketaconazole and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, MO). Norcocaine nitroxide was synthesized from norcocaine according to method of Rauckman et al. (1982a). Structure was confirmed by ESR, and the purity was found to be 90% by TLC and HPLC. Cimetidine and SKF 525A (2-diethylamilonoate 2,2-diphenyl valerate) were a generous gift from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Diazinon (phosphothioic acid O,O-diethyl O-(6-methyl-2(1-methylethyl)-4-pyrimidinyl) ester) was purchased from Chem Service, Inc. (West Chester, PA), and sodium phenobarbital, U.S.P., N.F. was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA).

Animals and treatments. Male ICR mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 32 to 34 g were housed five per polycarbonate cage on corn cob bedding and given free access to food (Purina 5001, Ralston Mills, St. Louis, MO) and water. The animals were kept in temperature- and humidity-controlled animal quarters (temperature, 22 ± 2°C; humidity, 55 to 65%) with a 12-hr light/dark cycle. Mice were euthanized by CO2 asphyxiation. Intracardiac blood was collected immediately after the cessation of respiration. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Norcocaine nitroxide was administered in a single dose ranging from 10 to 50 mg/kg i.p. Depending on the experiment, some mice were pretreated with sodium phenobarbital (80 mg/kg/day i.p., for 4 days), cimetidine (100 mg/kg i.p., 1 hr before and 1 hr after norcocaine nitroxide treatment), diazinon (asingle 30-min pretreatment of 10 mg/kg i.p.), SKF 525A (a single 15- or 30-min pretreatment of 50 mg/kg i.p.), treoleandomycin (100 mg/kg i.p., 2 hr before and 2 hr after the norcocaine nitroxide dose), chloramphenicol (a single 1-hr pretreatment of 100 mg/kg i.p.) or ketaconazole (a single 30-min pretreatment of 100 mg/kg i.p.). Some mice received, for comparison purposes, a single dose of cocaine, 50 mg/kg i.p. Treatment groups ranged from 5 to 10 mice each. Saline was used as the vehicle for cocaine, sodium phenobarbital, SKF 525A, cimetidine, ketoconazole and chloramphenicol, and corn oil was used as the vehicle for norcocaine nitroxide, diazinon and treoleandomycin.

Serum ALT measurements. Serum ALT activity was measured by the method of Bergmeyer et al. (1978) using an ALT 20 kit (Sigma Diagnostics, Inc., St. Louis, MO).

Histopathology. Liver was harvested, fixed in neutral buffered 10% formalin for at least 24 hr, trimmed, processed, embedded in paraffin, sectioned at 4 to 6 μm and stained with hematoxylin and eosin for light microscopic evaluation. For electron microscopy, tissues were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, washed in 0.1 M cacodylate buffer and post-fixed in buffered 1% osmium tetroxide for 1 hr. Samples were then dehydrated in a graded ethanol series at 10-min intervals followed by a 2% uranyl acetate en bloc stain overnight. Ethanol-dehydrated samples were washed twice in 100% acetone, infiltrated with Embed 812 resin and polymerized at 60°C for 2 days. Ultrathin sections were collected on carbon-coated 0.2% formvar grids and poststained with 2% aqueous uranyl acetate followed by Reynold’s lead citrate. Photomicrographs of stained sections were taken on a Hitachi H-7000 electron microscope at 75 kV.

Statistical analysis. Serum ALT activity data were analyzed by a one-way analysis of variance followed by a Student Neuman-Keuls’ post-hoc test. Groups were considered significantly different when P < .05. Because of the log-normal distribution of serum ALT activity values, data were log transformed for statistical comparisons.

Results

Time course of norcocaine nitroxide toxicity. The hepatic effects of the administration of a single dose of norcocaine nitroxide (50 mg/kg i.p.) were evaluated over time. Clinical observation of the animals after norcocaine nitroxide treatment found no evidence of CNS stimulation, such as occurs with a comparable dose of cocaine. Serum ALT activities were significantly elevated in response to norcocaine nitroxide, reaching peak levels 12–18 hr after treatment and declining thereafter (fig. 1). Serum ALT activities followed a similar time course in mice treated with cocaine (50 mg/kg i.p.) for comparison. Although the serum ALT activity levels were higher in cocaine-treated mice at each time-point, none of the differences was statistically significant (P > .05).

