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Increased Toxicity of Cocaine on Human Hepatocytes Induced by Ethanol: Role of GSH

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ABSTRACT. Increased toxicity of cocaine to human hepatocytes is observed when cells are simultaneously incubated with ethanol. Ethanol might exacerbate cocaine hepatocyte toxicity by three different pathways: a) by increasing the oxidative metabolism of cocaine and hence the oxidative damage; b) by the formation of a more toxic metabolite, namely cocaethylene; or c) by decreasing the defence mechanisms of the cell (i.e. GSH). In the present study, experiments were conducted to investigate the feasibility of these hypotheses. In hepatocytes preincubated for 48 hr with ethanol, neither significant changes in cocaine metabolism nor cytotoxicity were found despite differences in hepatocyte *p*-nitrophenol hydroxylase (largely *CYP2E1* activity). Cocaethylene, the transesterification product of cocaine and ethanol, was found to be more toxic than cocaine for human hepatocytes (3x). However, the small amount formed when human hepatocytes were incubated with cocaine and ethanol would hardly explain the increased toxicity observed. On the other hand, the simultaneous presence of cocaine and ethanol caused a sustained decline in the intracellular GSH content that was larger than that observed in cocaine- or ethanol-treated cultures. Parallel to this phenomenon, a significant increase in lipid peroxidation was observed, as compared to cells treated with equimolar amounts of cocaine, ethanol, or cocaethylene. Finally, depletion of hepatocyte GSH with diethylmaleate down to levels similar to those found in ethanol-treated cells made hepatocytes more susceptible to cocaine. Taken together, the results of this research suggest that by decreasing GSH levels, ethanol makes human hepatocytes more sensitive to cocaine-induced oxidative damage. BIOCHEM PHARMACOL **58**;10:1579–1585, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cocaine, cocaethylene; ethanol; human hepatocytes; toxicity; GSH

Hepatotoxicity secondary to cocaine use has been well documented in experimental animals and man [1–3]. Most cocaine users are concurrent ethanol consumers, because the euphorigenic effects of the alkaloid are increased and prolonged by ethanol [4]. Data from both epidemiological studies and animal research strongly suggest that cocaine-induced liver damage can be potentiated by alcohol [5–10], and direct evidence of this increased toxicity has been observed in cultured human hepatocytes incubated with ethanol and cocaine [11]. Cocaine is metabolised mainly (ca. 90%) by plasma and liver esterases, yielding the pharmacologically and toxicologically inactive metabolites benzoylecgonine, ecgonine, and ecgonine methyl ester. Only a minor part is *N*-demethylated to norcocaine (mediated by *CYP3A* in humans, *CYP2B* in rats) [12–14]. This metabolite can be oxidised to *N*-hydroxynorcocaine and then to the free radical norcocaine nitroxide. As this compound can be easily back-reduced in the cell, it has been hypothesised that in its presence the cell enters a redox cycling with the final result of GSH depletion, lipid peroxidation, and cytotoxicity [15–19].

The toxicity of cocaine is greatly enhanced by *CYP*¶ induction [20]. Therefore, it is conceivable that by inducing the expression of biotransformation enzymes, ethanol could increase the oxidative metabolism of cocaine. A second possibility is that cocaine ethyl ester (cocaethylene) could be formed by transesterification in the presence of ethanol [21–25]. Indeed, this ethanol-dependent metabolite has been detected in humans simultaneously consuming cocaine and ethanol [26–28]. Cocaethylene has been claimed to be responsible for this increased hepatic toxicity [26, 29–32], as this compound can easily enter the metabolic oxidative pathway of cocaine. Finally, it is also possible that ethanol acting indirectly (i.e. by decreasing GSH content) impairs the defence mechanisms of cells.

In the present report, we examine the feasibility of the above-mentioned toxicity mechanisms in human hepato-

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¶ *Abbreviations:* *CYP*, cytochrome P-450; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DEM, diethylmaleate; and *p*-NPh, *p*-nitrophenol hydroxylase activity.

