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COCAINE HEPATOTOXICITY: TWO DIFFERENT TOXICITY MECHANISMS FOR PHENOBARBITAL-INDUCED AND NON-INDUCED RAT HEPATOCYTES

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Abstract—Hepatocytes isolated from both phenobarbital-induced and control rats were short-term cultured and exposed to cocaine (8–2000 μM) for varying times. Intracellular lactate dehydrogenase activity, free calcium levels ($[Ca^{2+}]_i$), reduced glutathione (GSH) and lipid peroxidation were investigated to evaluate the toxic effect of cocaine on hepatocytes. Cytochrome P450 induction by phenobarbital potentiated the *in vitro* cytotoxicity of cocaine by a factor of 13 (IC_{50} = 84 μM in induced cells vs 1100 μM in non-induced cells). This difference in the susceptibility of the two types of hepatocytes to cocaine correlated well with the activity of cytochrome P450 2B1/2. Rapid depletion of GSH, reaching 30% of the control levels, and massive lipid peroxidation thereafter were the two most remarkable phenomena preceding cell death in phenobarbital-induced hepatocytes. On the other hand, a sustained rise in $[Ca^{2+}]_i$ starting 2 hr after incubation with cocaine was the most noteworthy finding in non-induced liver cells. We suggest two different pathways for cocaine hepatotoxicity: in phenobarbital-induced hepatocytes oxidative metabolism of the drug causes GSH depletion, subsequent extensive lipid peroxidation and cell death, at low concentrations of cocaine. In non-induced hepatocytes these changes are less relevant, and the major alteration caused by cocaine is a non-transient rise in $[Ca^{2+}]_i$ that is evident at higher concentrations of the drug.

Cocaine is a natural alkaloid that acts pharmacologically on the peripheral and central nervous systems. It is both a local anesthetic and a sympathomimetic agent [1]. However, this drug is better known for its stimulant and euphoriant effects. The dramatic increase in its abuse in the last decade has been accompanied by an increase in the number of cocaine toxicity reports. Acute cocaine intoxication is predominantly associated with effects on the cardiovascular, neuromuscular and central nervous systems [2, 4]. However, there is increasing clinical evidence showing that the liver is also a target of cocaine toxicity. Abusive doses of cocaine can produce striking rises in serum aminotransferases and precipitate massive, perivenous, midzonal or periportal parenchymal necrosis [5–12]. The principal hepatic lesion resulting from cocaine in humans, coagulative necrosis, is similar to the one described earlier in experimental animals [13, 14].

Cocaine is biotransformed in the liver mainly via hydrolytic cleavage, which produces stable non-toxic metabolites. However, a small fraction of the drug is N-oxidized by cytochrome P450 and FAD-containing monooxygenases [15–17]. Previous evidence suggested that an electrophilic intermediate generated during this N-oxidative biotransformation could be responsible for the cytotoxic effect of cocaine [18, 19].

To explain cocaine hepatotoxicity two basic mechanisms have been postulated. First, as a result of redox cycling between *N*-hydroxynorcocaine and norcocaine nitroxide, superoxide anion could be produced at the expense of NADPH. The formation of oxygen species would initiate lipid peroxidation, and subsequent cell membrane damage [15, 20]. Although there is a good deal of evidence supporting this hypothesis [13, 21, 22], a number of recent reports question this assumption [23–25]. Secondly, it has been suggested that the nitrosonium ion generated during oxidation of norcocaine could bind both critical cellular constituents and reduced glutathione (GSH†) [26]. Nevertheless, although several studies have demonstrated covalent binding of cocaine metabolites [27, 28], the target macromolecule(s) have not yet been identified.

Sustained elevation of intracellular free calcium ($[Ca^{2+}]_i$) levels is frequently one of the primary events in the hepatotoxicity of many xenobiotics. The resting $[Ca^{2+}]_i$ in hepatocytes is about 100–200 nM, and its regulation appears to be important for a wide variety of cellular processes such as hormonal response, enzymatic activation and cell division. A dysfunction in any of the systems involved in its homeostasis may result in a non-physiological sustained increase in $[Ca^{2+}]_i$ which can lead to cell injury [29]. A recent review [30] postulated that cocaine could have an effect on $[Ca^{2+}]_i$ homeostasis due to interaction of cocaine metabolites with critical thiol groups of the plasma membrane or microsomal Ca^{2+}-ATPases; however, this has not yet been

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† Abbreviations: GSH, reduced glutathione; $[Ca^{2+}]_i$, intracellular free calcium; LDH, lactate dehydrogenase; IC_{50} and IC_{10}, toxic concentrations that cause a 50% and 10% inhibitory effect, respectively; PROD, 7-pentoxyresorufin *O*-dealkylase; TBA, thiobarbituric acid; BSA, bovine serum albumin.

demonstrated. To our knowledge, the only previous published report [31] failed to show any alteration in basal $[Ca^{2+}]_i$ induced by cocaine or lidocaine in rat hepatocytes.