Light microscopic examination of liver tissue collected 6 hr after administration of norcocaine nitroxide showed mild acute degeneration and necrosis of the hepatocytes of the central one-third to one-half of the lobules. At the periphery of the affected areas, which were sharply demarcated from the remainder of the lobules, the hepatocytes were slightly swollen, and the cytoplasm was slightly pale and finely granular. Among these cells were a few necrotic hepatocytes that had amorphous eosinophilic cytoplasm and fragmented nuclei. Moderate numbers of subcapsular lobules contained small discrete centrilobular foci in which the hepatocytes had more pronounced degenerative changes, and these areas contained a higher proportion of necrotic hepatocytes.

At 12 hr, the cytoplasm of central to midzonal hepatocytes was severely swollen, pale and finely granular (cloudy swelling) with necrosis of a few individual hepatocytes in this zone (fig. 2, top panel). This change was not uniformly distributed throughout the liver, and centrilobular hepatocytes adjacent to these areas were more severely affected.

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Fig. 1. Time course of serum ALT activities after a single dose of norcocaine nitroxide. Serum ALT activities were measured at varying times after a single dose of norcocaine nitroxide or cocaine, 50 mg/kg i.p. Results are expressed as mean ± S.E.M., N = 5 to 10 animals. Untreated control mice had serum ALT activities of 62 ± 16 I.U./l. Mice treated with saline (vehicle for cocaine) had serum ALT activities of xx ± xx I.U./l. Serum ALT activities for treated mice at all time-points (except 6 hr for norcocaine nitroxide) were significantly increased compared with controls, P < .05. There were no significant differences between serum ALT values for norcocaine nitroxide- and cocaine-treated mice.
to the central vein were only slightly altered, resembling those in mice euthanized at 6 hr. Also, the subcapsular foci of hepatocellular degeneration and necrosis contained a higher proportion of necrotic hepatocytes, and these were regularly accompanied by neutrophils. At 18 hr, centrilobular to midzonal hepatocellular degeneration and necrosis were more extensive and severe (fig. 2, bottom panel). In many lobules, necrosis of the affected areas was essentially complete except for a narrow zone of hepatocytes immediately adjacent to the central vein. In affected areas, we observed mild Kupffer cell swelling and accumulation of small numbers of neutrophils. At 24 hr, lesions ranged from very similar to the more severe lesions at 18 hr to very slight; most lobules were indistinguishable from normal, and there were only a few subcapsular foci of degenerating and necrotic hepatocytes. Lesions at 48 hr were similar to those at 24 hr. Histopathologic examination of livers from mice treated with an equivalent dose of cocaine revealed hepatic necrosis that was similar in intralobular distribution and severity (not shown).

Liver collected from mice 12 hr after a single dose of norcocaine nitroxide (50 mg/kg i.p.) was processed and examined by TEM. As in previous experiments, liver from vehicle (corn oil)-treated mice served as control. TEM showed condensation of the nucleus and extensive vacuolation of hepatocytes after norcocaine nitroxide treatment, whereas liver cells from control mice contained rounded nuclei and numerous mitochondria. In order to examine early changes after norcocaine nitroxide treatment, we euthanized additional groups of mice from 30 to 120 min after administration. As early as 30 min after injection, we observed dilation of the nuclear membrane, presence of vacuoles caused by dilation of the cytocapillary network and dissociation of ribosomes from the rough endoplasmic reticulum. (fig. 3). Mitochondrial swelling was apparent by 60 min, and by 120 min, mitochondria were undergoing condensation, cristae were dissociating and the matrix became condensed (not shown).

**Dose-response relationship for norcocaine nitroxide hepatotoxicity.** Liver toxicity (based on serum ALT activity) caused by norcocaine nitroxide treatment increased with the dose administered (fig. 4). Mean serum ALT activities 12 hr after administration for mice injected with 10 or 20 mg/kg norcocaine nitroxide were not significantly different from controls, whereas mice injected with 30, 40 or 50 mg/kg...
norcocaine nitroxide had significantly elevated serum ALT activity levels (P ≤ .05). Serum ALT activities were highest in mice injected with 50 mg/kg i.p., but these results were not significantly different from those in mice injected with 40 mg/kg.