TABLE 1. Human liver samples

Biopsy	Sex	Age	Cell viability (%)	Yield (10^6 cells/g)
1	Woman	56	95	24.3
2	Woman	64	93	38.8
3	Woman	72	93	18.3
4	Woman	73	91	28.7
5	Man	71	92	26.3
6	Woman	18	85	32.0
7	Woman	74	91	25.5
8	Woman	52	89	16.7
9	Woman	70	93	28.8

cytes exposed to cocaine and ethanol. The evidence obtained in favour of the first two hypotheses (cocaethylene formation and increased oxidative metabolism of cocaine) does not, however, allow us to conclude that these two mechanisms are key events in cocaine–ethanol hepatocyte toxicity. Instead, the experimental results suggest that by decreasing GSH levels, ethanol makes human hepatocytes more sensitive to cocaine toxicity.

MATERIALS AND METHODS

Chemicals

Cocaine HCl (96–98%, as assayed by HPLC) was obtained from the Spanish Regulatory Office of Narcotics (Dirección General de Estupefacientes). Cocaethylene, benzoylecgonine, and ecgonine methyl ester were obtained from RBI. Culture media were from GIBCO BRL. Absolute ethanol and other reagents were of analytical grade.

Isolation and Culture of Human Hepatocytes

Surgical liver biopsies weighing 1–3 g (N = 9, Table 1) were obtained from patients undergoing cholecystectomy after informed consent. Patients had no known liver pathology nor had they received medication during the weeks prior to surgery. None of the patients were habitual consumers of alcohol or other drugs. Hepatocytes were isolated by microperfusion of the tissue as described elsewhere [33]. Cellular viability, estimated by the dye exclusion test with 0.4% trypan blue in saline, was higher than 85%. Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 $\mu g/cm^2$) at a density of 8×10^4 viable cells/cm^2 and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% newborn bovine serum, 50 mU/mL penicillin, 50 μg/mL streptomycin, 0.2% BSA, and 10 nM insulin. One hour later, the medium was changed, and after 24 hr, cells were shifted to serum-free, hormone-supplemented medium (10 nM insulin and 10 nM dexamethasone). The medium was changed daily. As a key requirement of *in vitro* cytotoxicity studies is the metabolic competence of cultured cells, the suitability of the hepatocyte cultures was tested as previously described [34, 35]. The cultures used in this research fulfilled the criteria.

Measurement of p*-Nitrophenol Hydroxylase Activity in Hepatocytes*

The activity of *CYP2E1* was determined by measuring the rate of hydroxylation of *p*-nitrophenol. Plates were washed with saline to remove traces of phenol red and incubated with 0.5 mM *p*-nitrophenol up to 60 min (linear response). Formation of 4-nitrocatechol medium was assessed spectrophotometrically in culture medium as described in detail [36].

Cytotoxicity Assays

Cells were seeded on fibronectin-coated 96-well microtiter plates at a density of 25×10^3 viable cells/well. After the various treatments with the compounds, viability was assessed by the MTT test as described in detail elsewhere [37]. Reduced glutathione was fluorimetrically determined in cell homogenates by the reaction with *o*-phtaldialdehyde, according to Hissin and Hilf [38], as described in detail [39]. Lipid peroxidation was assayed in aliquots of culture medium after reaction with thiobarbituric acid [40]. The product of the reaction was extracted with *n*-butanol and measured spectrophotometrically, as described in detail [39].