In the present study we have investigated the effect of cocaine on $[Ca^{2+}]_i$ in both phenobarbital-induced and non-induced rat hepatocytes, along with its effect on cell viability, GSH levels and lipid peroxidation. In addition, the activity of cytochrome P450 2B1/2, which plays a central role in the bioactivation of cocaine in rat hepatocytes [32, 33], was evaluated. The results show that while in phenobarbital-induced hepatocytes depletion of GSH and lipid peroxidation are the major toxic events taking place at low concentrations of cocaine, these effects are less relevant in non-induced hepatocytes, where non-transient elevations of $[Ca^{2+}]_i$ are the major toxic phenomenon preceding cell death.

MATERIALS AND METHODS

Materials. Cocaine·HCl (96–98% as assayed by HPLC) was obtained from the Spanish Regulatory Office of Narcotics (Dirección General de Estupefacientes, Madrid). Collagenase was from Boehringer (Mannheim, Germany). Calf serum was from Gibco (Paisley, U.K.) and culture media were from Flow (Irvine, U.K.). Phenobarbital·Na was from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fluo-3-AM (cell permeant) and Pluronic F-127 were obtained from Molecular Probes, Inc. (OR, U.S.A.) All other reagents used in this study were of analytical grade.

Isolation of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley-derived rats (180–250 g) by perfusion of the liver with collagenase as described elsewhere [34]. Hepatocytes were resuspended in Ham F-12/Leibovitz-15 medium supplemented with 2% newborn calf serum, 50 mU/mL penicillin, 50 μg/mL streptomycin, 0.2% bovine serum albumin (BSA) and 10^{-8} M insulin. Viability was estimated by the dye exclusion test with 0.4% Trypan blue in saline. Hepatocytes were seeded on fibronectin-coated culture plates (3.5 μg/cm^2) at a density of 80×10^3 cells/cm^2. Unattached cells were removed by changing the medium 1 hr after seeding. Following 3 hr adaptation to culture, cells were exposed to selected concentrations of cocaine. In some experiments, rats were administered phenobarbital, 80 mg/kg i.p., for 3 consecutive days prior to hepatocyte isolation.

Measurement of $[Ca^{2+}]_i$ in cultured hepatocytes. In order to investigate changes in $[Ca^{2+}]_i$ the fluorescence indicator Fluo-3 was used [35, 36]. To measure short-term Ca^{2+} changes, hepatocytes were first incubated (30 min, 37°) in culture medium containing 5 μM Fluo-3-AM and 0.075% (w/v) Pluronic F-127 (1/200 dilution from the stock solution in dimethyl sulfoxide).

The quality of Fluo-3 loading was monitored with a fluorescence microscope (Olympus BHS). The cells showed a diffuse and uniform green fluorescence, without spots or brighter intensity areas. Assuming a cytoplasmic hepatocyte volume of 5.5 μL/10^7 cells [37], the average intracellular Fluo-3 concentration was estimated to be 800 μM. The cells were then washed three times with HEPES-buffered Krebs–Henseleit solution (10 mM HEPES, pH = 7.4, 25°) containing 1% BSA. Immediately thereafter, fluorescence was measured with a multiwell plate scanner fluorimeter (Ex: 485 ± 22 nm, Em: 530 ± 30 nm, Cytofluor 2300 System, Millipore). Once a stable fluorescence baseline was reached, cocaine was added to selected wells, and fluorescence was registered at discrete time intervals for a maximum of 60 min. The fluorescent signal was stable during the course of measurements. Using these experimental conditions, we have not found any significant cytotoxicity caused by Fluo-3.

For determination of long-term Ca^{2+} changes, hepatocytes were first incubated with increasing concentrations of cocaine for 1, 2, 5 and 20 hr, followed by loading of Fluo-3 as described above and subsequent recording of the fluorescence. Using this approach we avoided the tendency of Fluo-3 to leak out of loaded cells with time, and we ensured that the amount of dye entrapped in the cells and its intracellular distribution were similar during the entire duration of the experiment. The loading efficiency at the later time points was similar in both cocaine-incubated and control cells, allowing calcium measurements even in cultures with only 20–25% of living hepatocytes.

