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COCAETHYLENE HEPATOTOXICITY IN MICE

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Abstract—Cocaethylene is a novel metabolite of cocaine formed in the presence of ethanol. When administered to ICR male mice in dosages ranging from 10 to 50 mg/kg, i.p., cocaethylene was found to produce dose-dependent hepatic necrosis in the midlobular zone (zone 2). Severity of the lesion was maximal 12–24 hr after administration. A transient but significant decrease in hepatic glutathione content was observed 1 hr after cocaethylene administration. Pretreatment with the cytochrome P450 inhibitors cimetidine (200 mg/kg, i.p., in divided doses) or SKF 525A (50 mg/kg, i.p.) diminished toxicity. Pretreatment of mice with the esterase inhibitor diazinon (10 mg/kg, i.p.) increased cocaethylene hepatotoxicity, as did pretreatment with the cytochrome P450 inducing agents phenobarbital (80 mg/kg/day, i.p., for 3 days) or β-naphthoflavone (40 mg/kg/day, i.p., for 3 days). Phenobarbital pretreatment also caused a shift in the morphologic site of necrosis from midzonal to peripheral lobular (zone 1) regions. The type of hepatic lesion produced by cocaethylene, its morphologic distribution (including the shift with phenobarbital treatment), the potency of cocaethylene in producing this effect, and the apparent requirement of oxidative metabolism for hepatoxicity were all remarkably similar to observations with its parent compound, cocaine, in this and earlier studies. This suggests that these compounds produce liver toxicity through the same or similar mechanisms.

Cocaethylene (benzoylecgonine ethyl ester) is a unique transesterification metabolite of cocaine formed in the presence of ethanol [1]. It has been found in the urine of individuals using cocaine and ethanol in combination [2, 3] and in post-mortem blood, brain, and liver samples from medical examiner cases with positive blood screens for ethanol and cocaine [1, 4]. Concentrations of cocaethylene in post-mortem specimens were in many cases comparable to those of cocaine, indicating that cocaethylene may be a quantitatively important metabolite of cocaine when ethanol is present.

Limited studies conducted to date find that cocaethylene possesses at least some of the pharmacologic effects of cocaine. It has equal or similar potency with cocaine in its affinity for dopamine transporter sites in rat and human brain tissue [1, 4], its inhibition of dopamine uptake into rat striatal synaptosomes [4, 5], and in producing behavioral effects in rats [4, 5]. Cocaethylene is also of the same approximate potency as cocaine in maintaining responding in self-administration studies in rhesus monkeys [4]. Based upon these observations, it has been proposed that cocaethylene formed during combined cocaine and ethanol use may play a significant role in the influence of ethanol on neurobehavioral effects of cocaine [1, 4, 5].

It is possible that cocaethylene also contributes to the cocaine–ethanol interaction involving liver toxicity. Numerous studies have described hepatocellular necrosis in laboratory animals (principally mice) administered cocaine [see Refs. 6–8 for reviews], and there are several clinical reports of cocaine-induced liver damage [9–12]. Studies in mice have shown that acute pretreatment with ethanol potentiates the hepatotoxicity of cocaine [13]. The mechanism for this interaction is unknown, but limited observations have indicated that mice, like humans, form cocaethylene in the liver when cocaine and ethanol are present [14]. Cocaethylene, if hepatotoxic, could conceivably cause or contribute to the ethanol-enhanced hepatic necrosis from cocaine. As an initial step in evaluating this possibility, a study was conducted to determine the hepatotoxic potential of cocaethylene using the mouse as an experimental model.

METHODS

(−)-Cocaethylene fumarate was obtained from the National Institute on Drug Abuse (Rockville, MD). Cocaine hydrochloride and β-naphthoflavone were purchased from the Sigma Chemical Co. (St. Louis, MO), and sodium phenobarbital, U.S.P., N.F. was purchased from the Spectrum Chemical Mfg. Corp. (Gardena, CA). Diazinon (phosphothiotic acid *O*,*O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester) was obtained from Chem Service, Inc. (West Chester, PA). Cimetidine and SKF 525A (2-diethylaminoethyl 2,2-diphenylvalerate) were gifts from Smith Kline & French Laboratories (Philadelphia, PA).