Metabolite Analysis

Cocaine metabolism was monitored in aliquots of culture medium of hepatocytes incubated for 24 hr with the drug. Samples were enzymatically deconjugated using 50 mU/mL β-glucuronidase and 30 mU/mL arylsulphatase in 0.1 M acetate buffer (pH 4.5) for 4 hr at 37° in the presence of 120 mM NaF to prevent cocaine hydrolysis. The samples were then processed as previously described by Lau *et al.* [41]; briefly, samples were extracted with a 1:10 v/v mixture of 1 M borate buffer: ethyl acetate. The organic layer was evaporated to dryness and the residue was suspended in mobile phase. The mobile phase consisted of a 65:35 v/v mixture of solvents, designated A and B. Solvent A was 31 mM sodium acetate buffer, pH 5.1, containing 0.15 mM tetramethylammonium phosphate, while Solvent B consisted of methanol and acetonitrile (42.9:57.1, v/v). Cocaine metabolites were identified on the basis of their retention times and UV spectra compared to standards. A peak eluting at 2.5 min that had a slightly higher retention time than ecgonine methyl ester, had the same UV spectra, and appeared only in cocaethylene was tentatively assigned to ecgonine ethyl ester.

The metabolite concentrations were determined by interpolation of obtained areas in their respective calibration curves. The flow rate was set at 2 mL/min and a C18 reversed-phase HPLC with a Spherisorb ODS2 5-μm 20 × 0.46-cm column was used. The UV-visible detector was set at 230 nm.

TABLE 2. Metabolites detected by HPLC analysis of culture supernatants

Compound	pmol/mg cell prot/hr		
	Benzoylecgonine	Ecgonine methyl ester	Ecgonine ethyl ester
Cocaine	1050	820	ND
Cocaine + EtOH	973	705	ND
Cocaethylene	622	ND	570

Hepatocytes were incubated for 24 hr with 250 μM cocaine or cocaethylene. Ethanol, when present, was added at 100 mM. (ND = below the detection limit).

RESULTS

Metabolism of Cocaine by Cultured Human Hepatocytes

Hepatocytes were incubated for 24 hr with 0.25 mM cocaine, 0.25 mM cocaethylene, or 0.25 mM cocaine plus 100 mM ethanol, and aliquots of the culture medium were analysed by HPLC. In general, cocaine is poorly metabolised by cultured human hepatocytes (ca. 1% of initially added drug). Two peaks, corresponding to benzoylecgonine and ecgonine methyl ester, are normally detected in HPLC chromatograms of culture media (Table 2). Analysis of culture media of cells incubated with cocaethylene showed the presence of a first peak (benzoylecgonine, retention time (RT) = 1.6 min) followed by a peak with an RT of 2.5 min. As no standard was available, the identification of this peak was not straightforward. However, based on the fact that this compound eluted at a slightly higher retention time than benzoylecgonine methyl ester, showed the same UV spectra, and appeared only in cocaethylene-incubated hepatocytes, we tentatively assigned it to ecgonine ethyl ester. We did not detect appreciable formation of cocaine as a result of transesterification of cocaethylene. The supernatants of cultures were incubated with increasing concentrations of cocaine (0.25 to 6 mM) plus ethanol (100 mM) and analysed by GC-MS to quantify the formation of cocaethylene. Despite the fact that the cells were metabolically active and showed biotransformation activities, only small amounts of cocaine were transesterified to cocaethylene by cells (less than 1%).

Cytotoxicity of Cocaine in Human Hepatocytes Pre-exposed to Ethanol

Hepatocyte cultures were pre-exposed for 48 hr to 100 mM ethanol and the *p*-nitrophenol hydroxylase (P4502E1) activity was determined. When compared with controls, hepatocytes induced with ethanol showed 2- to 3-fold *2E1* activity. In parallel plates, the toxicity of cocaine (IC_{50}) was investigated. The results (summarised in Fig. 1) showed no clear differences in cocaine toxicity despite the differences in *2E1* activity.

