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Mitochondrial involvement in cocaine-treated rat hepatocytes: effect of N-acetylcysteine and deferoxamine

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1 The cytotoxicity of cocaine (0–1000 μM), was studied on parameters related to the mitochondrial role and the cascade of events that lead to apoptosis in hepatocyte cultures from phenobarbitone (PB) pretreated rats.

2 Cytotoxicity was dose-dependent and LDH leakage was significantly enhanced above 100 μM cocaine. Apoptosis was visualized by DNA fragmentation on agarose gel, and appeared at 50 and 100 μM cocaine. Cocaine induced biphasic changes in mitochondrial transmembrane potential and significantly increased the mitochondrial release of cytochrome *c*, the caspase-3 like DEVDase activity and the level of 20 kDa subunit, a product of pro-caspase-3 cleavage.

3 The protective effect of N-acetylcysteine (NAC) and deferoxamine (DFO) on all these parameters confirmed the involvement of oxygen radicals in cocaine-induced necrosis/apoptosis.

4 We conclude: first, that the biphasic changes recorded in mitochondrial inner membrane potential by the effect of cocaine, were parallel to apoptosis; second, that caspase-3 activity and cleavage to it p20 subunit increased sharply in parallel to the translocation of cytochrome *c* from mitochondria to cytosol; and third, that the antioxidants, NAC or DFO exerted a noticeable protective role in counteracting the cytotoxicity of cocaine, these effects being more pronounced in the case of DFO than NAC.

5 These findings demonstrate that cocaine cytotoxicity involves mitochondrial damage.
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Keywords: Cocaine cytotoxicity; oxidative stress; mitochondria; cytochrome *c*; caspase-3; apoptosis; antioxidants

Abbreviations: CMXRos, Chloromethyl-X-rosamine; DFO, deferoxamine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithioerithitol; EDTA, ethylen diamino tetraacetic acid; FCS, foetal calf serum, LDH, lactate dehydrogenase; NAC, N-acetylcysteine; $\Delta\Psi$m, mitochondrial transmembrane potential; PB, phenobarbitone; ROS, reactive oxygen species; PMSF, phenyl methyl sulphonyl fluoride.

Introduction

Experimental and clinical evidence has demonstrated that cocaine is an hepatotoxic drug that significantly alters liver function and induces liver injury characterized by an unevenly distributed intraacinar necrosis (Marks & Chappel, 1967; Kanel *et al*., 1990; Cascales *et al*., 1994). Cocaine hepatotoxicity is mediated by a series of sequential oxidations catalyzed by cytochrome P450 and flavin-mono-oxygenases (Kloss *et al*., 1984; Boelsterli & Godlin, 1991). It is well known that phenobarbitone (PB) by inducing the expression of P-450 microsomal mono-oxygenases responsible for cocaine oxidative metabolism (Cascales *et al*., 1994; Powell *et al*., 1994) enhances the rate of cocaine oxidation and consequently the hepatotoxicity of this drug. Two major pathways have been postulated to explain cocaine-induced hepatotoxicity, as the result of oxidative metabolism: (a) either through depletion of cellular reducing equivalents (NADPH) and generation of reactive oxygen species (ROS), superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), during the redox cycling between N-hydroxynorcocaine and norcocaine nitroxide: or (b) through the production of the nitrosonium ion or other as yet unidentified reactive metabolites that bind covalently to cellular macromolecules (Kloss *et al*., 1984; Bouis & Boelsterli, 1990; Boelsterli & Godlin, 1991; Jover *et al*., 1993; Ponsoda *et al*., 1999).

In a previous study we demonstrated that apoptosis occurs in liver following the *in vivo* administration of cocaine to mice either pre-treated or non-pretreated with PB (Cascales *et al*., 1994). Apoptosis was also detected in cultures of hepatocytes from PB-pretreated rats (Zaragoza *et al*., 2000). Several experiments have demonstrated that cocaine can induce apoptosis in cultured foetal neurons (Nassogne *et al*., 1997), mouse thymocyte populations (Wu *et al*., 1997) and foetal myocardial cells (Xiao *et al*., 2000). Pathological studies have implicated oxidative damage in the mechanisms of cocaine-induced liver injury (Godlin & Boelsterli, 1991; Boelsterli *et al*., 1993) Recent data of our group (Díez-Fernández *et al*., 1999) have also shown that reactive oxygen species contribute directly or indirectly to cocaine-induced apoptosis in cultured hepatocytes. Actually, although many factors can be involved in apoptosis, ROS generated as subproducts derived from the metabolism of toxic agents are important mediators in this

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form of cell death (Gardner *et al*., 1997; Hildeman *et al*., 1999).