Light microscopic examination of livers from mice given 10 mg/kg norcocaine nitroxide revealed slightly increased eosinophilia of centriflobular hepatocytes in a few lobules. Subcapsular lobules contained a few small foci of pale, swollen hepatocytes in the central zone. At a dose of 20 mg/kg, there was increased eosinophilia of the centriflobular hepatocytes of most lobules, and many subcapsular lobules contained centriflobular foci of hepatocellular necrosis accompanied by moderate accumulation of neutrophils and adjacent pale, swollen hepatocytes. A dose of 30 mg/kg resulted in changes similar to those in mice treated with 20 mg/kg except that eosinophilia of the centriflobular hepatocytes was more pronounced, and there were a few pale, swollen hepatocytes at the periphery of these areas. Mice administered 40 mg/kg norcocaine nitroxide had distinctly increased eosinophilia of the centriflobular hepatocytes, and at the periphery of these areas and extending into the midlobular zone, the hepatocytes were severely pale, swollen and finely granular. Hepatocellular necrosis was present in many lobules and was extensive in some. Changes in mice given 50 mg/kg were similar to those in mice treated with 40 mg/kg norcocaine nitroxide (not shown).

Effects of enzyme induction and inhibition on norcocaine nitroxide toxicity. Serum ALT activities and hepatic lesions were evaluated in mice 12 hr after a single dose of norcocaine nitroxide, with or without pretreatment with enzyme inducers or inhibitors (fig. 5). Serum ALT activities were not significantly affected by phenobarbital pretreatment. However, pretreatment with phenobarbital shifted the site of necrosis from the midzonal and centriflobular regions (zones 2 and 3, respectively) to the periportal region (zone 1) of the lobule (fig. 6).

Pretreatment with P450 enzyme inhibitors significantly decreased the hepatotoxicity of norcocaine nitroxide. In mice pretreated with inhibitors relatively nonspecific as to P450 isozyme (SKF 525A and cimetidine) the increase in serum ALT activities from subsequent norcocaine nitroxide administration (50 mg/kg i.p.) was virtually abolished (fig. 5). Histopathologic examination of liver sections from these mice confirmed the protection afforded by P450 inhibitor pretreatment (a representative section from a mouse pretreated with SKF 525A is shown in fig. 6). Preliminary follow-up experiments were conducted in which mice were pretreated with P450 inhibitors selective for CYP3A (troleandomycin and ketoconazole) and CYP2B1/2 (chloramphenicol). Each of these pretreatments reduced by about 10-fold the serum ALT response to norcocaine nitroxide (data not shown). In contrast to the protection afforded by P450 inhibitor pretreatment, the esterase inhibitor diazinon greatly exacerbated norcocaine nitroxide hepatotoxicity. Serum ALT activities were approximately doubled by diazinon pretreatment (fig. 5), and liver sections from these mice showed massive midzonal and centriflobular necrosis (not shown). Perhaps as a consequence of this increased liver injury, diazinon pretreatment resulted in a 30% mortality compared with no mortality observed in the other treatment groups, including mice treated with diazinon alone (data not shown).

Discussion

Norcocaine nitroxide was found to produce in mice dose-dependent hepatotoxicity remarkably similar to that produced by cocaine. Previous reports have indicated that significant elevations of serum ALT activities result from cocaine doses greater than 30 mg/kg i.p. in this mouse strain (Roberts et al., 1992b). With this as a basis for comparison, the results shown in figure 4 suggest that cocaine and norcocaine nitroxide are similar in potency. This interpretation must be made cautiously, however, given the uncertainty regarding the fate of an administered dose of the nitroxide derivative of cocaine. Previous studies have shown that although norcocaine nitroxide is stable in solution, it may be
nitroxide dose (fig. 3). In particular, there are pronounced nitroxide liver injury is how rapidly it develops. Electron as well as from cocaine. It appears to be relevant for toxicity from the nitroxide metabolite never been explained, but the underlying mechanism ap-

Norcocaine nitroxide in the liver relative to that of cocaine may be greater than is implied by the comparison presented here.

Norcocaine nitroxide and cocaine were found to produce essentially the same lesion: hepatocellular necrosis predominantly in the midzonal region. Previous studies have shown that phenobarbital induction results in an apparent shift in the cocaine lesion from the midzonal or centrilobular region (depending on the mouse strain) to the periportal region (Roth et al., 1992; Powell et al., 1991). The same shift was observed in this study for hepatic necrosis from norcocaine nitroxide. The basis for the shift in the cocaine lesion has never been explained, but the underlying mechanism appears to be relevant for toxicity from the nitroxide metabolite as well as from cocaine.