After finishing the measurements, the fluorescence corresponding to $[Ca^{2+}]_i$ levels was calibrated. Cytosolic Fluo-3 was released from cells into medium containing 2 mM $CaCl_2$, by treating cells for 4 min with 30 μM digitonin (8 mM stock solution in dimethyl sulfoxide), and maximal fluorescence (F_{max}) was recorded. Next, 10 mM EGTA (100 mM stock solution in NaOH 0.5 M) was added to quench Ca^{2+} and to elevate pH > 8.5, and minimal fluorescence (F_{min}) was recorded. $[Ca^{2+}]_i$ was calculated for any previous fluorescence signal (F) using a K_d of 400 nM for Fluo-3-Ca [35], according to the following equation:

$$[Ca^{2+}]_i = K_d \times [(F\text{-}F_{min})/(F_{max}\text{-}F)].$$

Quantification of intracellular GSH. GSH was fluorimetrically measured according to the method of Hissin and Hilf [38] adapted to our experimental conditions. Cells were detached by scraping and homogenized by ultrasound. The resulting homogenates were deproteinated with 5% trichloroacetic acid containing 2 mM EDTA and centrifuged 30 min × 10,000 rpm. Aliquots of 50 μL were transferred to 96-well microtiter plates and mixed sequentially with: 15 μL 1 M NaOH (to neutralize the acid supernatant to pH 7.5), 175 μL of a 0.1 M sodium phosphate buffer pH 8, containing 5 mM EDTA, and 10 μL of *o*-phthaldialdehyde (stock solution 10 mg/mL in methanol for spectroscopy). The reaction was allowed to proceed for 15–20 min at room temperature in the dark. The fluorescence was measured in a multiwell fluorimeter (Ex. 355 ± 35 nm, Em. 460 ± 25 nm). Known amounts of GSH (50–5000 pmol/well) were used as reference standards.

Measurement of lipid peroxidation. Aldehydes generated after lipid peroxidation were measured by reaction with thiobarbituric acid (TBA) essentially

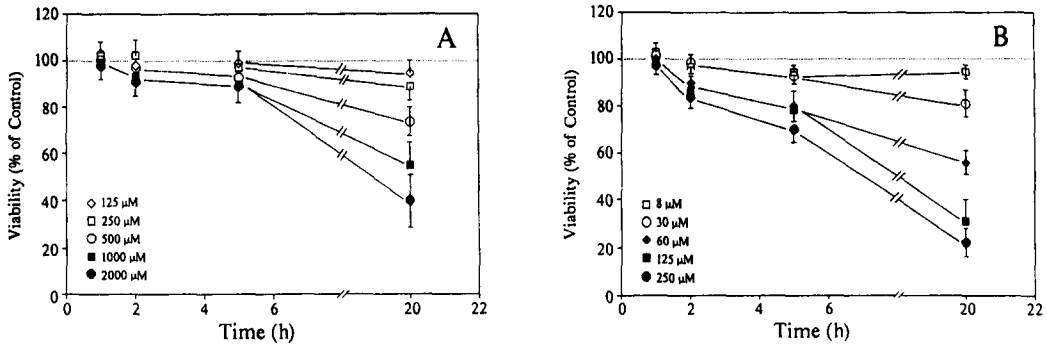

Fig. 1. Cytotoxicity of cocaine in non-induced (A) and phenobarbital-induced (B) rat hepatocytes. Cultured cells were incubated with the drug for varying times and intracellular LDH activity was measured as a cytotoxicity endpoint. Results are expressed as percentage of controls. Data are means ± SEM of six (A) and five (B) independent experiments.

as described by Masugi and Nakamura [39]. Briefly, cell-free culture medium (250 µL) was mixed with 100 µL SDS (7%), 1 mL HCl (0.1 N), 150 µL phosphotungstic acid (1%) and 500 µL aqueous TBA solution (0.67%). The mixture was heated for 60 min in a boiling water bath and, after cooling, 1 mL of *n*-butanol was added and mixed vigorously. The tubes were centrifuged for 10 min at 3000 rpm, and the fluorescence of the organic phase was measured at 530 ± 25 nm excitation and 590 ± 35 nm emission. Malondialdehyde bis (dimethylacetal) dilutions were used as standards.

Other biochemical assays. Cytochrome P450 2B1/2 activity was determined by measuring 7-pentoxyresorufin *O*-dealkylase activity (PROD) in intact cultured hepatocytes as described in detail [40]. Intracellular lactate dehydrogenase (LDH) activity was determined after cocaine incubation as an index of cell viability [41]. Cellular protein was evaluated by the method of Lowry *et al.* [42] using BSA as standard.

