Biochemical Pharmacology, Vol. 58, pp. 797–805, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00168-9

Cocaine Cytotoxicity in Hepatocyte Cultures from Phenobarbital-Induced Rats: Involvement of Reactive Oxygen Species and Expression of Antioxidant Defense Systems

Carmen Díez-Fernández, Asunción Zaragoza,* Alberto M. Alvarez† and María Cascales*‡*

*Instituto de Bioquímica (CSIC–UCM) and †Centro de Citometría de Flujo, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

ABSTRACT. The present study was designed to investigate whether cocaine modifies the production of reactive oxygen species, affects cellular enzyme-mediated antioxidant defense systems and, subsequently, promotes apoptosis and/or necrosis of hepatocytes. Primary cultures of hepatocytes isolated from phenobarbital-induced rats were exposed to cocaine (0–1000 μM) for 24 hr, and cell death (apoptosis or necrosis), antioxidant enzyme activities and mRNA levels, and peroxide generation were determined. Cocaine cytotoxicity by apoptosis was observed by detecting apoptotic nuclei using optic microscopy and by measurement of the hypodiploid peak (<2C) in DNA histograms obtained by flow cytometry. Necrosis was evidenced by lactate dehydrogenase (LDH) leakage, and peroxide production was quantified with 2′,7′-dichlorodihydrofluorescein diacetate. Low concentrations of cocaine (less than 100 μM) resulted in an increase in dichlorofluorescein fluorescence, associated with an enhancement in apoptotic cell death and sharp decreases in the enzyme activities and RNAs of catalase and manganese-superoxide dismutase (Mn-SOD). The progressive decrease in peroxide production in cell cultures detected in the range of 250–1000 μM cocaine was associated with increases in LDH leakage and decreases in the percentage of apoptotic cells, accompanied by low levels in catalase and Mn-SOD enzyme activities and mRNAs, without apparent changes in apoptosis. These data indicate that oxygen radicals may contribute directly or indirectly to cocaine-induced apoptosis in cultured hepatocytes. We conclude that, in primary hepatocyte cultures, cocaine-induced cell death by necrosis was dependent on cocaine concentration, while cell death by apoptosis was parallel to peroxide concentration. The down-regulation of the gene expression of antioxidant enzyme systems should be one of the mechanisms involved in cocaine toxicity. BIOCHEM PHARMACOL **58**;5:797–805, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cocaine cytotoxicity; oxidative stress; peroxides; antioxidant enzyme expression; cell death; apoptosis/necrosis

Cocaine is a stimulant of the central nervous system, whose widespread abuse has become one of the major medical and social problems. Experimental and clinical evidence has shown the hepatotoxic effect of cocaine in laboratory animals and in humans [1–3]. The principal hepatic lesion resulting from cocaine in humans, coagulative necrosis, is similar to that described earlier in experimental animals. It has been proposed that cocaine hepatotoxicity is mediated by a series of sequential oxidations catalyzed by cytochrome P450 isoforms and flavin-containing monooxygenases [4, 5], and it is well known that PB§ induces P-450 microsomal monooxygenase isozymes responsible for cocaine oxidative metabolism [6, 7].

The *N*-demethylation to norcocaine is the first step in the bioactivation of cocaine. The toxicity of the drug is the result of further oxidative metabolism, either through redox cycling between *N*-hydroxynorcocaine and norcocaine nitroxide leading to NADPH depletion as well as the generation of superoxide anion ($O_2^{-\cdot}$) and H_2O_2, or the production of a still to be identified reactive metabolite, derived from cocaine nitroxide and norcocaine nitrosonium [4, 5, 8, 9]. Pathological studies have implicated oxidative damage

‡ Corresponding author: María Cascales, Ph.D., Instituto de Bioquímica (CSIC-UCM), Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal sn, 28040 Madrid, Spain. Tel. 34-15436262; FAX 34-13941782; E-mail: cascales@eucmax.sim.ucm.es

Received 20 November 1998; accepted 5 March 1999.

§ *Abbreviations:* PB, phenobarbital; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SOD, superoxide dismutase; LDH, lactate dehydrogenase; DCF, dichlorofluorescein; DCFH, dichlorodihydrofluorescein; DCFH-DA, dichlorodihydrofluorescein diacetate; and PI, propidium iodide.

in the mechanisms of cocaine-induced liver injury [10, 11] and have also shown that mitochondrial injury is involved in these processes [12]. However, there is no direct evidence of ROS production by isolated hepatocytes in culture treated with this drug.

