Cocaine Hepatotoxicity: Influence of Hepatic Enzyme Inducing and Inhibiting Agents on the Site of Necrosis

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Cocaine-induced hepatotoxicity has been reported in human beings and is well documented in mice. One interesting feature of this toxicity appears to be common to both species is an apparent shift in the intrasacral site of necrosis under circumstances known to alter cocaine metabolism. However, the evidence in human subjects is limited, and studies elucidating the mechanism of this phenomenon cannot be performed in human beings. Although future studies in mice may define the basis of this mechanism, the current evidence is a somewhat fragmented composite of studies using different mouse strains and enzyme-inducing agents. Therefore a comprehensive pathologic investigation was initiated for the purpose of identifying and establishing an animal model suitable for studying this phenomenon. In naive ICR mice a single 60 mg/kg dose of cocaine was found to produce midzonal (zone 2) coagulative necrosis. In mice whose oxidative metabolism had been increased with β-ionone or in which esterase metabolism had been inhibited by diazepam, the severity of the toxicity was increased but the intrasacral origin of the lesion did not change. However, when the oxidative microsomal metabolism of ICR mice was induced by phenobarbital or β-naphthoflavone, the acinar zone affected was dramatically different. Phenobarbital induction produced zone 1 necrosis, whereas β-naphthoflavone induction caused necrosis in zone 3. The site of necrosis corresponded with the distribution of cocaine, and its metabolites were identified with colloidal gold-conjugated antibody probes. The results of this study suggest that the agents shifting the location of cocaine-induced hepatic necrosis alter the intrasacral site of protein binding of cocaine and its metabolites. (HEPATOLOGY 1992;15:934-940.)

The ability of cocaine to produce hepatic necrosis in mice is well documented (1-5). Several lines of evidence indicate that this effect of cocaine is produced by an N-oxidative metabolite or metabolites. For example, it has been observed that metabolites derived from the tropene nitrogen (i.e., norcocaine and N-hydroxynorcocaine) are generally more potent than cocaine in producing liver toxicity, whereas metabolites formed from ester hydrolysis are inactive (6-8). Also, increased hepatotoxicity from cocaine results if its oxidative metabolism is increased, either by pretreatment of mice with a cytochrome P-450-inducing agent (e.g., phenobarbital) or an inhibitor of competing esterase activity (e.g., diazepam) (2, 3, 6). Conversely, liver toxicity is diminished when mice are pretreated with agents that inhibit cytochrome P-450 activity such as SKF 525-A, cimetidine or metyrapone (3, 7, 9).

Some agents that increase oxidative metabolism of cocaine, in addition to increasing the extent of hepatic necrosis, may alter the acinar zone at which it occurs. Whereas hepatic necrosis in naive mice treated with a single dose of cocaine has typically been described as either midzonal (zone 2) or perivenular (zone 3) (1, 3, 9, 10), the necrosis in mice pretreated with phenobarbital has primarily been reported as periportal (zone 1) (2, 6, 11). These observations suggest that an unusual shift in the morphologic site of cocaine-induced liver injury may occur after treatment with phenobarbital (and perhaps other agents that alter cocaine metabolism), but a clear description of this phenomenon has been hampered by the absence of a systematic examination. Simultaneous variables of pretreatment agent, cocaine dosing regimen (single vs. multiple doses), and mouse strain in existing studies confound the determination of the contribution of each to changes in the site of liver lesions resulting from cocaine administration.

The objective of this study was to examine the effects of hepatic enzyme-inducing and -inhibiting agents on the lesions produced by a single dose of cocaine in a single mouse strain (ICR). In addition to the classical cytochrome P-450 inducers phenobarbital and β-naphthoflavone, β-ionone was selected for study because it has been observed to produce perhaps the most striking increases in cocaine hepatotoxicity in male mice.
(11). Diazinon, which is thought to increase liver toxicity from cocaine through inhibition of esterase activity (6-8), was also included in the study. To facilitate comparisons with previous studies, lesions were evaluated at 24 hr. Earlier time points were also examined to determine the influence of the pretreatment agents on the initial intrahepatic site of necrotic lesions. Studies were also conducted to determine the relationship between the site of necrosis and intrahepatic distribution of cocaine and its metabolites.