Statistics. Experiments were carried out using three to six different cultures as detailed in Table 1. Each determination was carried out on four plates or eight wells in each culture, and the results shown are the means ± SEM. To calculate IC_{10} and IC_{50} values (concentrations that produce a 10% and 50% inhibitory effect, respectively), the typical sigmoid dose–effect curves were linearized using the LOGIT transformation and the IC values were interpolated mathematically.

RESULTS

Cytotoxicity of cocaine to cultured rat hepatocytes

Hepatocytes were exposed to increasing concentrations of cocaine for varying times, and intracellular LDH activity was measured as an index of cell viability. Figure 1 shows the cytotoxicity of cocaine to cultured hepatocytes from both non-induced (A) and phenobarbital-induced (B) rats. The effect of cocaine on non-induced hepatocytes was scarcely evident during the first 5 hr of exposure, but after 20 hr exposure, cytotoxicity was marked at

Table 1. $[Ca^{2+}]_i$, GSH content, levels of TBA-reacting substances (TBA-RS) and PROD activity of non-induced (N) and phenobarbital-induced (Ph) rat hepatocytes at different times of culture

	Time of culture (hr)			
	4	6	9	24
PROD (pmol/mg)				
N	4.9 ± 0.3 (5)	—	—	1.2 ± 0.1 (4)
Ph	27.2 ± 2.6 (5)	—	—	5.8 ± 0.7 (5)
$[Ca^{2+}]_i$ (nM)				
N	290 ± 28 (5)	301 ± 38 (6)	230 ± 23 (6)	262 ± 26 (6)
Ph	250 ± 10 (5)	214 ± 19 (6)	207 ± 11 (6)	192 ± 21 (5)
GSH (nmol/mg)				
N	13 ± 2 (4)	15 ± 2 (4)	18 ± 3 (5)	46 ± 6 (5)
Ph	23 ± 4 (5)	27 ± 4 (6)	27 ± 4 (6)	37 ± 4 (5)
TBA-RS (pmol/mg)				
N	162 ± 47 (3)	148 ± 26 (3)	162 ± 32 (3)	155 ± 37 (3)
Ph	372 ± 50 (4)	297 ± 45 (4)	395 ± 73 (4)	390 ± 88 (4)

Values are means ± SEM of (N) different rat hepatocyte cultures.

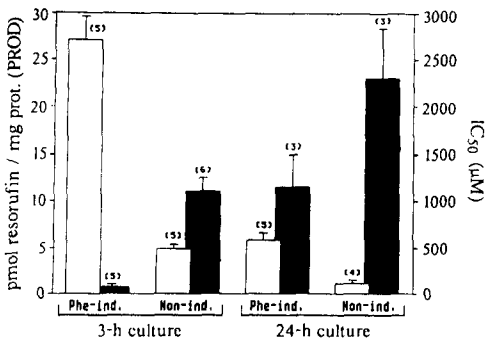

Fig. 2. Comparison between pentoxyresorufin O-dealkylation (P450 2B1/2 activity) and IC_{50} values of cocaine. Hepatocytes from either non-induced or induced rats were exposed to cocaine for 20 hr. Treatments began after 3 or 24 hr of culture. At the beginning of treatment the activity of cytochrome P450 2B1/2 was measured as PROD activity (white bars), and at the end of treatment the intracellular LDH activity was measured to estimate the cytotoxicity and calculate the IC_{50} values (black bars). Data are means ± SEM of (N) independent experiments.

concentrations ≥ 500 μM. In contrast, the cytotoxicity of cocaine to induced hepatocytes was clearly noticeable after 5 hr incubation at concentrations ≥ 60 μM. The average IC_{50} and IC_{10} values for cocaine in non-induced hepatocytes were 1100 and 220 μM, respectively, after 20 hr incubation, whereas in induced hepatocytes these values decreased to 84 and 22 μM, respectively.

The cytotoxicity of cocaine correlated well with the activity of cytochrome P450 2B1/2, the major isoforms involved in the oxidative catabolism of cocaine in the rat [33]. In phenobarbital-induced hepatocyte cultures (3 hr) this activity was 5.5 times that found in non-induced cells (Table 1). Parallel to an elevated cytochrome P450 2B1/2 activity, we also found higher cytotoxicity of cocaine in induced hepatocytes (IC_{50} value 15 times lower than the IC_{50} of non-induced hepatocytes, Fig. 2). It is noteworthy that 24 hr later, when the cytochrome P450 2B1/2 activity had spontaneously decreased in both induced and non-induced cultured hepatocytes (Table 1), the cytotoxicity of cocaine also decreased (IC_{50} increased to 1000–2000 μM), and the large difference in cocaine cytotoxicity between induced and non-induced hepatocytes disappeared (Fig. 2).