ICR male mice, 20–24 g body weight, were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Mice were housed five per cage on corn cob bedding in polycarbonate and stainless steel cages. The animal quarters were temperature

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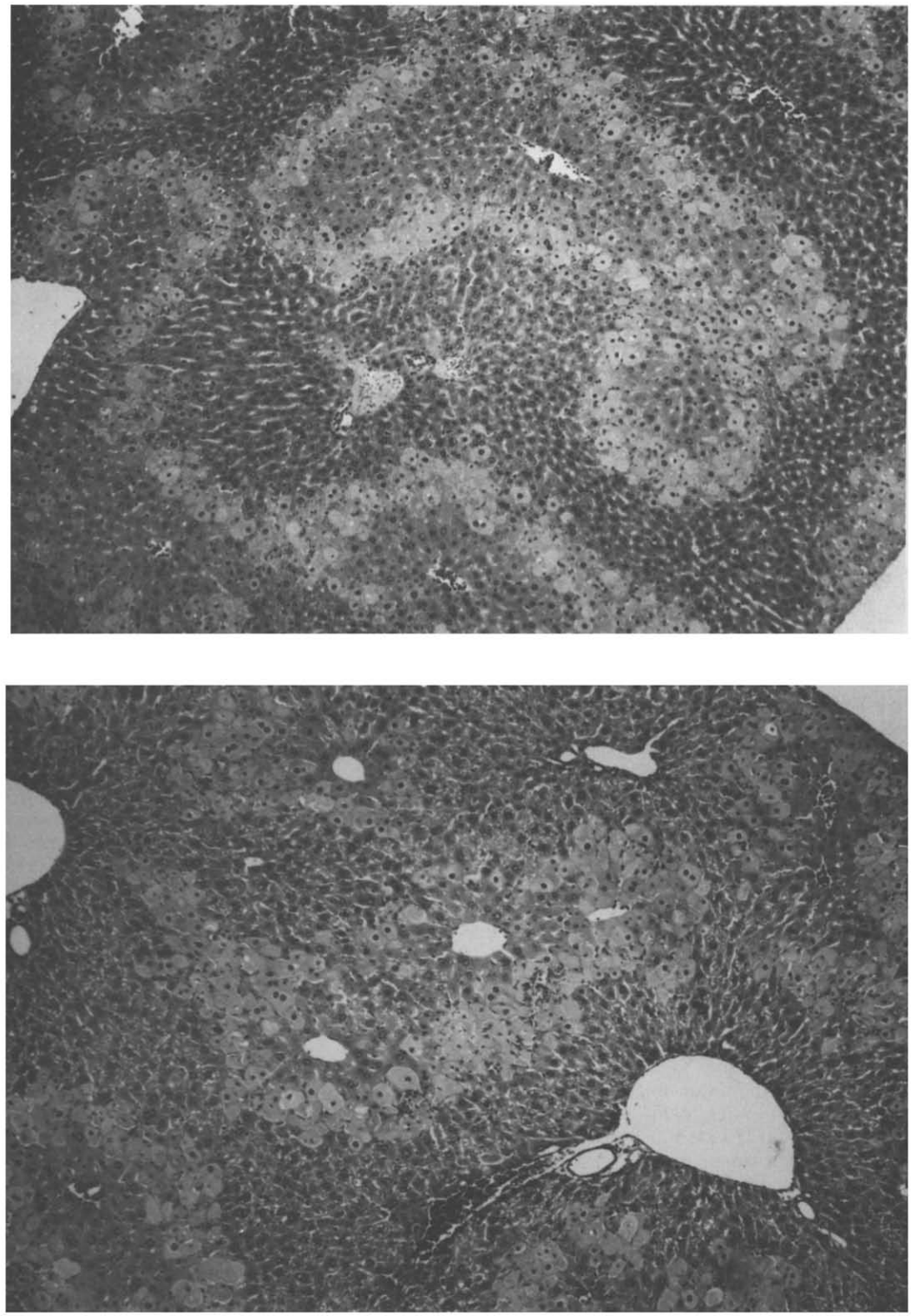