Toxicity of Cocaethylene in Cultured Human Hepatocytes

Cultured human hepatocytes were exposed to increasing concentrations of cocaine and cocaethylene (0.1 to 10 mM) for 24 hr. At the end of treatment, viability was measured by the MTT test. The toxicity of cocaethylene was moderately higher than that of cocaine, as can be observed in the concentration–effect curves (Fig. 2). When cytotoxicity was quantified, the IC_{50} of cocaethylene was lower than that of cocaine (2.70 ± 0.39 mM, N = 5 vs 3.52 ± 0.29 mM, N = 5; $P < 0.05$; evaluated for five different cell preparations with the Student's *t*-test, paired data), thus indicating that cocaethylene was moderately more cytotoxic to human hepatocytes than cocaine.

Changes in GSH Content in Hepatocytes Incubated with Cocaine and Ethanol

To explore the potential effects of cocaine alone or in combination with ethanol on human hepatocyte GSH, cells were incubated for 24 hr with 1 mM cocaine, 1 mM cocaethylene, 100 mM ethanol, or 1 mM cocaine plus 100

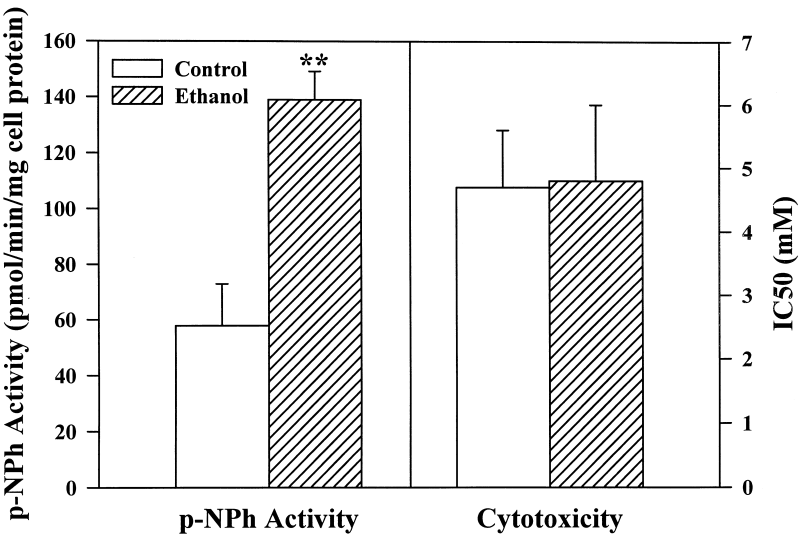

FIG. 1. Cytotoxicity of cocaine in human hepatocytes pre-exposed to ethanol. Hepatocytes were pre-incubated with 100 mM ethanol for 48 hr. After ethanol was removed, variable concentrations of cocaine were added to cultures and 24 hr later the cytotoxicity (IC_{50}) was determined by the MTT test (left panel). In parallel, the activity of CYP2E1 (*p*-nitrophenol hydroxylation) was measured (right panel). Control cultures were not preincubated with ethanol. Bars represent mean values ± SD of four different experiments. **, $P < 0.01$, evaluated with Student's *t*-test. *p*-NPh, *p*-nitrophenol hydroxylase.

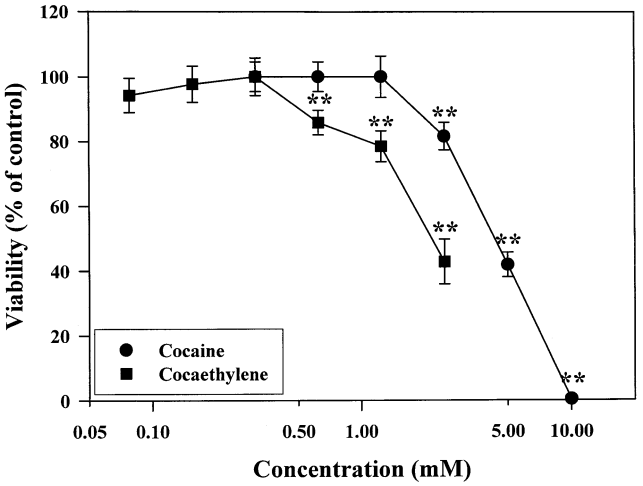

FIG. 2. Toxicity of cocaine and cocaethylene to human hepatocytes. Human hepatocytes were exposed to increasing concentrations of cocaine or cocaethylene for 24 hr. At the end of treatment, viability was measured by the MTT test. Data represented are mean values ± SD of five microwells of a representative culture. **P < 0.01, evaluated with Dunnett's test.