$[Ca^{2+}]_i$ *levels after incubation with cocaine*

The average $[Ca^{2+}]_i$ in undisturbed cultured hepatocytes was 243 ± 16 nM (mean ± SEM, N = 10). This value is in agreement with those previously reported by others [43, 44]. No changes in $[Ca^{2+}]_i$ were observed during short-term incubations of hepatocytes with cocaine (Fig. 3A and B), but they became evident after long-term incubations.

Non-induced rat hepatocytes showed a dose-dependent increase in $[Ca^{2+}]_i$ after 2 hr incubation. The maximal $[Ca^{2+}]_i$ was twice as high as the control and was found after 5 hr incubation with 2000 μM cocaine (Fig. 3A). In spite of this, cell viability had not significantly decreased (Fig. 1A). In induced rat hepatocytes, changes in $[Ca^{2+}]_i$ occurred at much lower cocaine concentrations (Fig. 3B) and with a concomitant loss of cell viability (Fig. 1B).

The measured increase in $[Ca^{2+}]_i$ was not transient in nature. Its concentration remained elevated even after 20 hr incubation.

GSH depletion by cocaine

Cocaine caused rapid depletion of GSH in both non-induced and induced rat hepatocytes, clearly noticeable after 30 min (Fig. 4). Maximal depletion was reached after 2–5 hr incubation with the drug, and subsequently the general tendency of cells was to replenish GSH.

Despite the extensive cytotoxicity caused by the highest concentrations of cocaine assayed (1000 and 2000 μM) in non-induced hepatocytes, the effect of this drug on GSH levels was moderate and they never decreased beyond 60% of control values (Fig. 4A). In contrast, the depletion caused by cocaine in induced rat hepatocytes was much greater (down to 30% of controls) and more sustained (Fig. 4B) at

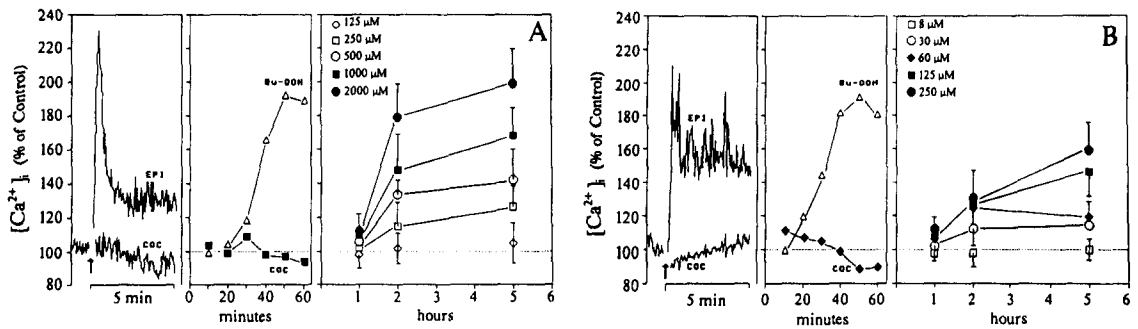

Fig. 3. Changes in $[Ca^{2+}]_i$ caused by cocaine in hepatocytes from non-induced (A) or induced (B) rats. Short-term and long-term changes in $[Ca^{2+}]_i$ were measured after cell exposure to cocaine according to the experimental procedure described in Materials and Methods. (A) and (B) include the effect of 10 μM epinephrine (EPI) and 250 μM *tert*-butyl hydroperoxide (Bu-OOH) to demonstrate the responsiveness of hepatocytes during short-term $[Ca^{2+}]_i$ measurements (0–5 and 10–60 min). Data shown in these short-term $[Ca^{2+}]_i$ measurements are from a representative experiment; COC = cocaine 1000 (A) and 60 (B) μM. Data shown in long-term $[Ca^{2+}]_i$ measurements are means ± SEM of five to six different cell preparations, and are expressed as percentage of controls (see Table 1).