Cells are protected from the damaging effects of superoxide anion by conversion to H_2O_2 via SODs, and H_2O_2 can be rapidly converted to water via glutathione peroxidase and/or catalase [13, 14]. The imbalance of these enzyme systems results in an excess of ROS generation, which leads to depletion of glutathione, lipoperoxidation, altered enzyme activity, DNA damage, etc., which in turn promote cell death by apoptosis or necrosis [15–17]. Apoptosis is characterized by cell shrinkage, membrane blebbing, nuclear pyknosis, chromatin condensation, and genomic fragmentation. In contrast, necrosis is caused by passive cell swelling, injury to cytoplasmic organelles including mitochondria, rapid collapse of internal homeostasis, membrane lysis, and release of cellular contents. Both apoptosis and necrosis represent the extreme ends of damage and can occur simultaneously in cell cultures exposed to the same stimulus [18, 19].

Previous studies in our laboratory showed that apoptosis occurs in mouse liver following the *in vivo* administration of cocaine to mice pretreated or not with PB [7]. It has also been demonstrated that cocaine produced apoptosis in mouse thymocyte populations [20] and that *in vitro* exposure of fetal mouse neurons to cocaine resulted in apoptosis [21]. However, studies of apoptotic changes in hepatocyte cultures incubated with the drug and the relationship between apoptosis, the level of peroxides, and the expression of antioxidant cellular defense systems have not yet been reported. Although many factors can be involved in apoptosis, this form of cell death is known to be triggered by the intracellular generation of ROS as subproducts derived from the metabolism of toxic agents [22, 23]. On the other hand, it has been described that SOD and catalase protect cells from inducers of apoptosis, suggesting a role for ROS in regulating this mode of cell death [15, 24, 25]. These considerations prompted us to investigate the way in which the oxidative metabolism of cocaine can affect the mode of cell death. Moreover, as the system involved in antioxidant cell defense protects cells from oxidative stress, cocaine-induced changes in the activity and gene expression of the enzymes involved in the elimination of ROS were also evaluated.

MATERIALS AND METHODS

Reagents

Tissue culture media were from BioWhittaker. Standard analytical grade laboratory reagents were obtained from Merck. Collagenase was from Boehringer. [α-^{32}P]dCTP (3000 Ci/mmol) and the multiprimer DNA-labeling system kit were purchased from Amersham. Agarose was from Hispanagar. DCFH-DA was obtained from Molecular Probes and PI was from Sigma Chemical. HCl cocaine (99.5% purity, checked by HPLC) was obtained from the Drug Restriction Service Ministry of Health in Spain.

Animals

Two-month-old male Wistar rats with an average body weight of 180–230 g were used for the cell preparations. All 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 Institutes of Health. Rats were supplied with food and water *ad lib*. and exposed to a 12-hr light-dark cycle. Since pretreatment of rats with PB is required for toxicity to be enhanced [7], the animals were injected intraperitoneally with sodium phenobarbital at a dose of 80 mg/kg/day for 5 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 [26] and cell viability, determined by trypan blue exclusion, was always greater than 90%. Freshly isolated hepatocytes (1.5×10^6) were seeded into 60×15-mm culture dishes (Becton Dickinson) in 3 mL Dulbecco's modified Eagle's medium supplemented with 100 IU/mL penicillin, 50 μg/mL of streptomicin, 50 μg/mL of gentamicin, and 10% fetal bovine serum. After 3-hr incubation at 37° in a humidified 5% CO_2-95% air atmosphere, the medium was replaced with fresh medium supplemented with 0.1% BSA.

Exposure to Cocaine and LDH Leakage Assay

Following a 24-hr preculture period, hepatocytes were incubated in the presence of cocaine. Cocaine HCl was dissolved in fresh medium and precultured hepatocytes were exposed to the drug at a dose range of 0–1000 μM for 24 hr. Cytotoxicity was measured using the index of LDH [27] leakage from damaged hepatocytes according to Vasault [28], and was expressed as a percentage of total cellular activity.

Determination of Intracellular Generation of Peroxides

Production of peroxides was monitored by flow cytometry using DCFH-DA [29]. This dye is a stable non-polar compound that readily diffuses into cells. Once inside the cells, the acetate groups are cleaved from the molecule by intracellular esterases to yield DCFH, which is trapped within the cells. H_2O_2 or low-molecular-weight peroxides, produced by the cells in the presence of cellular peroxidases, oxidize DCFH, releasing the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxide produced by the cells. Following incubation with cocaine, hepatocytes from culture dishes were washed with PBS and immediately detached with trypsin/EDTA, then incubated with agitation for 30 min in

2 mL of PBS containing 5 μM DCFH-DA at 37°. The cells were washed twice with PBS to remove the extracellular DCFH-DA, followed by analysis on a FACScan flow cytometer (Becton Dickinson) (excitation: 488 nm; emission: 525 nm). Because identification of non-viable and late apoptotic cells is essential for obtaining accurate data, PI (10 μg/mL) was added to each tube 10 min before flow cytometry analysis to ensure that only live and early apoptotic cells were analyzed. PI treatment differentiates viable and non-viable cells since non-viable cells permit the entrance of this dye into the cells. For DCFH analysis, only PI-negative (viable) cells were acquired. Hepatocyte autofluorescence was detected by running unstained cells, and only hepatocytes with fluorescence higher than basal were quantified as peroxide-generating cells (|-M1-| peak). A laser scanning confocal microscope was used to analyze localizations of DCF fluorescence in hepatocytes. Confocal imaging was performed using an MRC 1000 confocal microscope (BioRad).