Several studies have also shown that mitochondrial alterations are involved in these processes (Masini *et al*., 1997), and that structural and functional changes in mitochondria are impaired by cocaine and its metabolites (Devi & Chan, 1996; Yuan & Acosta, 1996; Xiao *et al*., 2000). Recent studies suggest that mitochondria could be the main sensor in apoptosis, since the release of mitochondrial factors in the cytosol, such as cytochrome *c*, can initiate the activation cascade of caspases (Liu *et al*., 1996; Green & Reed, 1998), a crucial event in the apoptotic death program. The activity of these proteases, related to interleukin-1β-converting enzyme (ICE) is involved in the multiple steps, such as cell shrinkage, membrane blebbing and chromatin degradation, that constitute apoptosis.

It is well known that exogenous antioxidants play an important role against oxidant injury induced by excess of ROS generation. The hepatoprotective action of N-acetylcysteine (NAC), a thiol containing a compound that acts as a nucleophile and as a precursor of reduced glutathione, is well defined (Cotgreave, 1997); and the effect of deferoxamine (DFO), an iron chelating agent that consequently prevents ROS generation by inhibiting Fenton reaction, has also been well studied (Susa *et al*., 1997; Zaragoza *et al*., 2000). The purpose of the present investigation was to find, in a model of cocaine cytotoxicity, using rat hepatocyte cultures, a potential relationship between apoptosis and a series of related parameters: the presence of cytochrome *c* in the cytosol, caspase activity and the levels of 20 kDa caspase subunit, and mitochondrial transmembrane potential ($\Delta\Psi_m$). Afterwards, the effect of NAC or DFO as antioxidants coadministrated with cocaine, was evaluated in the same experimental conditions to determine the way by which these substances protect against the toxic effects of cocaine.

Methods

Reagents

Tissue culture media were obtained from Biowhittaker (Walkesville, U.S.A.). Standard analytical grade laboratory reagents were obtained from Merck (Germany). Collagenase was obtained from Boehringer (Manheim, Germany). Polyclonal antibodies for cytochrome *c* and caspase-3 were purchased from Santa Cruz Biotechnology U.S.A.). Agarose was from Hispanagar (Barcelona, Spain). Chloromethyl-X-rosamine (CMXRos) and the 7-amino-4-methylcoumarin-derived substrate Z-DEVD-AMC were obtained from Molecular Probes (U.S.A.). Rnase was obtained from Boehringer (Mannheim, Germany). Proteinase K was obtained from Sigma Chemical Co (U.S.A.).

Phenobarbitone, N-acetylcysteine and deferoxamine were from Sigma Chem Co (U.S.A.). HCl cocaine (99.5% purity, checked by HPLC), was obtained from the Drug Restriction Service Ministry of Health in Spain, proteinase K.

Animals

Two month old male Wistar rats with an average body weight of 180–230 g were used for the cell preparations. Animals received care as outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health. Rats were supplied with food and water *ad libitum* and exposed to a 12-h light–dark cycle. Since pre-treatment of rats with phenobarbitone (PB) is required for toxicity to be enhanced (Cascales *et al*., 1994), 80 mg kg^{-1} day^{-1} of sodium phenobarbitone was injected intraperitoneally 2 days previous to the isolation of hepatocytes.

Isolation and culture of hepatocytes

Hepatocytes were isolated by perfusion of the liver with collagenase as described elsewhere (Seglen, 1993; Díez-Fernández *et al*., 1998) and cell viability, determined by trypan blue exclusion, was always greater than 90%. 1.5×10^6 freshly isolated hepatocytes were seeded into 60×15 mm culture dishes (Becton–Dickinson) in 3 ml Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin and 10% foetal calf serum (FCS). After 3 h incubation at 37°C in a humidified 5% CO_2–95% air atmosphere, the medium was replaced with fresh medium supplemented by 0.1% bovine serum albumin (B.S.A.).

Exposure to cocaine and antioxidants and LDH leakage assay

Isolated hepatocytes from PB-pretreated rats were pre-incubated for 24 h either in the presence or absence of 0.5 mM NAC or 1.5 mM DFO, and exposed for another 24 h to increasing concentrations of cocaine from 0–1000 μM together with 0.5 mM NAC or 1.5 mM DFO. The concentrations of NAC and DFO were calculated by selecting the highest dose of each antioxidant that protects cells without increasing the toxicity. As an index of cell toxicity and membrane lysis LDH leakage was measured (Welder & Acosta, 1994). Cocaine HCl was dissolved in fresh medium and hepatocytes were exposed to the drug at a dose range of 0–1000 μM for 24 h. NAC and DFO were dissolved in fresh medium and added to precultured hepatocytes at doses of 0.5 mM and 1.5 mM, respectively (Zaragoza *et al*., 2000). Cytotoxicity was measured using the index of lactate dehydrogenase (LDH) (Welder & Acosta, 1994) leakage from damaged hepatocytes according to Vasault (1987), and was expressed as a percentage of total cellular activity.