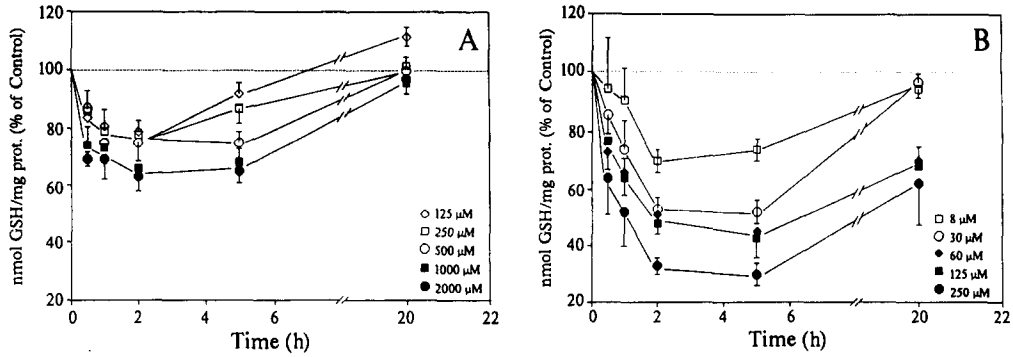

Fig. 4. Time course of GSH depletion by cocaine in non-induced (A) and induced (B) rat hepatocytes. Cultured cells were exposed to cocaine, and at the indicated time points GSH content was measured. Results are expressed as percentage of controls (see Table 1). Data are means ± SEM of four to six different cell preparations.

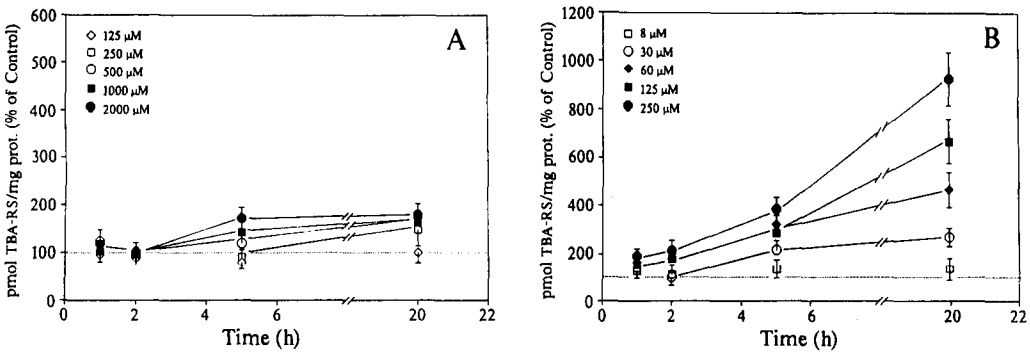

Fig. 5. Effect of cocaine on lipid peroxidation in hepatocytes from non-induced (A) and phenobarbital-induced (B) rats. Cultured cells were exposed to cocaine, and at the indicated time points the TBA-reacting substances were measured in the culture medium. Results are expressed as percentage of controls (see Table 1). Data are means ± SEM of three to four different cell preparations.

lower concentrations of the drug. The decrease in GSH preceded cytotoxicity, but non-cytotoxic concentrations of cocaine (i.e. 8 μM) were also able to significantly decrease cellular GSH.

Lipid peroxidation in rat hepatocyte cultures exposed to cocaine

Cultured rat hepatocytes do not accumulate end products of lipid peroxidation like malondialdehyde. They excrete these substances into the extracellular medium, where they can be detected shortly after they are produced [45]. Cocaine caused barely measurable lipid peroxidation in non-induced rat hepatocytes (Fig. 5A). In contrast, the peroxidation of cellular lipids was notably enhanced by cocaine in induced rat hepatocytes (Fig. 5B). Substances reacting with TBA, which result from the peroxidative process, gradually accumulated in the medium after a lag phase of 1–2 hr, when phenobarbital-induced hepatocytes were exposed to concentrations of cocaine in the 30–250 μM range.

Time-course of $[Ca^{2+}]_i$, *GSH content, lipid peroxidation and cell viability in induced and non-induced hepatocytes exposed to cocaine*

Figure 6 summarizes the time-course of $[Ca^{2+}]_i$ GSH and lipid peroxidation in both non-induced (A) and induced (B) rat hepatocytes after addition of cocaine to the culture medium. The common feature of the two experiments was the decrease in cell viability (50%) after 20 hr incubation.

A sustained rise in $[Ca^{2+}]_i$ was the most notable finding in non-induced rat hepatocytes incubated with 1000 μM cocaine (Fig. 6A). The depletion of GSH and lipid peroxidation was much less marked in these cultures, and cell death was detected after 20 hr treatment. On the other hand, the rapid depletion of GSH and increased lipid peroxidation were the two most remarkable phenomena in induced hepatocytes incubated with 60 μM cocaine, whereas the rise in $[Ca^{2+}]_i$ was less marked, and led to a rapid decrease in cell viability (5 hr, Fig. 6B).