Fig. 1. Midzonal hepatocellular necrosis from cocaethylene and cocaine in naive mice. Representative sections from mice administered a single dose of (top panel) cocaethylene or (bottom panel) cocaine, 50 mg/kg, i.p., are shown. Livers were removed 24 hr post-administration for histopathologic examination. Sections were stained with hematoxylin and eosin.

and humidity controlled, with a 12-hr light/dark cycle. Mice had free access to food (Purina 5001, Ralston Mills, St. Louis, MO) and water.

β-Naphthoflavone and diazinon were dissolved in corn oil prior to administration; all other drugs and chemicals were administered in saline. All compounds were administered by the i.p. route. Pretreatment regimens consisted of: phenobarbital, 80 mg/kg/day for 3 days; β-naphthoflavone, 40 mg/kg/day for 3 days; SKF 525A, 50 mg/kg, in a single dose 15 min before cocaethylene administration; cimetidine, 100 mg/kg, 1 hr before and 1 hr after administration of cocaethylene; and diazinon, 10 mg/kg, in a single dose 30 min before the cocaethylene dose. HPLC assay [14] was used to confirm the accuracy of cocaethylene concentrations in dose solutions.

At specified times following the administration of cocaethylene or cocaine, mice were euthanized by carbon dioxide asphyxiation. In some experiments, livers were collected and fixed in neutral buffered 10% formalin. Tissues were trimmed, processed routinely, embedded in paraffin, sectioned at 4–6 μm, and stained with hematoxylin and eosin for examination by light microscopy. The extent of hepatic necrosis from cocaethylene or cocaine administration was also evaluated by measurement of serum alanine aminotransferase (ALT*) activity. Serum ALT activity was measured according to the method of Bergmeyer *et al.* [15] using a commercially available kit (Sigma Diagnostics, Inc., St. Louis, MO).

Hepatic reduced glutathione (GSH) concentrations were measured by high performance liquid chromatography using electrochemical detection according to the method of Allison and Shoup [16]. To correct for any diurnal fluctuations in hepatic GSH levels, results are expressed as a percent of the mean hepatic GSH concentration in concurrently sacrificed, saline-treated controls.

Statistical comparisons were made using an analysis of variance and a Student–Newman–Keuls *post hoc* test. Serum ALT values were log transformed prior to statistical analysis. In all cases, differences among treatment groups were considered statistically significant when $P \leq 0.05$.

RESULTS

Hepatocellular necrosis and degeneration were observed following a single dose of cocaethylene, 50 mg/kg, i.p., in male ICR mice. The lesion was located in the midzonal (zone 2) intralobular region (Fig. 1, top panel). This lesion was identical to that produced by cocaine, also administered as a single dose of 50 mg/kg, i.p. (Fig. 1, bottom panel). Livers removed at various time intervals following the cocaethylene dose showed that the severity of the hepatic injury peaked within 12–24 hr. In mice euthanized 6 hr following administration of cocaethylene, midzonal hepatocytes were slightly to moderately swollen, with paler, slightly granular to finely vacuolated cytoplasm; occasional hepatocytes had undergone coagulative necrosis, having densely eosinophilic cytoplasm with pyknotic and fragmented nuclei. By 12 hr, swelling had progressed to coagulative necrosis in most mice, with affected cells arranged in thin bands at the mid-one-third of each lobule. Coagulative necrosis with few widely scattered swollen cells was present in distinct bands in midlobular zones 24 hr post-administration. A similar time–course for progression of the lesion was observed in cocaine-treated mice. The lesion from cocaethylene began to resolve within 48 hr. At this time, fewer swollen and necrotic cells were observed in midlobular zones, and scattered mitotic figures were present among these cells. Measurement of serum alanine aminotransferase (ALT) activities following either cocaethylene or cocaine administration found peak serum levels 12 hr following the dose, with a return to near control values by 48 hr (Fig. 2).