Analysis of DNA fragmentation

Hepatocytes (1.5×10^6 cells) were treated with lysis buffer (0.1 M Tris/HCl, pH 7.8, 10 mM EDTA and 0.5% lauroyl sarcosine) for 30 min at 41°C and centrifuged at $30{,}000 \times g$ for 15 min at 41°C. The supernatant was digested with RNase (10 mg/ml) for 30 min at 37°C followed by the treatment with 250 mg ml^{-1} proteinase K for 45 min at 50°C. DNA was extracted with phenol-chloroform, ethanol-precipitated in the presence of 0.5 M NaCl and stored at −20°C overnight. DNA was collected by centrifugation and resuspended in 10 mM Tris, 1 mM EDTA, pH 8 buffer. Gel electrophoresis was performed at constant voltage (30 V) for 8 h through a 1.8% agarose gel containing 0.5 mg ml^{-1} of ethidium bromide and the DNA was visualized using an

ultraviolet light source (Cascales *et al*., 1994; McGahon *et al*., 1995).

Determination of changes in mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was monitored by flow cytometry using chloromethyl-X-rosamine (CMXRos). This dye, a derivative of rhodamine 123, permeates the plasma membrane and specifically associates with actively respiring mitochondria (Poot & Pierce, 1999). Following the incubation with cocaine, in the presence or absence of antioxidant, cultured hepatocytes were washed with phosphate buffer saline (PBS) and immediately detached with Trypsin/EDTA and incubated with agitation for 30 min in 2 ml of PBS containing 100 nM CMXRos at 37°C. The cells were washed twice with PBS, followed by analysis on a FACScan flow cytometer (Becton-Dickinson).

Caspase activity

Caspase-3 activity (Z-DEVD-AMC cleavage) was assayed essentially as previously described (Vanags *et al*., 1996). Hepatocytes were lysed in 25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X-100, pH 7.4. The fluorimetric assay was performed with a substrate concentration of 40 μM. Cleavage was followed in reaction buffer (50 mM HEPES, pH 7.4, 10 mM dithiothreitol (DTT), 1% sucrose, 0.1% CHAPS) over a period of 30 min (excitation: 350 nm; emission: 450 nm). The Ac-DEVD-CHO inhibitor was used to confirm that the observed fluorescence signal in cells was due to the activity of caspase-3-like proteases. Protein estimation was made according to Bradford (1976), using bovine serum albumin (BSA) as standard.

Western blotting

Caspase-3 cleavage and cytoplasmic cytochrome *c* content were analysed by immunoblot. To detect the release of cytochrome *c* from the mitochondria to the cytosol, hepatocytes were harvested after treatments, resuspended in lysis buffer (mM: HEPES 20, pH 7.4, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, DTT 1, phenyl methyl sulfonyl fluoride (PMSF); (μM): leupeptin 10, and aprotinin containing 250 mM sucrose) 10 on ice and disrupted by 40 strokes of a glass homogenizer. Cells were then centrifuged at $750 \times g$ for 10 min at 4°C; the resultant supernatant was centrifuged at $12{,}000 \times g$ for 30 min at 4°C to remove mitochondria, and the final supernatant was used as the cytosol fraction for Western blot analysis. For caspase-3 Western blot analysis, cells were lysed in a buffer containing 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF and 40 μM leupeptin for 15 min on ice (Hames & Rickwood, 1990).

Equal amounts of protein were boiled in Laemmli's buffer for 10 min, and were separated in 15% SDS-polyacrylamide gels. After transblotting, the nitrocellulose membranes were blocked in 5% non-fat milk, and then probed using polyclonal antibodies against cytochrome *c* and caspase-3 (Santa Cruz Biotechnology). After incubation with primary and appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), membranes were developed using the ECL-reagent (Amersham).

Statistical analysis

All results were expressed as the means ± s.d.mean of four observations (four animals) in duplicate. Time-course data were compared by using Student *t*-test: (1) values against its corresponding control (no cocaine). NAC or DFO data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student-Newman-Keuls *post hoc* test; (2) differences due to NAC; (3) differences due to DFO. Differences we considered significant at $P < 0.05$.

Results

Figure 1 shows the effect of either NAC or DFO on the percentages of LDH released by hepatocytes when incubated with increasing concentrations of cocaine (0, 50, 100, 500 and 1000 μM). In the experimental conditions of the present study, the cytotoxic effect of cocaine was dose-dependent, and the differences versus control (no cocaine) were significant ($P < 0.05$) at concentrations above 500 μM of cocaine. The effect of 0.5 mM NAC decreased significantly the release of LDH at 500 and 1000 μM cocaine to 70% ($P < 0.05$) and 64% ($P < 0.05$), respectively. In the case of DFO, the protective effect was also significant ($P < 0.05$), 67 and 69%, at the same cocaine concentrations, respectively.