**MATERIALS AND METHODS**

Cocaine hydrochloride, β-ionone, and β-naphthoflavone were purchased from Sigma Chemical Co. (St. Louis, MO). 4-[3H]-cocaine (4.5 Ci/mmol) was obtained from the National Institute on Drug Abuse (Rockville, MD). Sodium phenobarbital, U.S.P., N.F., was purchased from Spectrum Medical Chemical Corp. (Gardena, CA), and diazinon (phenothion, O,O-dimethyl O-(6-methyl-2-(1-methylthyl)-4-pyrimidinyl) ester) was obtained from Chem Service, Inc. (West Chester, PA). SKF 525A (2-diethylaminomethyl 2,2-diphenylvalerate) was a generous gift of Smith Kline & French Laboratories (Philadelphia, PA). All drugs and chemicals were used as supplied. Male ICR mice weighing 20 to 25 gm were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were housed 5/cage on corn-oil bedding in temperature- and humidity-controlled animal quarters with a 12 hr light-dark cycle. Rodent chow (Purina 5001;Ralston Mills, St. Louis, MO) and water were provided ad libitum.

Pretreatment regimens were sodium phenobarbital, 80 mg/kg intraperitoneally, for 3 days; β-naphthoflavone, 40 mg/kg intraperitoneally, for 3 days; β-ionone, 6 mg/mouse subcutaneously, in two doses, 72 and 48 hr before treatment; diazinon, 10 mg/kg 30 min before treatment; or SKF 525A, 50 mg/kg intraperitoneally, 30 min before treatment. After pretreatment, mice were administered a single intraperitoneal dose of cocaine hydrochloride. Cocaine and phenobarbital were administered in saline solution; β-ionone, β-naphthoflavone and diazinon were administered in corn oil. Mice administered saline or corn oil vehicle alone were served as controls. Each of the study groups consisted of 10 mice. Before initiation of the study, all procedures were assessed and approved by the Institutional Animal Care and Use Committee.

At specified times after administration of cocaine, mice were euthanized 24 hr after cocaine dosage. Immediately after cessation of respiration from CO2 asphyxiation, a 1:1 to 1.0 ml intracardiac blood sample was taken. Serum ALT activity was determined in these samples according to the method of Bergmeyer et al. (15) with a commercially available kit (Sigma Diagnostics, Inc., St. Louis, MO). Statistical comparisons of serum ALT activities were made by ANOVA with log-transformed data.

**RESULTS**

Hepatic lesions and serum ALT activities were evaluated in male ICR mice 24 hr after a single dose of cocaine, with or without enzyme inducer or inhibitor pretreatment. Serum ALT activities resulting from cocaine administration (60 mg/kg intraperitoneally) were increased by pretreatment with the cytochrome P-450-inducing agents phenobarbital and β-ionone, but not β-naphthoflavone (Table 1). The esterase inhibitor diazinon increased not only the hepatotoxicity of cocaine, but also its acute toxicity related to stimulant effects. Rapid mortality from diazinon-enhanced acute toxicity of cocaine necessitated a reduction in cocaine dosage from 60 to 30 mg/kg. At this lesser cocaine dose, diazinon produced an approximate fivefold increase in serum ALT activities (Table 1).
Fig. 1. Cocaine-induced hepatic necrosis at 24 hr in naive mice. Necrosis is primarily in zone 2. Large arrow indicates portal triads; small arrow indicates central veins (H&E). Bar = 50 μm.

Fig. 2. Cocaine-induced hepatic necrosis at 24 hr in β-ionone-pretreated mice. Necrosis is in zone 2. Large arrows indicate portal triads; small arrow indicates central veins (H&E). Bar = 50 μm.