Perhaps one of the most striking features of norcocaine nitroxide liver injury is how rapidly it develops. Electron microscopic examination of the liver shows dramatic changes in subcellular structure within 30 min of the norcocaine nitroxide dose (fig. 3). In particular, there are pronounced changes in mitochondria and endoplasmic reticulum. Previous studies in our laboratory and others have found swelling of both smooth and rough endoplasmic reticulum and loss of ribosomes after cocaine administration (Gottfried et al., 1986; Mehanny and Abdel-Rahman, 1991; Roth et al., 1992; Powers et al., 1992). Recent studies have reported mitochondrial inner membrane permeability changes and lipid peroxidation from cocaine in rats (Masini et al., 1996), although reports of alterations in mitochondrial morphology after cocaine treatment have been inconsistent (Gottfried et al., 1986; Powers et al., 1992). Overall, observations regarding the nature and timing of the histopathologic response to norcocaine nitroxide observed in this study appear to be consistent with a role for this metabolite in cocaine hepatotoxicity.

The importance of P450 activity in the bioactivation of cocaine has been well established, and some of the partici-

Fig. 6. Effect of pretreatment with phenobarbital or SKF 525A on hepatotoxicity in mice 12 hr after a single dose of norcocaine nitroxide (50 mg/kg i.p.). Top panel) Liver from a mouse pretreated with phenobarbital. Hepatocellular degeneration and necrosis are confined to the periportal region. Bottom panel) Liver from a mouse pretreated with SKF 525A, showing normal morphology. C, central; P, portal. Hematoxylin-eosin stain. Bar = 40 μm. Original magnification, ×400.
and Goldlin, 1991) (see fig. 7). Some experimental observations argue against this hypothesis. For example, no superoxide formation was detected during the metabolism of N-hydroxynorcocaine to norcocaine nitroxide by rat liver microsomes (Lloyd et al., 1993), and studies using rat hepatocytes have found that preventing the lipid peroxidation from cocaine by cotreatment with an antioxidant (α-tocopherol polyethylene glycol 1000 succinate) has no effect on cytotoxicity (Goldlin and Boelsterli, 1991). On the other hand, generation of reactive oxygen species appears to be important in the toxicity of cocaine in hepatocyte cultures (Goldlin and Boelsterli, 1991; Boelsterli et al., 1993), which suggests that oxidative damage is a key feature of cocaine hepatotoxicity but that it occurs through nonperoxidative mechanisms. Another hypothesis holds that norcocaine nitroxide is further oxidized to a reactive metabolite that binds to critical subcellular targets (Evans, 1983) (fig. 7). In regard to this, Charkoudian and Schuster (1985) have proposed that the nitroxide undergoes a one-electron oxidation to a nitronium, a species that is characteristically highly reactive.

The results of the present study are consistent with both of these proposed mechanisms, including the requirement for P450 enzyme activity. In the case of the reactive metabolite mechanism, the required P450 would presumably be the enzyme that oxidizes the nitroxide to the reactive species. For the futile redox cycle mechanism, norcocaine nitroxide toxicity could be blocked by inhibiting the P450 enzyme responsible for oxidizing N-hydroxynorcocaine to norcocaine nitroxide. Even though the nitroxide was administered directly in these studies, an appropriate P450 inhibitor would break the cycle by preventing oxidation of N-hydroxynorcocaine formed from reduction of the administered nitroxide.

In conclusion, although there is ample evidence that terminal oxidative metabolites of cocaine are responsible for its hepatotoxic effects, there has been little direct study of the toxicological properties of these metabolites. This study confirms the widespread assumption that norcocaine nitroxide is hepatotoxic and indicates that both P450 and esterase activities are important determinants in its toxicity.

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References


Shuster L, Quimby F, Bates A and Thompson ML (1977) Liver damage from cocaine by cotreatment with an antioxidant (α-tocopherol polyethylene glycol 1000 succinate) has no effect on lipid peroxidation from cocaine by cotreatment with an antioxidant (α-tocopherol polyethylene glycol 1000 succinate) has no effect on lipid peroxidation. Life Sci 20:1035–1042.


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