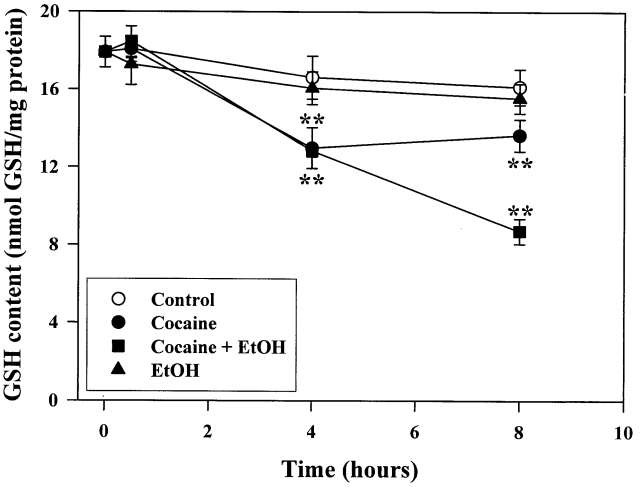

FIG. 3. Time–course of GSH levels in hepatocytes incubated with cocaine and ethanol. Hepatocytes were incubated with 1 mM cocaine, 1 mM cocaethylene, 100 mM ethanol, or 1 mM cocaine plus 100 mM ethanol (EtOH) and the levels of intracellular GSH were monitored at regular time intervals. Data represented are mean values ± SD of four plates of a representative culture. **P < 0.01, evaluated with Dunnett's test for each time.

mM ethanol. Intracellular GSH was then monitored at regular time intervals. The concentration of ethanol used in the experiment produced only a moderate decrease in GSH during the first 8 hr, one not significant when compared with controls. Cocaine decreased intracellular GSH by ca. 20% after 4 hr incubation (control values 16.9 ± 0.8 nmol/mg cell protein). Interestingly, the simultaneous presence of ethanol and cocaine in culture medium caused a sustained decrease in GSH in cultured human hepatocytes, which reached 50% of control values after 8 hr incubation (Fig. 3).

Oxidative Stress Elicited by Cocaine in Presence of Ethanol

The levels of intracellular GSH in human hepatocytes incubated for 24 hr with the xenobiotics showed an interesting pattern. In cultures treated with cocaine or ethanol alone, GSH decreased moderately (ca. 20%); cells treated for 24 hr with cocaethylene had somewhat lower levels of GSH than the controls (ca. 30%). Interestingly, in cells treated simultaneously with cocaine and ethanol, the levels of GSH further decreased to 50% of initial values (Fig. 4). In the same experiment, lipid peroxidation was measured as thiobarbituric acid-reactive substances accumulating in 24-hr culture medium. In cultures containing ethanol, lipid peroxidation was moderate and similar to controls. Cocaine and cocaethylene significantly increased the concentration of lipid peroxidation products in the culture media of hepatocytes. However, when cells were incubated both with cocaine and ethanol, a substantial increase in the concentration of lipid peroxidation end products was observed (268 ± 31, N = 3; Fig. 4). This behaviour was also seen in cells exposed to ethanol for 48 hr and then exposed to cocaine or cocaine plus ethanol. GSH content was 70 ± 6% of controls in cocaine-treated cells, while this value decreased to 20 ± 4% of controls when cells were incubated with cocaine and ethanol (data not shown).