Microscopically, cocaine (60 mg/kg, intraperitoneally) produced midzonal (zone 2) grade 3 coagulative necrosis in livers of naive mice (Fig. 1). Necrotic cells were densely eosinophilic, with pyknotic or fragmented nuclei, and formed a band encompassing 30% to 40% of the parenchyma. In 65% to 75% of acini, a 1- or 2-cell-thick band of swollen hepatocytes with finely granular cytoplasm and slightly shrunken nuclei with dense chromatin was located next to the most perivenular border of the necrotic cells. In the remaining acini, swollen cells (grade 1 injury) extended to the central veins. In all cases, zone 1 hepatocytes were spared.

In mice pretreated with the cytochrome P-450 inducer β-ionone, the pattern of necrosis from cocaine was essentially the same as that observed in naive mice (Fig. 2); i.e., grade 3 injury was observed in a band of hepatocytes located in acinar zone 2 involving 25% of the parenchyma. A wider band of swollen cells (grade 2 injury) encompassing 10% to 15% of the parenchyma was present in adjacent perivenular (zone 3) regions. Mice pretreated with β-naphthosilavone also experienced hepatic necrosis from cocaine, but the necrotic cells were located in zone 3 rather than in zone 2 (Fig. 3). Hepatocytes in this zone, accounting for 25% to 33% of the parenchyma, had undergone grade 3 injury. These cells were densely stained and shrunk or fragmented, with pyknotic or fragmented nuclei. They were surrounded by a very thin band (1 to 2 cells wide) of swollen, pale-staining hepatocytes (grade 2 injury). As with naive and β-ionone–induced mice administered cocaine, the zone 1 cells appeared normal. A pattern of necrosis strikingly different from that seen after cocaine administration was seen in mice pretreated with phenobarbital; grade 3 injury was observed in zone 1 rather than in zones 2 or 3. In most cases, limiting-plate hepatocytes were spared, but immediately adjacent to these cells was a wide zone of coagulative necrosis that typically involved 33% to 50% of the parenchyma (Fig. 4). Narrow bands, 1 or 2 cells wide, of pale-staining swollen hepatocytes, located in zone 2, bordered the areas of coagulative necrosis.

A reduced dosage of cocaine (30 mg/kg, intraperitoneally) was tested with and without pretreatment with diazoxin as described above. The hepatic lesion resulting from this lower dose of cocaine differed from that of the higher dose only in the extent of necrosis. Diazoxin pretreatment did not alter the site of necrosis (Fig. 5) but did increase its severity, resulting in grade 3 cellular injury. Lesions were not observed in livers from control mice that received saline solution or corn oil.

To more closely examine the origins of the different lesions observed 24 hr after cocaine administration, additional studies were conducted in which liver specimens were taken at earlier time points after the cocaine dose (15 min, 30 min, 45 min, 1 hr, 3 hr, 6 hr, and 12 hr). In mice treated with cocaine without prior enzyme induction, slight hepatocellular swelling (grade 1 injury)
was detected by light microscopy in zones 2 and 3 as early as 30 min after the dose (Table 2; Fig. 6). The cells most severely affected over time were those in the midzonal region (i.e., those in zone 2). Grade 2 injury appeared in these cells at 6 hr, and grade 3 injury with cellular swelling (grade 2) was observed in zone 2 12 hr after administration. With transmission electron microscopy (TEM), minimal swelling of rough and smooth endoplasmic reticulum of zone 2 hepatocytes was observed at 15 min; it had progressively increased in severity 30 and 60 min after cocaine administration. Mitochondrial alterations were not detected during these times.