Incubation of hepatocytes from induced rats with

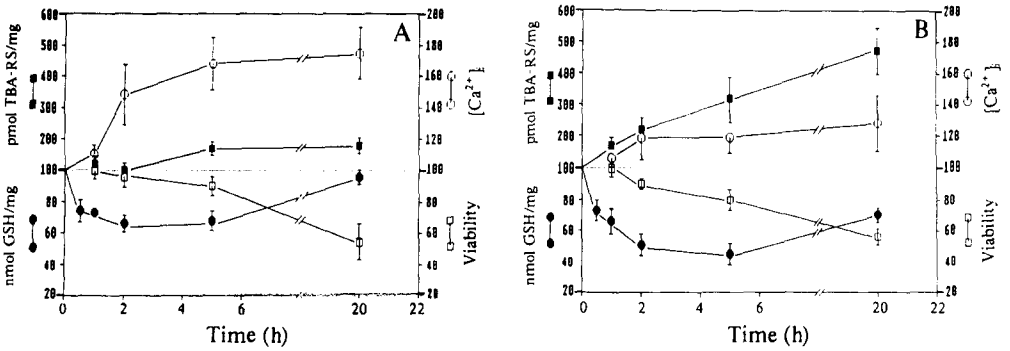

Fig. 6. Comparative time course of $[Ca^{2+}]_i$, GSH content and lipid peroxidation in non-induced (A) and induced hepatocytes (B) exposed to cocaine. Cells were exposed to a concentration of the drug able to reduce cell viability by 50% after 20 hr; 1000 (A) and 60 (B) μM. After variable time intervals, LDH activity, $[Ca^{2+}]_i$ GSH and TBA-reacting substances were evaluated. Results are expressed as percentage of controls (see Table 1). Data are means ± SEM of three to six different cell preparations.

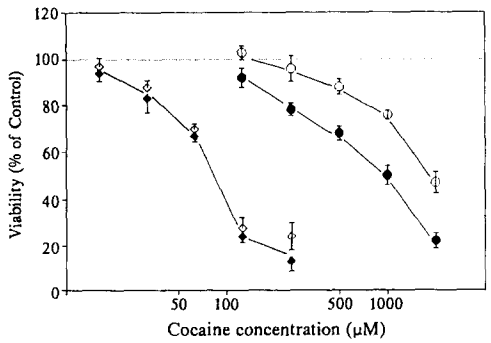

Fig. 7. Protective effect of verapamil against cocaine-induced cytotoxicity. Hepatocytes from non-induced (circles) and phenobarbital-induced (diamonds) rats were exposed to increasing concentrations of cocaine in presence (open symbols) or absence (filled symbols) of 25 μM verapamil. After 24 hr incubation intracellular LDH activity was measured as index of cell viability. Data are means ± SD of two different cell preparations.

the calcium channel blocker verapamil (25 μM) and with increasing concentrations of cocaine showed no protective effect against cocaine cytotoxicity (IC_{50} 83 μM cocaine vs 86 μM cocaine + verapamil). In contrast, in non-induced cells, the use of verapamil markedly reduced the toxicity of cocaine, shifting the IC_{50} from 950 to 1965 μM (Fig. 7).

DISCUSSION

The key molecular events involved in cocaine-induced hepatotoxicity are now beginning to be understood. In spite of this, our present knowledge on this subject is still incomplete. In an attempt to understand better the mechanism of hepatotoxicity we have investigated, both in phenobarbital-induced and non-induced rat hepatocytes, the major biochemical events preceding cell death after incubating cells with cocaine.

The N-demethylation of cocaine to norcocaine seems to be the first step towards that hepatotoxicity of the drug [18]. This metabolite is further oxidized to *N*-hydroxynorcocaine and norcocaine nitroxide. It has been postulated that both metabolites participate in a redox-cycling reaction causing NADPH depletion and generating reactive oxygen species [15]. One interesting finding of this work is that the sensitivity of hepatocytes to cocaine correlated well with the *in vitro* activity of cytochrome P450 2B1/2, the P450 isozymes involved in the N-demethylation of cocaine in rat hepatocytes [33]. Moreover, when this activity decreased in cultured cells, the cytotoxicity of cocaine also decreased. These results explain and support former studies [25, 46] reporting that cocaine cytotoxicity was increased in the hepatocytes of rats previously treated with phenobarbital.