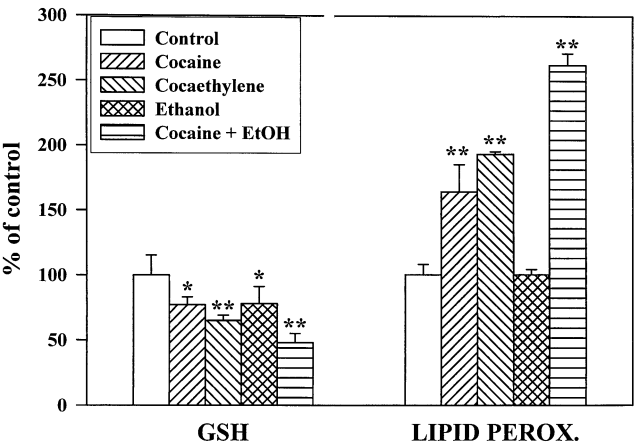

FIG. 4. Increased lipid peroxidation in hepatocytes incubated with cocaine and ethanol. The levels of intracellular GSH of human hepatocytes incubated with 1 mM cocaine, 1 mM cocaethylene, 100 mM ethanol, or 1 mM cocaine plus 100 mM ethanol (EtOH) were determined 24 hr later (left panel). Lipid peroxidation was measured in parallel cultures as thiobarbituric-acid-reactive substances (TBARS) in culture medium (right panel). Control values were 23.52 ± 3.58 nmol GSH/mg cell protein and 1053.67 ± 88.51 pmol TBARS/plate. Bars represent mean values ± SD of four plates of a representative culture. **P < 0.01; *P < 0.05, evaluated with Dunnett's test.