Cocaine-induced apoptosis was identified on the basis of the occurrence of internucleosomal DNA cleavage on agarose gel electophoresis. The effect of cocaine on the integrity of DNA and the corresponding electrophoretic pattern of the DNA extracted from cocaine-treated hepatocytes is shown in Figure 2. The characteristic formation of oligonucleosome-sized fragments of DNA as ladders of multiples of ≈200 bp on agarose gels, the biochemical hallmark of apoptosis, was detected in cells incubated in the presence of increasing concentrations of cocaine (from 50 to 500 μM). The

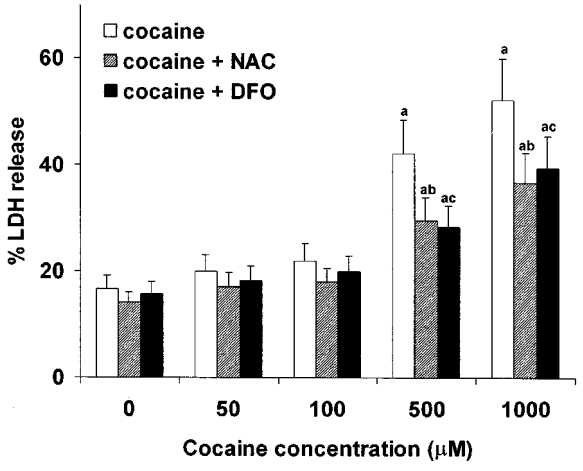

Figure 1 Effect of 0.5 mM NAC and 1.5 mM DFO on cytotoxicity of cocaine in cultured hepatocytes isolated from PB-induced rats. Data are expressed as mean ± s.d.mean of four independent experiments from four rats and were compared by a two-way ANOVA test. Pairwise comparisons were conducted by a Student Newman-Keuls *post hoc* test. (a) Values against their controls; (b) differences due to NAC; (c) differences due to DFO. $P < 0.05$ was considered the level of significance.

protective effect of antioxidants on apoptosis induced by cocaine is also shown in Figure 2. The coincubation of hepatocytes with cocaine and NAC or DFO, at the concentrations above mentioned, diminished the DNA ladders observed in hepatocytes treated with cocaine. As Figure 2 shows, the effect against apoptosis was much more pronounced in the case of DFO.

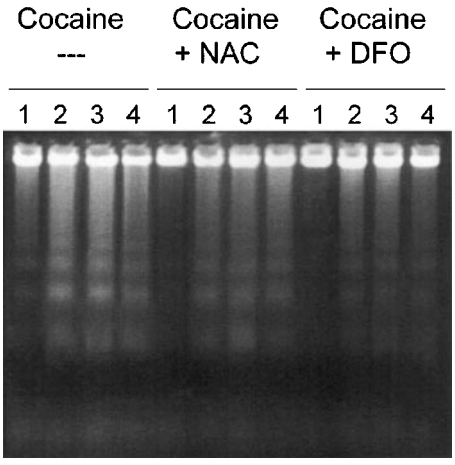

Figure 2 Analysis of internucleosomal DNA cleavage on agarose gel electophoresis in primary cultures of rat hepatocytes incubated with cocaine in the presence of 0.5 mM NAC or 1.5 mM DFO. Lanes 1, 2, 3 and 4 correspond to 0, 50, 100 and 500 μM cocaine, respectively.

For better understanding the mechanisms underlying cocaine-induced apoptosis in hepatocytes, we examined the effect of this drug on cytochrome *c* translocation from the mitochondrial inter membrane space into the sytosol in these cells. The representative Western blot analysis showed that the polyclonal antibody for cytochrome *c* detected a single band at the expected size of 15 kDa. After cocaine treatment, there was an increase in cytochrome *c* levels in the cytosolic fraction of cells incubated in the presence of doses of 100 μM, diminishing at higher concentrations (500 and 1000 μM). Densitometric scanning of the signals for various independent experiments, revealed that with 100 μM cocaine cytosolic cytochrome *c* levels were increased by 8 fold. The addition of NAC to the culture medium diminished cocaine-induced translocation of cytochrome *c* in hepatocytes at 100 and 1000 μM cocaine (Figure 3A). These changes were significantly different when compared to cocaine alone at a concentration of 100 μM (49%, $P<0.05$, of the corresponding value with no antioxidant). The DFO effect was even more pronounced against cytochrome *c* release from the mitochondria induced by cocaine (Figure 3B). This iron chelating agent decreased significantly cytochrome *c* levels in the cytosol at all cocaine concentrations with the following results: 56% ($P<0.05$); 33% ($P<0.05$); 55% ($P<0.05$); and 69% ($P<0.05$), for 0, 100, 500 and 1000 μM cocaine, respectively.