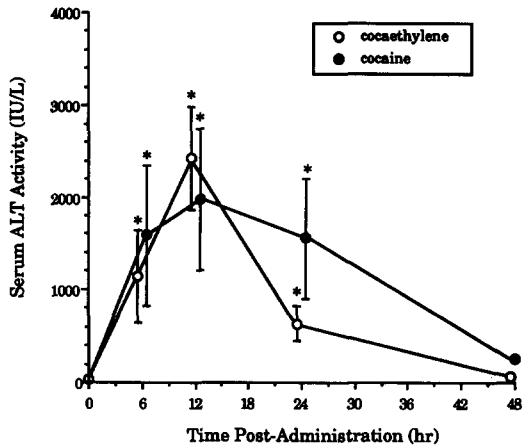

Fig. 2. Time course of serum alanine aminotransferase (ALT) activity following cocaethylene or cocaine administration. Results are means ± SEM, N = 6. Cocaethylene and cocaine were each administered at a dose of 50 mg/kg, i.p. The serum ALT activity for saline-treated controls was 27 ± 3 IU/L. Key: (*) significantly different from saline-treated controls ($P < 0.05$).

In a subsequent experiment, mice were administered single doses of cocaethylene from 10 to 50 mg/kg, i.p., and euthanized 12 hr later for evaluation of the extent of hepatic necrosis. Higher doses of cocaethylene were precluded by acute toxicity leading to mortality. Lethality in these cases was preceded by signs suggesting excessive central nervous system stimulation (e.g. hyperactivity and convulsions), and typically occurred within 15 min of the cocaethylene dose. Histopathologic examination found severe lesions in mice treated with 40 or 50 mg/kg cocaethylene. Thin bands of swollen hepatocytes with fewer necrotic cells were present in midlobular zones of mice that received a 30 mg/kg dose. No significant lesions were detected by light microscopy in mice that received 20 or 10 mg/kg doses of cocaethylene. For comparison purposes, separate groups of mice were treated with the same doses of cocaine. As with cocaethylene, hepatic

* Abbreviations: ALT, alanine aminotransferase; and GSH, reduced glutathione.

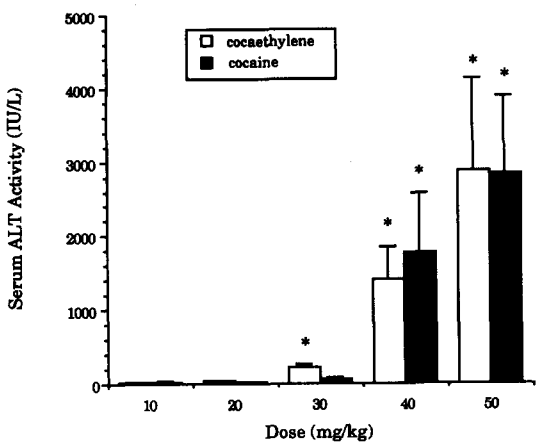

Fig. 3. Dose–response relationship for serum alanine aminotransferase (ALT) activity. Results are means ± SEM, N = 10. Cocaethylene or cocaine was administered at 50 mg/kg, i.p. Serum ALT activities were measured 12 hr following the dose. Key: (*) significantly different from saline-treated controls ($P < 0.05$).

necrosis from cocaine was dose dependent with little or no hepatic necrosis at doses of less than 30 mg/kg. Measurement of serum ALT activities found similar, dose-related increases for both cocaethylene and cocaine (Fig. 3).

Pretreatment with agents that induce or inhibit cytochrome P450 activity had significant effects on the hepatotoxicity of cocaethylene. Pretreatment with both cytochrome P450 inducers phenobarbital and β-naphthoflavone increased the extent of hepatic necrosis as indicated by serum ALT activity (Fig. 4). In contrast, the cytochrome P450 inhibitors SKF 525A and cimetidine nearly abolished the hepatotoxicity. Hepatic necrosis from cocaethylene could also be increased by pretreatment with the esterase inhibitor diazinon. Interestingly, the intralobular site of hepatic necrosis shifted when mice were pretreated with phenobarbital. Instead of occurring in the midzonal region, as in naive mice, the lesion developed in the peripheral lobular (periportal or zone 1) region in phenobarbital-pretreated mice (Fig. 5).