β-Iodonine pretreatment produced little alteration in the progression of the hepatic changes leading to necrosis, other than perhaps a slight delay in the first signs of swelling observed by light microscopy (Table 2). In mice pretreated with β-naphthoflavone, 30% to 50% of the parenchyma (i.e., hepatocytes in zones 2 and 3) became swollen at early time points. In these mice, the most severe swelling and necrosis did not occur among cells at the outer margin (closer to the portal triads) of the affected areas as in naïve mice administered cocaine. Rather, injury occurred in cells closer to the central vein. Initial signs of hepatotoxicity from cocaine were markedly delayed in mice pretreated with phenobarbital. Swelling of hepatocytes was not observed by light microscopy until 3 hr after cocaine administration. Affected cells were located in zone 1, although limiting plate hepatocytes were not involved (Fig. 7). TEM revealed swelling of the endoplasmic reticulum in zone 1 cells somewhat earlier, at 2 hr after the cocaine dose, but no effects were observed at 1 hr or after shorter time periods. Swelling among zone 1 cells increased in severity with time, and grade 3 necrosis was observed in this region by 12 hr after the cocaine dose. No lesions were detected by light microscopy or TEM in saline solution–treated controls at any time point.

The intralobular distribution of cocaine and metabolites was examined by immunohistochemistry in liver sections taken 6 hr after cocaine administration. Silver-enhanced gold staining indicative of anti-cocaine antibody binding was localized in zone 2 in naïve mice, zone 1 in phenobarbital-pretreated mice, and zone 3 in β-naphthoflavone-pretreated animals (Fig. 8), corresponding to the sites of hepatic necrosis in these animals. No staining was observed in control slides incubated with normal rabbit serum or PBS in lieu of primary antibody. When mice were pretreated with the cytochrome P-450 inhibitor SKF 525A (50 mg/kg) before cocaine administration, this staining at 6 hr was not observed (not shown), suggesting that the staining under the circumstances of these experiments reflected the presence of an oxidative metabolite of cocaine. HPLC measurement of liver concentrations of cocaine and its oxidative metabolites, norcocaine and...
Fig. 6. Earliest cocaine-induced hepatocellular injury observed in naive mice 30 min after cocaine dose. Swelling is confined to hepatocytes in zones 2 and 3. Large arrow indicates a portal triad; small arrow indicates a central vein (H&E). Bar = 100 μm.

Fig. 7. Earliest hepatocellular changes caused by cocaine in phenobarbital-induced mice 180 min after cocaine dose. Swelling is confined to hepatocytes in zones 1 and 2. Large arrow indicates a portal triad; small arrow indicates central vein (H&E). Bar = 100 μm.

Table 2. Liver lesions produced by cocaine in naive and pretreated ICR mice examined by light microscopy

<table>
<thead>
<tr>
<th>Time after cocaine dose</th>
<th>None</th>
<th>β-Ionone</th>
<th>β-Naphthoflavone</th>
<th>Phenobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>30 min</td>
<td>Minimal swelling in zones 2 and 3</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>45 min</td>
<td>Minimal swelling in zones 2 and 3</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>1 hr</td>
<td>Minimal swelling in zones 2 and 3</td>
<td>Minimal swelling in zones 2 and 3</td>
<td>Minimal swelling in zones 2 and 3</td>
<td>Zone 1 swelling, sparing limiting plate, extending to midzonal region</td>
</tr>
<tr>
<td>3 hr</td>
<td>Swelling in zones 2 and 3; most severe at midzonal region</td>
<td>Swelling in zones 2 and 3; some necrosis in zone 2</td>
<td>Swelling in zones 2 and 3; some necrosis in zone 2</td>
<td>Zone 1 swelling, sparing limiting plate, extending to midzonal region</td>
</tr>
<tr>
<td>6 hr</td>
<td>Swelling in zones 2 and 3; some necrosis in zone 2</td>
<td>Swelling in zones 2 and 3; some necrosis in zone 2</td>
<td>Swelling in zones 2 and 3; some necrosis in zone 2</td>
<td>Zone 1 swelling and necrosis, sparing limiting plate, extending to midzonal region</td>
</tr>
<tr>
<td>12 hr</td>
<td>Swelling in zones 2 and 3; necrosis in zone 2</td>
<td>Swelling in zones 2 and 3; necrosis in zone 2</td>
<td>Swelling in zones 2 and 3; necrosis in zone 2</td>
<td>Zone 1 swelling and necrosis, sparing limiting plate, extending to midzonal region</td>
</tr>
</tbody>
</table>