Using fluorometric assay, we detected that caspase-3-like DEVDase activity increased progressively and significantly in cocaine-treated hepatocytes as the drug concentration increased, from 0 to 1000 μM. Thus, if we consider 100% for the control with no cocaine, the values obtained were

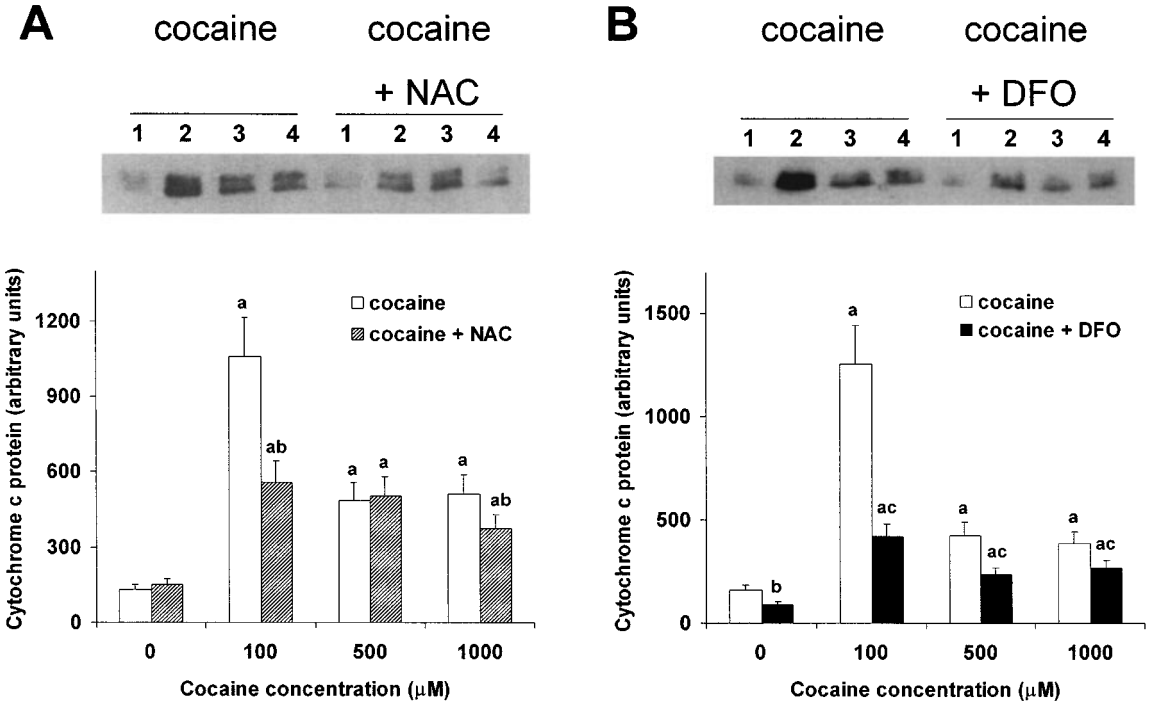

Figure 3 Western blot analysis of cytosolic levels of cytochrome *c* in hepatocytes incubated with cocaine in the presence of 0.5 mM NAD or 1.5 mM DFO. 1, 2, 3 and 4 correspond to 0, 100, 500 and 1000 μM cocaine. A and B shows the differences due to NAC and DFO, respectively. Data are expressed as mean $\pm$ s.d.mean of four independent experiments from four rats and were compared by a two-way ANOVA test. Pairwise comparisons were conducted by a Student Newman-Keuls *post hoc* test. (a) Values against their controls; (b) differences due to NAC; (c) differences due to DFO. $P<0.05$ was considered the level of significance.

156% ($P<0.05$), 193% ($P<0.05$) and 280% ($P<0.05$) for 100, 500 and 1000 μM of cocaine, respectively (Figure 4). Coincubation of cocaine with NAC or DFO (Figure 4) decreased the effect of cocaine on caspase-3-like DEVase activity.

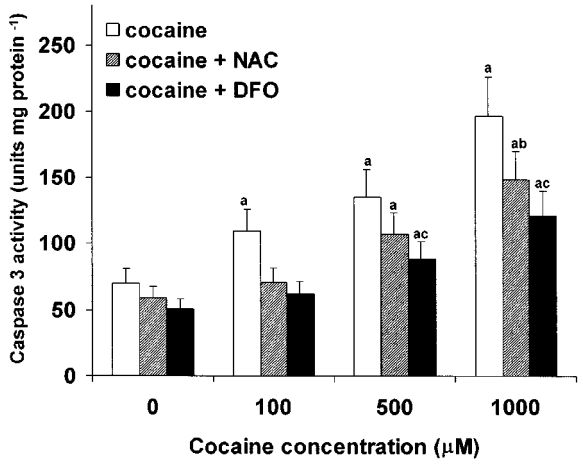

Figure 4 Caspase 3 activity in primary cultures of hepatocytes incubated with cocaine in the presence of 0.5 mM NAC or 1.5 mM DFO. Results obtained as fluorescence units (350 nm excitation and 450 nm emission) mg of protein^{-1}, are expressed as mean$\pm$s.d.mean of four independent experiments from four rats and were compared by a two-way ANOVA test. Pairwise comparisons were conducted by a Student Newman-Keuls *post hoc* test. (a) values against their controls; (b) differences due to NAC; (c) differences due to DFO. $P<0.05$ was considered the level of significance.