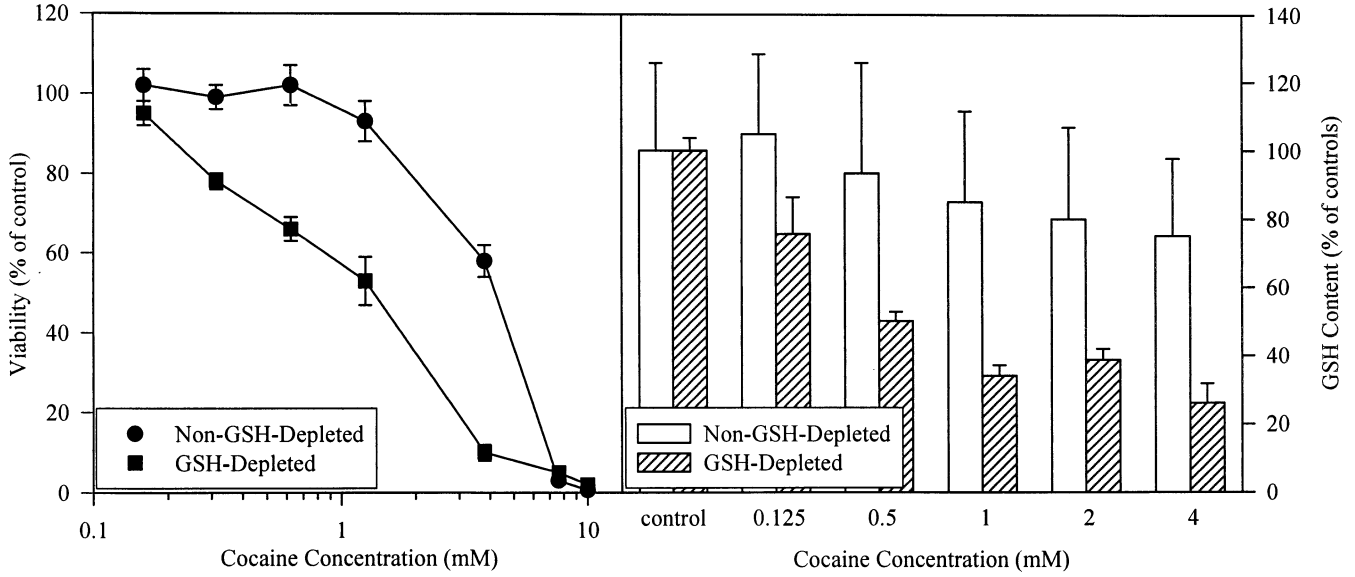

FIG. 5. Changes in cocaine toxicity after DEM pretreatment. Cultured human hepatocytes were treated with 0.5 mM DEM for 30 min. Medium was renewed and cocaine was added at increasing concentrations to cultures. After 24 hr incubation, the MTT test (left panel) and GSH content (right panel) were assayed. Results were normalised as a percentage of controls (no cocaine treatment, no DEM pretreatment). Bars represent mean values ± SD of five (MTT) or three (GSH) plates of a representative culture.

Cocaine Toxicity after GSH Depletion

To further investigate the role of GSH in cocaine toxicity, cultures were incubated for 30 min with 0.5 mM DEM. This treatment caused a transient rapid decrease in intracellular GSH levels. Monolayers were then washed with PBS to remove DEM and cells were subsequently incubated with increasing concentrations of cocaine. Twenty-four hours later, cytotoxicity and GSH content were determined. As shown in Fig. 5, non-DEM-treated cells did recover their previous GSH levels despite the presence of cocaine in the incubation media. Conversely, cocaine was clearly more toxic in GSH-depleted cells (IC_{50} = 3.40 ± 0.31, N = 5 vs 1.17 ± 0.33, N = 5; $P < 0.01$ between control and DEM-treated cultures; Student's *t*-test, paired data), a phenomenon that was paralleled by sustained low levels of GSH content in cells pretreated with DEM.

DISCUSSION

The concurrent consumption of cocaine and alcohol is a factor that appears to increase the risk of hepatocellular damage. Epidemiological data in humans as well as experiments with laboratory animals support this view [5–10]. Evidence of this toxic phenomenon was also found in cultured human hepatocytes exposed to cocaine and a non-cytotoxic concentration of ethanol [11]. Cocaine causes toxicity to hepatocytes by different mechanisms [20]. It is generally accepted that *N*-demethylation to norcocaine by *CYP3A* in humans or *CYP2B* in rats [12–14] can be followed by oxidation to *N*-hydroxynorcocaine and norcocaine nitroxide, and by the production of reactive oxygen species which are ultimately responsible for cell death [15–19, 42, 43]. In the presence of ethanol, cocaine becomes more toxic and this could be due to ethanol acting by different mechanisms: a) by increasing the oxidative metabolism of cocaine; b) by facilitating the formation of cocaethylene by transesterification; or c) by decreasing the defence mechanisms of the cell (i.e. lowering the GSH level), and thus making cells more vulnerable to drug-induced oxidative stress.

To investigate the feasibility of these mechanisms, we examined, first of all, whether the metabolism and cytotoxicity of cocaine in hepatocytes changed in cells pretreated with ethanol. The results given in Fig. 1 and Table 2 do not support the idea that short-term ethanol treatment alters cocaine metabolism by modifying the expression of CYP enzymes. Ethanol is essentially a *CYP2E1* inducer in human hepatocytes [34], although some authors found it also able to induce *CYP3A4* [44]. Despite differences in *p*-nitrophenol hydroxylase activity (largely *CYP2E1*), no changes in cocaine cytotoxicity were observed (Fig. 1). In agreement with this view, no correlation has been found between the levels of ethanol-inducible *CYP* and a cocaine-potentiated lethality in rats treated with alcohol [45]. CYP3A in man and mice and CYP2B1 in the rat are the enzymes that lead the metabolism of cocaine towards the formation of toxic metabolites [12–14], and none of these enzymes are the major forms induced by ethanol in human hepatocytes [34]. Moreover, there is no evidence supporting the involvement of *CYP2E1* in the formation of toxic metabolites of cocaine [45].