Cocaine dose was 60 mg/kg, intraperitoneally; n = 10

Pretreatment dosages: phenobarbital, 60 mg/kg intraperitoneally for 3 days; β-naphthoflavone, 40 mg/kg intraperitoneally for 3 days; β-ionone, 6 mg/mouse subcutaneously 48 and 72 hr before treatment.

N-hydroxynorcocaine, after a cocaine dose showed peak concentrations 15 min after administration; these were followed by a rapid decline. None was detectable in the liver 6 hr after the dose, indicating that residual cocaine in the liver at this time point resulting in antibody binding might represent in large part reactive metabolite(s) from cocaine bound to protein. With a radiolabeled dose of cocaine, the loss of antibody staining 6 hr after administration with SKF 525A pretreatment was found to be accompanied by a significant decrease in covalent (irreversible) binding of radiolabel to hepatic protein. In mice without pretreatment, covalent binding of cocaine at this time point was measured to be 2.29 ± 0.42 nmol/mg hepatic protein (mean ± S.E.M., n = 5), whereas the binding in SKF 525A-pretreated mice was 0.46 ± 0.11 nmol cocaine/mg hepatic protein, a loss of about 80%.

DISCUSSION

Cocaine hepatotoxicity has been observed in several studies in mice (1-5, 16), and clinical reports (17-19) indicate that cocaine can produce liver injury in humans. The principal hepatic lesion resulting from cocaine in mice and humans is the same—coagulative necrosis. Although the precise mechanism of cocaine hepatotoxicity is unknown, studies in mice clearly indicate that it results from the formation through N-oxidation of a toxic metabolite (16). The ability of both mouse and human liver to generate reactive metabolites from cocaine through cytochrome P-450 activity (20) suggests that liver toxicity in both species occurs through the same mechanism.

A remarkable feature of liver toxicity from cocaine in mice and humans is the apparent shift of the site of hepatocellular injury under different circumstances. To
some extent, differences in the site of necrosis observed in mouse studies may reflect differences in the strain used and the cocaine dosing regimen employed. Genetics and exposure conditions might also contribute to the differences in humans. However, there is reason to suspect that an important determinant of the site of cocaine necrosis is the influence of other drugs. Evidence for the ability of specific drugs to alter the site of hepatic necrosis from cocaine is strongest in mice. In general, studies conducted in mice have found that a single dose of cocaine of sufficient size produces necrosis in acinar zones 2 or 3 (1, 3, 9, 10). Phenobarbital-pretreated mice, in contrast, typically exhibit necrosis from cocaine in zone 1 (2, 6, 11). The few reports of cocaine hepatotoxicity in humans in which the lesion is described seem to be consistent with this pattern. The single report of cocaine hepatotoxicity in an individual with measurable levels of barbiturates (phenobarbital and secobarbital) described zone 1 necrosis (17), whereas the three cases in which zone 3 and zone 2 and 3 necrosis had no evidence of prior barbiturate use (18, 19).