A second finding was that only hepatocytes from induced animals exposed to cocaine showed substantial depletion of GSH followed by a dramatic increase in lipid peroxidation. These two events point to oxidative stress elicited by cocaine [15]. In phenobarbital-induced hepatocytes, depletion of GSH was faster, greater and more sustained than in non-induced cells. Lipid peroxidation began shortly after GSH depletion (2–5 hr after addition of cocaine) and paralleled the loss of cellular viability. The greater the GSH depletion caused by cocaine, the greater the cytotoxicity. Thus, it is understandable that in hepatocytes with high cytochrome P450 2B1/2 activity extensive oxidative metabolism of cocaine takes place, overwhelming the GSH defence systems and increasing lipid peroxidation. Accordingly, we found that in non-induced hepatocytes, similar concentrations of cocaine neither caused a severe depletion of GSH nor increased lipid peroxidation.

Many xenobiotics can disturb calcium homeostasis, thus leading to a sustained rise in intracellular $[Ca^{2+}]_i$, which then obliterates the responses to hormone stimulation, compromises mitochondrial function and ATP production, alters cytoskeletal organization, and activates irreversible catabolic

processes [29]. We have explored whether a similar mechanism could be involved in cocaine hepatotoxicity, as was recently hypothesized [25, 30, 47]. The studies of Engelking *et al.* [31] failed to demonstrate any significant change in $[Ca^{2+}]_i$ in rat hepatocytes briefly exposed to cocaine (2 min). We have been able to show that cocaine significantly changed $[Ca^{2+}]_i$ in both induced and non-induced rat hepatocytes after a few hours of incubation. Interestingly, the $[Ca^{2+}]_i$ elevation preceded cell death in non-induced cells. On the other hand, the rise in $[Ca^{2+}]_i$ found in induced hepatocytes was more modest and was first evident when cells started to die.

The comparison between $[Ca^{2+}]_i$, GSH and lipid peroxidation in hepatocytes incubated with a IC_{50} cytotoxic concentration of cocaine suggests a sequential mechanism presumably involved in the killing of hepatocytes by cocaine (Fig. 6). In hepatocytes with elevated cytochrome P450 2B1/2 activity, GSH depletion and lipid peroxidation were the two most remarkable phenomena at low concentrations of cocaine. These two events are compatible with the previously postulated toxic mechanisms of cocaine: (1) redox cycling between *N*-hydroxynorcocaine and norcocaine nitroxide; (2) production of reactive oxygen species at the expense of NADPH; and (3) depletion of glutathione followed by lipid peroxidation [15, 20]. The rise in $[Ca^{2+}]_i$ observed in these cells was less significant and the use of calcium channel blockers (verapamil) did not prevent cell death caused by cocaine. These two facts suggest that the elevation of cellular calcium is not the event finally responsible for the toxicity of cocaine in induced hepatocytes.

The rise in $[Ca^{2+}]_i$ was the most remarkable event in non-induced rat hepatocytes (Fig. 5A). Both GSH depletion and lipid peroxidation were much less marked in these cells, where low cytochrome P450 2B1/2 activity ensures lower rates of cocaine oxidation. $[Ca^{2+}]_i$ reached levels ≥ 400 nM, similar to those found in hepatocytes after hormonal stimulation [48–50] (Fig. 5A), but in contrast to the transient elevation elicited by hormonal stimulation, the effects of cocaine were long-lasting. Cocaine, as a local anesthetic, has its main site of action in the cell membrane, where it alters permeability to potassium and sodium ions [1]. In the same way, it is conceivable that when the concentration of cocaine is high enough (i.e. after overdosage), it could interfere directly with the sodium pumps and indirectly with Na^+/Ca^{2+} exchange [51], thus impairing the extrusion of calcium ions and calcium homeostasis in hepatocytes. The protection elicited by verapamil against cocaine toxicity in these cells lends support to this hypothesis. This mechanism of toxicity in non-induced cells appears effective at elevated concentrations of cocaine. The relevance of this mechanism should be considered in certain cases, i.e. after high and/or repetitive cocaine doses in man [52] or in studies in animals after high acute doses [53].

Taken together, the results of this study suggest two different pathways for cocaine hepatotoxicity. In phenobarbital-induced hepatocytes the greater oxidative metabolism of the drug makes it possible for low concentrations of cocaine to cause marked depletion of GSH and subsequent extensive lipid peroxidation. In non-induced hepatocytes these effects are not relevant, and the major alteration is the non-transient rise in $[Ca^{2+}]_i$ at more elevated concentrations of cocaine.

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