Activation of the caspase involves the proteolytic cleavage of the precursor at specific Asp residues into a 20 kDa and a 10 kDa active heterodimer in cells undergoing apoptosis (Jaescke *et al*., 1998). To determine whether caspase-3 is processed to the active protease in hepatocytes, a rabbit polyclonal antibody which reacts with the p20 subunit, was probed, as shown in Figure 5A. Western blots analysis of protein samples clearly demonstrated that the content of the p20 subunit, calculated by arbitrary units, as expressed in Figure 5, sharply and significantly increased by the effect of cocaine. The increased values, expressed as percentages against control conditions (no cocaine), were as follows: 202% ($P<0.05$), 476% ($P<0.05$) and 453% ($P<0.05$) for doses of 100, 500 and 1000 μM of cocaine, respectively. Also shown in Figure 5 is that NAC or DFO, when incubated simultaneously to cocaine, decreased the p20 subunit content. The DFO effect was significant at all cocaine concentrations assayed (Figure 5B), whereas differences registered in the presence of NAC were less pronounced (Figure 5A).

Cocaine hepatotoxicity possibly involves mitochondrial injury. Modifications in mitochondrial transmembrane potential ($\Delta\Psi_m$) have also been correlated with the induction of cell death by apoptosis (Green & Reed, 1998). To assess whether cocaine induces mitochondrial changes in hepatocytes, we measured by flow cytometry the mitochondrial uptake of sensitive dye, CMXRos. Figure 6A shows the histograms in which fluorescence of this dye, detected with the FL3-H channel, is plotted against the number of cells. |-M2-| defines the peak of mitochondrial membrane potential (intense fluorescence). The quantification in arbitrary units of the |-M2-| peak is shown in Figure 6B. Treatment of

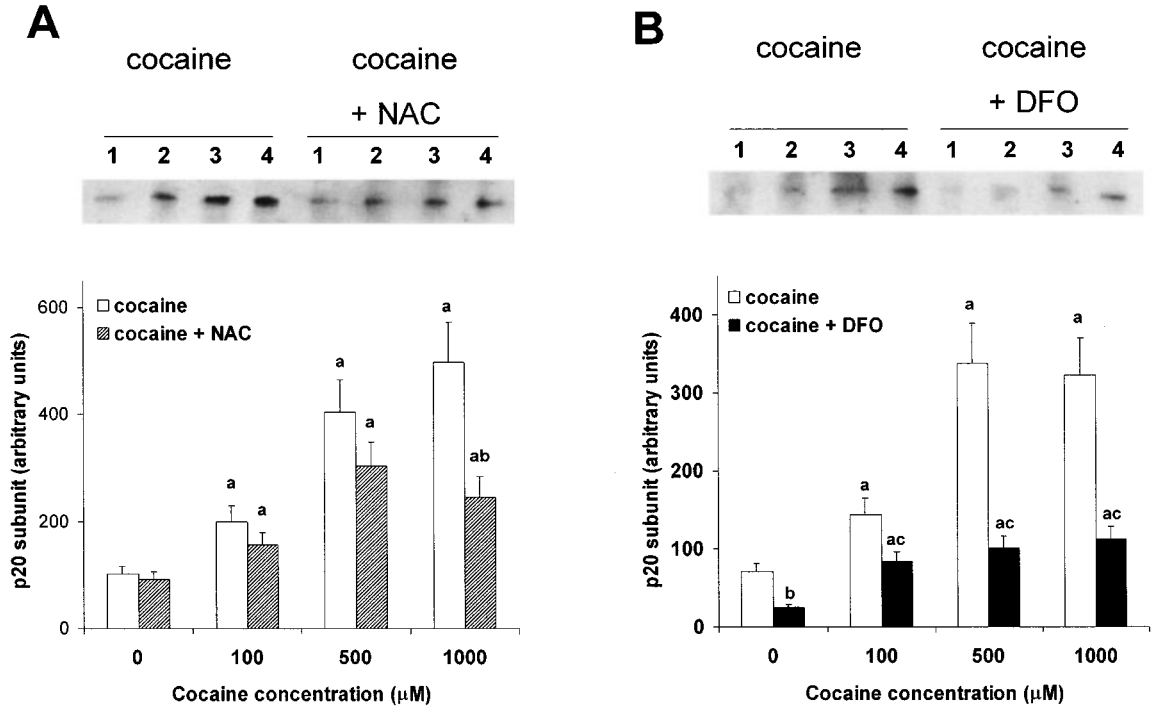

Figure 5 Western blots analysis of the levels of caspase-3 p20 subunit in primary cultures of hepatocytes incubated with cocaine in the presence of 0.5 mM NAC or 1.5 mM DFO. Results, obtained as arbitrary units, are the mean$\pm$s.d.mean of four experiments from four animals and were compared by a two-way ANOVA test. Pairwise comparisons were conducted by a Student Newman-Keuls *post hoc* test. (a) values against their controls; (b) differences due to NAC; (c) differences due to DFO. $P<0.05$ was considered the level of significance.