The objective of this investigation was to establish a model with which to study the apparent shifting hepatic lesion produced by cocaine. Although previous studies provided evidence for an effect of phenobarbital to alter the morphologic site of necrosis from cocaine, most lacked a suitable reference point to clearly demonstrate a shift in the lesion. That is, although periportal necrosis from cocaine was reported in phenobarbital-pretreated mice, there was no description of the lesion arising from a comparable dose in naive mice—either because this wasn’t included in the study or because the cocaine dose alone was not hepatotoxic under the conditions of the experiment. In two previous studies, lesions from a cocaine dose were compared in the same mouse strain with and without phenobarbital pretreatment. Unfortunately, differing results were obtained, and neither study observed the typical periportal necrosis from cocaine in phenobarbital-induced mice. Klose, Rosen and Rauckman (3) observed perivenular necrosis in both naive and phenobarbital-pretreated DBA/2Ha mice administered cocaine (30 mg/kg, intraperitoneally). Later, Powell, Connolly and Charles (10), using the same mouse strain, observed necrosis from cocaine (60 mg/kg, intraperitoneally) that was perivenular (zone 3) in naive mice but midzonal (zone 2) in phenobarbital-treated animals. The reason for the difference in the site of necrosis in phenobarbital-pretreated mice in these studies is uncertain, but might be due to differences in cocaine dosage or different phenobarbital pretreatment regimens.

In this study, cocaine administration to naive mice
resulted in zone 2 necrosis preceded by swelling of hepatocytes in acinar zones 2 and 3. A similar hepatic lesion and time course of hepatocellular changes after cocaine administration has been described by others (1, 21). Swelling of hepatocytes in response to cocaine in phenobarbital-induced mice occurred instead in zones 1 and 2 (excluding limiting-plate cells), with necrosis occurring exclusively in zone 1. This distinction in hepatocytes affected by cocaine between naive and phenobarbital-pretreated mice was apparent beginning with the earliest hepatic changes detectable by light microscopy and TEM. With β-naphthoflavone pretreatment, hepatocellular swelling from cocaine occurred in zone 3 and did not consistently extend to zone 2. Necrosis did not occur at the outer margin of the zone of affected cells (cells closest to zone 1) as in naive mice, but rather well within zone 3. A similar apparent shift in the site of necrosis to zone 3 has been observed previously, in response to chronic pretreatment with ethanol in studies of the same mouse strain (22). The other pretreatments, β-ionone and diazino, increased the apparent extent of necrosis but did not alter its location.

The shift in morphologic site of cocaine-induced necrosis caused by pretreatment with phenobarbital and β-naphthoflavone is unusual and no doubt has important mechanistic implications. Limited experiments conducted by immunohistochemistry found that the intracellular distribution of cocaine, metabolites or both corresponded closely with the sites of necrosis in saline solution, phenobarbital- and β-naphthoflavone-pretreated mice with zone 2, zone 1 and zone 3 lesions, respectively. The relative lack of specificity of the antibody toward cocaine and metabolites precludes any definitive conclusions about the nature of the cocaine species being detected in these studies. However, the ability of pretreatment with the cytochrome P-450 inhibitor SKF 525A to abolish the antibody binding 6 hr after cocaine administration indicates that the bound species is probably the product of oxidative cocaine metabolism. Irreversible binding of reactive metabolites of cocaine, measured separately through administration of a radiolabeled dose, was similarly inhibited by SKF 525A pretreatment, suggesting that the patterns of antibody binding represent localization of cocaine-reactive metabolites irreversibly bound to protein. If so, an ability of phenobarbital and β-naphthoflavone pretreatment to produce zone-specific shifts in the balance between bioactivation and detoxification is implied.

Clearly every individual who uses cocaine does not experience liver toxicity, and it is unlikely that susceptibility to cocaine hepatotoxicity is merely a function of dose alone. Studies in mice indicate that liver injury from cocaine can be greatly increased by the presence of other drugs, including commonly abused substances such as barbiturates, narcotics and ethanol (2, 22-24). It is reasonable to suspect that the same interactions apply to human beings. The shifting hepatic lesion resulting from cocaine use is not just an interesting phenomenon; it provides valuable clues as to the mechanisms by which other drugs alter the responsiveness of the liver to cocaine. In understanding this phenomenon, it is to be hoped that a better insight can be gained into the situations and circumstances under which cocaine poses a threat of liver toxicity in human subjects.

REFERENCES