Cocaethylene has been reported to be more toxic than cocaine in mice and rats [29–32], and its formation by esterase-catalysed transesterification of cocaine and ethanol has been described [21–24, 46]. Trace concentrations of cocaethylene have been documented in sera of individuals

simultaneously consuming cocaine and ethanol under controlled circumstances [27, 28]. Pharmacokinetic studies in humans have shown that the levels of cocaine and norcocaine can be increased by co-administration of cocaine and ethanol, with the formation of cocaethylene and norcocaethylene [28]. The results presented in Fig. 2 show that the intrinsic toxicity of cocaethylene to human hepatocytes was somewhat greater than that of cocaine. Given the small amount of cocaethylene formed by human hepatocytes when incubated with concentrations of cocaine and ethanol—even larger than those existing in blood after drug consumption—together with the fact that cocaethylene shows slightly more toxicity than cocaine, the formation of cocaethylene could hardly explain the observed toxicity.

There are at least two esterases potentially involved in the metabolism of cocaine [23]. The reverse reaction catalysed by Esterase 1 is responsible for cocaethylene formation from ethanol. This enzyme can hydrolyse cocaine to benzoylecgonine, but the hydrolysis is reduced by ca. 40% in the presence of ethanol. Esterase 2 is also active in hydrolysing cocaine, but is not influenced by ethanol. If ethanol is present in the incubation media, the metabolism of cocaine could shift from a main detoxification hydrolysis pathway producing the inactive benzoylecgonine and ecgonine methyl ester to the formation of the more toxic oxidative metabolites norcocaine and norcocaethylene [23, 24]. This mechanism is believed by some authors to operate *in vivo*. The data we obtained with human hepatocytes (hydrolysis of cocaine to benzoylecgonine is reduced only by 8% in the presence of ethanol) only partially supports this view (Table 2).

The experiment depicted in Fig. 3 shows that the association of cocaine and ethanol caused a decline in intracellular GSH that was larger than that observed in cocaine- or ethanol-treated cultures. These results suggested that GSH depletion could be at the root of cocaine-increased toxicity by ethanol. Confirming this view, the experiment presented in Fig. 4 clearly shows that exposure of human hepatocytes to cocaine and ethanol results not only in a decrease in GSH but also in a significant increase in lipid peroxidation, effects that were not observed when equimolar amounts of cocaethylene were added to cultures.

The toxic effects of ethanol arise mainly from the formation of acetaldehyde, but ethanol toxicity is also related to glutathione depletion, free radical-mediated toxicity, and lipid peroxidation phenomena in hepatocytes [47, 48]. The results presented in Fig. 4 indicate that GSH depletion and lipid peroxidation are more clearly noticeable when both cocaine and ethanol, but not cocaethylene, were present in incubation media. This points at a sustained GSH depletion as one major cause of cocaine/ethanol toxicity. To investigate whether a reduction in cellular GSH as observed during incubation with ethanol would be sufficient to explain the increased toxicity of cocaine, hepatocytes were partially GSH-depleted with DEM. This treatment causes a rapid decrease in GSH, but cells recover their control levels 24 hr later. Addition of cocaine to GSH-depleted cells resulted in an increased sensitivity of hepatocytes to cocaine (Fig. 5). Under these circumstances, cocaine caused sustained low levels of GSH that resulted in an increased cytotoxicity.

In addition to the observed effects of ethanol on hepatocytes when added together with cocaine, it is conceivable that ethanol-induced *CYP2E1* may also contribute to cocaine hepatotoxicity *in vivo* via ethanol-derived radical formation (hydroxyethyl radical, reactive oxygen species) and GSH depletion [47, 48]. Indeed, pretreatment of human hepatocytes with ethanol for 0–48 hr, followed by 48- to 72-hr exposure to cocaine + ethanol, showed a greater decrease in GSH than decreases observed in non-induced cells exposed either to ethanol or cocaine alone. Based on the experimental results presented here, it seems reasonable to assume that by decreasing GSH levels, ethanol contributes to make human hepatocytes more susceptible to intrinsic cocaine-induced oxidative damage [20].

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