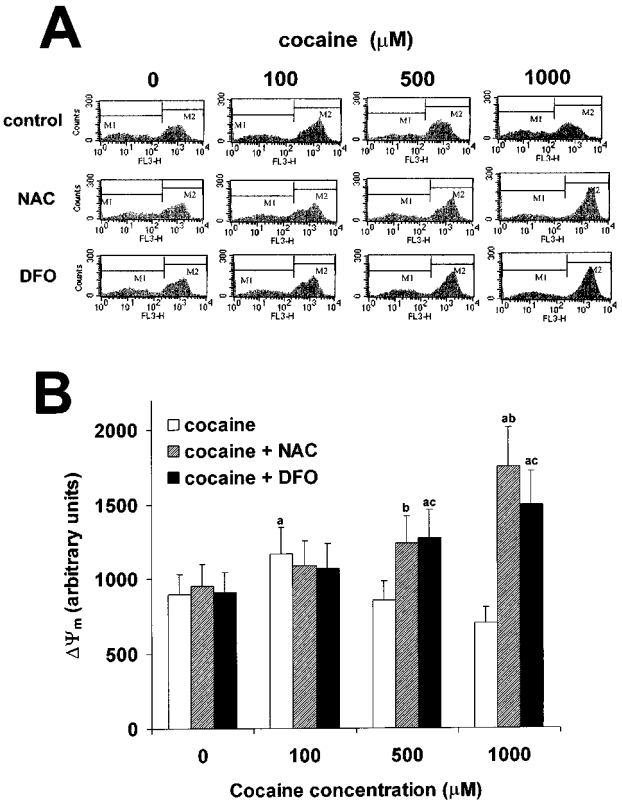

Figure 6 Flow cytometry analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in primary cultures of hepatocytes incubated with cocaine in the presence of 0.5 mM NAC or 1.5 mM DFO. ($\Delta\Psi_m$) was measured by flow cytometry using a membrane potential sensitive dye, CMXRos. Figure 6A shows the histograms in which fluorescence of this dye, detected with the FL3-H channel, is plotted against the number of cells. |-M2-| defines the peak of mitochondrial membrane potential (intense fluorescence). The quantification in arbitrary units of the |-M2-| peak is shown in Figure 6B. The results are the mean ± s.d.mean of four experiments from four animals and were compared by a two-way ANOVA test. Pairwise comparisons were conducted by a Student Newman-Keuls *post hoc* test. (a) Values against their controls; (b) differences due to NAC; (c) differences due to DFO. $P<0.05$ was considered the level of significance.

hepatocyte cultures with high doses of cocaine (500 & 1000 μM) produced a decline in $\Delta\Psi_m$, while a 100 μM concentration of the drug induced marked increases in CMXRos fluorescence. However the correlation between the changes in these two parameters dissapear in the presence of either of the antioxidants used since parallel dose-dependent increases in cytochrome *c* release from mitochondria were detected.

Discussion

It is well known that oxidative damage plays a role in the cytotoxicity of cocaine in the liver (Kloss *et al*., 1984; Perino *et al*., 1987; Boelsterli *et al*., 1991; Poet *et al*., 1996; Bornheim, 1998; Ponsoda *et al*., 1999), and in a recent *in vitro* study from our group, a relationship was proposed between the generation of ROS, through the oxidative metabolism of cocaine, and apoptosis (Díez-Fernández *et al*., 1999; Zaragoza *et al*., 2000). It has been suggested that cocaine cytotoxicity possibly involves mitochondrial damage (Yuan & Acosta, 1996; Devi & Chan, 1997; Masini *et al*., 1997), and that mitochondria plays an important role in regulating apoptotic cell death (Green & Reed, 1998). Taking into account these considerations, our present study was directed towards investigating the involvement of the mitochondrial in cocaine cytotoxicity and the mechanisms by which apoptosis is induced by this drug. The potential protective effect of antioxidant substances, such as NAC and DFO, on the extent of apoptosis and on those parameters involved in apoptosis machinery was also investigated.

Induction of apoptosis is accompanied by a fall in the mitochondrial membrane potential as a consequence of the opening of membrane permeability transition (PT) pore, since inhibitors of the PT pore prevent the induction of apoptosis by several direct stimuli (Zamzani *et al*., 1996). However, some studies have provided evidence that cytochrome *c* release and caspase activation can occur before or in the absence of a drop in mitochondrial inner transmembrane potential in some cells (Kluck *et al*., 1997; Finucane *et al*., 1999), suggesting that different regulatory mechanisms control the permeability of the inner and outer mitochondrial membranes. Moreover, other authors have observed an increase in $\Delta\Psi_m$ during apoptosis (Green & Reed, 1998). In the present study our results show that cocaine induced biphasic changes in $\Delta\Psi_m$ which peaked at 100 μM cocaine. This profile of changes was parallel to other apoptotic markers assayed in the present study, such as DNA phragmentation and cytochrome *c* release from mitochondria. Against the apoptogenic action of cocaine, the antioxidant NAC or DFO addition to the culture medium clearly decreased the extent of apoptosis; this protective effect was much more pronounced in the case of DFO.