Cocaethylene was examined for its effects on hepatic GSH concentrations in comparison with cocaine. Treatment with both compounds produced a modest but statistically significant decrease in hepatocellular GSH content 1 hr following administration (Fig. 6). Cocaine and cocaethylene were administered in the same dosage in this experiment, and their effects on hepatic GSH were nearly identical.

DISCUSSION

Cocaine in sufficient dosages produces hepatocellular swelling and necrosis in laboratory mice. This necrosis is typically midzonal or centrilobular in naive mice, but can be shifted to more peripheral zones by pretreatment of the animals with phenobarbital [17–19]. Limited histological descriptions in clinical reports of cocaine-related liver injury suggest that the same shift in lesion occurs in humans as well [11]. Hepatocellular damage appeared to be produced by a reactive metabolite of cocaine formed through sequential oxidation of the tropane nitrogen [6, 7]. This oxidative metabolism is mediated primarily by cytochrome P450 [7, 8]. Agents which increase oxidative metabolite formation from cocaine—inducers of cytochrome P450 or inhibitors of competing esteratic metabolism—increase liver necrosis from cocaine, while inhibitors of cytochrome P450, such as SKF 525A, cimetidine, and metyrapone, diminish toxicity [17, 20–23].

Studies of structure–toxicity relationships for cocaine and hepatic necrosis have found that relatively subtle changes in the cocaine molecule, other than those associated with oxidative metabolism, typically result in loss of hepatotoxicity [22, 24]. However, the present study indicates that the transesterification of cocaine from a benzoylecgonine methyl ester to the ethyl ester results in a metabolite with essentially equivalent hepatotoxicity. The midzonal hepatic swelling and necrosis from cocaethylene in naive mice appeared to be identical to the lesion produced by cocaine. Even the unusual shift in the intralobular sites of hepatic necrosis from midzonal to peripheral lobular regions in phenobarbital-pretreated mice was the same. Potency of cocaethylene in producing hepatic necrosis was also comparable to that of cocaine.

Experiments using inducers and inhibitors of cytochrome P450 indicate that the hepatotoxicity of cocaethylene, like that of cocaine, appears to be dependent upon oxidative metabolism of the compound. The ability of diazinon to increase cocaethylene hepatotoxicity, presumably by inhibiting competing, esteratic metabolism leading to non-toxic metabolites, is also consistent with a mechanism involving oxidative bioactivation. Similarities between the hepatotoxicity of cocaine and cocaethylene suggest that the same, or an analogous, reactive metabolite is responsible for the hepatocellular damage produced by both compounds. It is interesting to note that cocaethylene and cocaine in this study produced nearly identical, transient losses in hepatic glutathione content, though interpretation of this effect is difficult. A depression in hepatic glutathione concentrations by cocaine has been previously noted and proposed to result from the consumption of glutathione by a futile redox cycle involving cocaine metabolites [6] or through direct interaction of reactive cocaine metabolite(s) with glutathione [20]. At least a portion of the effect on hepatic glutathione content may also arise indirectly from cocaine-induced adrenergic stimulation of the liver [25]. All three of these proposed mechanisms could also reasonably apply to cocaethylene.

Ethanol, administered either as a single dose (1 hr) pretreatment or over several days in the diet, has been shown to potentiate cocaine-induced hepatic necrosis in mice [13, 26]. A number of potential mechanisms have been proposed for this interaction. The first to be suggested was an induction of cytochrome P450 responsible for cocaine bioac-

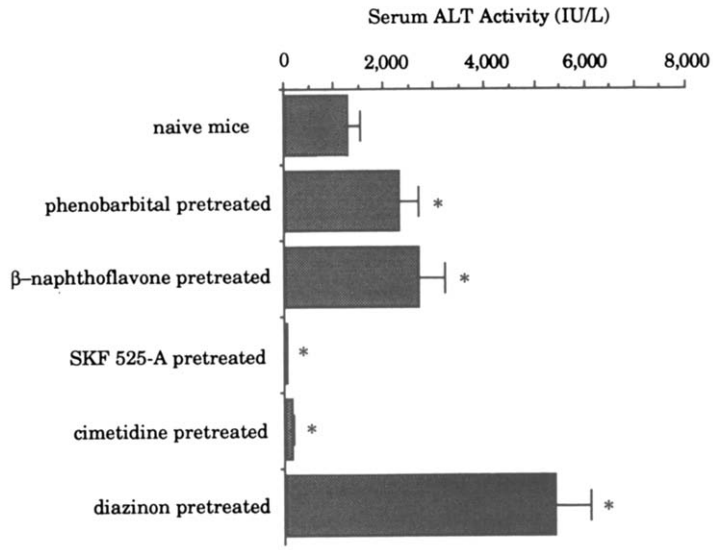

Fig. 4. Effects of pretreatment agents on serum alanine aminotransferase (ALT) activity following cocaethylene administration. Pretreatment regimens were: phenobarbital, 80 mg/kg/day, i.p., for 3 days; β-naphthoflavone, 40 mg/kg/day, i.p., for 3 days; SKF 525A, 50 mg/kg, i.p., as a single dose given 15 min before cocaethylene; cimetidine, 100 mg/kg, i.p., 1 hr before and 1 hr after the cocaethylene dose; and diazinon, 10 mg/kg, i.p., as a single dose given 30 min before cocaethylene. The cocaethylene dose was 35 mg/kg, i.p. Results are means ± SEM, N = 10. Serum ALT activities were measured 12 hr after the cocaethylene dose. Key: (*) significantly different from mice receiving cocaethylene alone ($P < 0.05$).