The central component of the specialized machinery required for apoptosis is a proteolytic system which involves a family of proteases: the caspases. A variety of key events in apoptosis focus on mitochondria (Green & Reed, 1998), including the release of caspase activators (such as cytochrome *c*), changes in electron transport and mitochondrial transmembrane potential, altered cellular oxidation-reduction, etc. The different signals that converge on mitochondria either trigger or inhibit these events, and their downstream effects delineate several major pathways in physiological cell death.

The translocation of proteins from the mitochondrial intermembrane space to cytosol is a common step in apoptogenic pathways (Vier *et al*., 1999). In the present study, cocaine-induced cytochrome *c* has been used to evaluate the extent of apoptosis. Our results show that cytochrome *c* appeared in the cytosol in hepatocytes of cocaine-treated cultures, with a sharp increase peaking at 100 μM cocaine (8 fold versus no cocaine). NAC, when incubated together with cocaine, significantly decreased this value to 49% ($P<0.05$), the effect of DFO being even more pronounced in restoring to normal the level of cytosolic cytochrome *c*. This restoration was statistically significant in the case of DFO, in all cocaine concentrations, with a minimum concentration required of 100 μM (33%, $P<0.05$). The pattern of changes of cytosolic cytochrome *c* induced by cocaine was parallel to both the extent of apoptosis and to $\Delta\Psi_m$.

The release of cytochrome *c* into the cytosol is a way to selectively activate caspase-3 (Vier *et al*., 1999). Because of the involvement of caspase-3 activity in the apoptogenic mechanisms, the cleavage and activation of inactive procaspase and the level of p20 subunit was then assayed. In our experiments, both caspase activity and the level of p20 caspase subunit, increased sharply and significantly as the cocaine concentration increased in a dose-dependent manner. The coincubation of cocaine with NAC or DFO decreased both apoptogenic parameters: caspase-3 activity and the level of p20 subunit, and the effect was more pronounced with DFO.

However, caspase activation occurred with 500 and 1000 μM cocaine concentrations at which was detected a decline in cytochrome *c* release to cytosol. In this respect, it is proposed that cytochrome *c* is only partially responsible for caspase activation. Other ways could be involved and caspase-3 may be activated independent of cytochrome *c* release (Vier *et al*., 1999; Porter & Janicke, 1999). It has been demonstrated that several oxygen species, including nitric oxide, can contribute to caspase activity (Brown & Borutaite, 1999). In previous experiments, we observed that the effect of cocaine (500 and 1000 μM) on caspase activation paralleled the cytoplasmic membrane lysis and decline in apoptotic parameters (Zaragoza *et al*., 2000). According to these results, it is suggested that this protease participates in necrotic cell death (Aguilar *et al*., 1996), since it has been described that necrosis can be prevented by caspase inhibitors (Shimizu *et al*., 1996; Jaeschke *et al*., 1998) and that increased DEVDase activity occurred in TNF-induced both apoptosis and necrosis (Faraco *et al*., 1999). However, the enhanced activity of caspase-3 at 1000 μM cocaine, could also be explained by the possibility that apoptotic cells in culture (in the absence of fagocitary cells) develop the morphology of a secondary necrosis and release their intracellular contents to the extracellular medium (Bonfoco *et al*., 1995).

The hepatoprotective action of NAC, as a GSH precursor, has been widely demonstrated (Cotgreave, 1997), as well as the antioxidant effect of DFO as an iron chelating agent (Susa *et al*., 1997). ROS have been reported to be intermediates in the signal transduction pathways during apoptosis, and they act as second messengers which can regulate the expression of various genes (Wedi *et al*., 1999). Our present results demonstrate that cocaine leads to cell death in rat hepatocyte cultures by inducing both apoptosis and necrosis. The two modes of cell death can occur simultaneously in the liver by the effect of hepatotoxic substances (Cascales *et al*., 1994). The effect of antioxidants NAC and DFO against cocaine cytotoxicity strongly suggests that the extent of apoptosis is parallel to peroxide production.

In the multifactorial process of apoptosis induced by cocaine in hepatocyte cultures, we have shown the following: (1) caspase-3 activity as well as protein p20 subunit level increased sharply, these increases being dose-dependent; (2) biphasic changes were recorded both in cytochrome *c* release and in mitochondrial inner membranes that were parallel to apoptotic cell death; and (3) the effect of classic antioxidants, NAC or DFO play an important protective role in counteracting the cytotoxic effect of cocaine. The protective effect was more pronounced in the case of DFO than NAC.

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