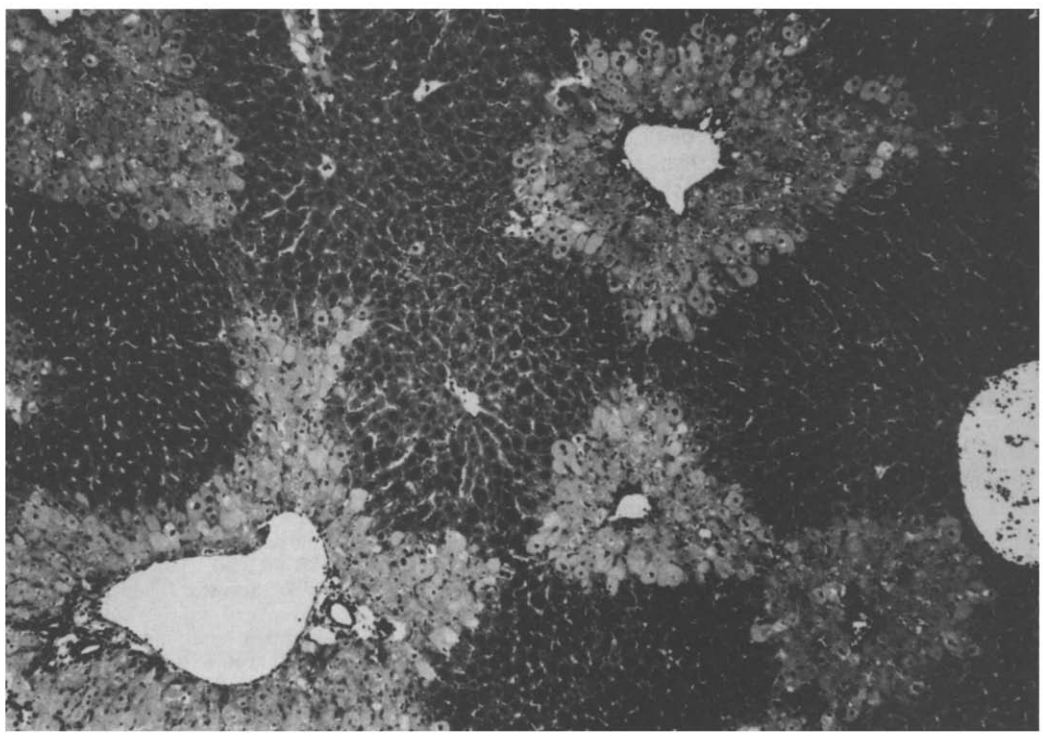

Fig. 5. Peripheral lobular hepatocellular necrosis from cocaethylene in phenobarbital-pretreated mice. Phenobarbital was administered at 80 mg/kg/day for 3 days, followed by a single dose of cocaethylene, 50 mg/kg, i.p. Livers were removed 24 hr after cocaethylene administration for histopathologic examination. A representative section is shown. Sections were stained with hematoxylin and eosin.

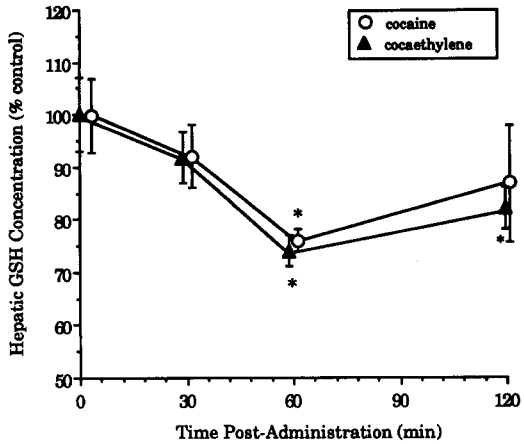

Fig. 6. Effects of cocaethylene and cocaine on hepatic reduced glutathione (GSH) concentrations. Cocaine or cocaethylene was administered at a dosage of 50 mg/kg, i.p. Results (mean ± SEM, N = 5) are expressed as a percentage of the mean hepatic GSH concentration in saline-treated controls (absolute value: 3.12 mg/g liver). Key: (*) significantly different from saline-treated controls ($P < 0.05$).

tivation [26]. This mechanism, however, does not easily explain acute interactions, and recent studies have provided evidence that the ethanol-inducible isozyme P450IIE1 is not responsible for the generation of reactive metabolite(s) from cocaine [27]. Still, ethanol induction of cytochrome P450 could be an important mechanism following repeated ethanol doses if a different cytochrome P450 isozyme population is involved, or if P450IIE1 induced by ethanol increases cocaine hepatotoxicity through an indirect mechanism such as the formation of reactive oxygen species [27]. Two other mechanisms have been proposed which have potential applicability to acute as well as subchronic ethanol pretreatment. One is based on an ethanol impairment of cocaine detoxification via hepatic glutathione suppression [13] and the second involves an ethanol-induced oxidant stress in the hepatocyte from xanthine oxidase stimulation [28].

The hepatotoxicity of cocaethylene demonstrated in this study, combined with other, recently published observations, raises the possibility of an additional mechanism for ethanol potentiation of cocaine-induced hepatic necrosis. Experiments by Dean and coworkers [29], using human liver tissue *in vitro*, have shown that the production of cocaethylene from cocaine in the presence of ethanol is accompanied by significantly diminished benzoylecgonine formation. This observation implies that the formation of cocaethylene through transesterification of cocaine occurs at the expense of ester hydrolysis to benzoylecgonine, a major detoxification pathway. Since the hepatotoxic potential of cocaethylene appears to be similar to its parent compound, cocaine, this ethanol-induced shift in the metabolism of cocaine from benzoylecgonine to cocaethylene formation means that the amount of cocaine ultimately converted to hepatotoxic metabolites is increased by the presence of ethanol. If this ethanol-induced shift in the metabolism of cocaine also occurs *in vivo*, it could play a significant role in ethanol–cocaine interactions following acute or chronic ethanol administration. Further exploration of this and other potential mechanisms for ethanol-potentiated cocaine hepatotoxicity is warranted, particularly in view of the very high frequency with which cocaine and ethanol are used together in drug abuse populations [30].

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