Analysis of Apoptosis

Following cocaine treatment, cultured hepatocytes were fixed in ice-cold methanol/acetic acid (3:1) for 5 min, stained with Hoechst 33258 (5 μg/mL) for 10 min, and washed with distilled water. Cells were mounted in a solution of 20 mM citric acid, 50 mM disodium ortophosphate and 50% glycerol (pH 5.5), and examined at a wavelength of excitation 330–380 and emission of 460 nm using an Olympus IMT-2 microscope with fluorescence attachment [30]. Quantification of apoptotic cell death was performed by measuring the hypodiploid peaks (<2C) in DNA multiploid histograms obtained by flow cytometry, as previously described [30, 31]. For the analysis of DNA content, cells were stained with PI, and the emitted fluorescence of DNA–PI complex was analyzed in a FACScan flow cytometer (Becton Dickinson) in the FL2-A channel. A double discriminator module was used to distinguish between signals coming from a single nucleus and those signals from products of nuclear aggregation. Data analysis was carried out by means of evaluation of single inputs (10^4 nuclei/assay).

Enzyme Activity Assays

Hepatocytes were collected from culture dishes, resuspended in PBS, and sonicated on ice. The solution was centrifuged at maximum speed for 15 min at 4° in a microcentrifuge and the supernatant used for enzyme activity assays. Antioxidant enzyme activities were measured as described [32]. Catalase activity was spectrophotometrically determined by measuring decreased absorbance at 240 nm using H_2O_2 as substrate [33]. Superoxide dismutase (Cu,Zn-SOD and Mn-SOD) activity was measured spectrophotometrically by monitoring the inhibition of the autoxidation of pirogallol [34]. Sodium cyanide (2 mM) was added to dissect Mn-SOD activity from that of Cu,Zn-SOD. Enzyme activity was expressed as units/mg protein. Protein estimation was made following Bradford [35], using BSA as standard.

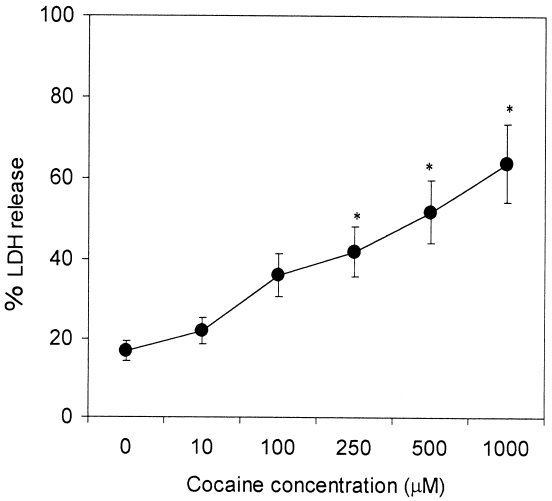

FIG. 1. Cytotoxic effects of cocaine in cultured hepatocytes derived from PB-induced rats. Hepatocytes were exposed to increasing concentrations of cocaine for 24 hr, and LDH leakage was measured as a cytotoxicity parameter and membrane lysis index. Results are expressed as the percentage of total cell LDH as described [27]. Data are expressed as means ± SD of four independent experiments from four rats. *P < 0.05.

RNA Extraction and Analysis

Total RNA (3×10^6 cells) was extracted following the guanidinium thiocyanate method [36] as described [37, 38]. After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde, the RNA was transferred to GeneScreenTM membranes (New England Nuclear). The relative levels of various mRNA transcripts were determined using catalase, Mn-SOD, and Cu,Zn-SOD cDNA probes [39], labeled with α^{32}P-dCTP using a multiprimer DNA-labeling system kit (Amersham). Quantification of the films was performed by a laser densitometer (Molecular Dynamics) using the hybridization with an 18S ribosomal RNA probe as an internal standard. The variability in the measurement of fold increase in mRNA, after quantification by scanning densitometry from the filters, was not greater than 15%.

Statistical Analysis

All results are expressed as the means ± SD of four observations (four animals) in duplicate. Statistical differences between two groups were analyzed by the Student's *t*-test; when more than two groups were compared, the Kruskal Wallis test (non-parametric ANOVA) was used. $P < 0.05$ was considered significant.

RESULTS

Isolated hepatocytes from PB-pretreated rats were exposed to increasing concentrations of cocaine from 0 to 1000 μM

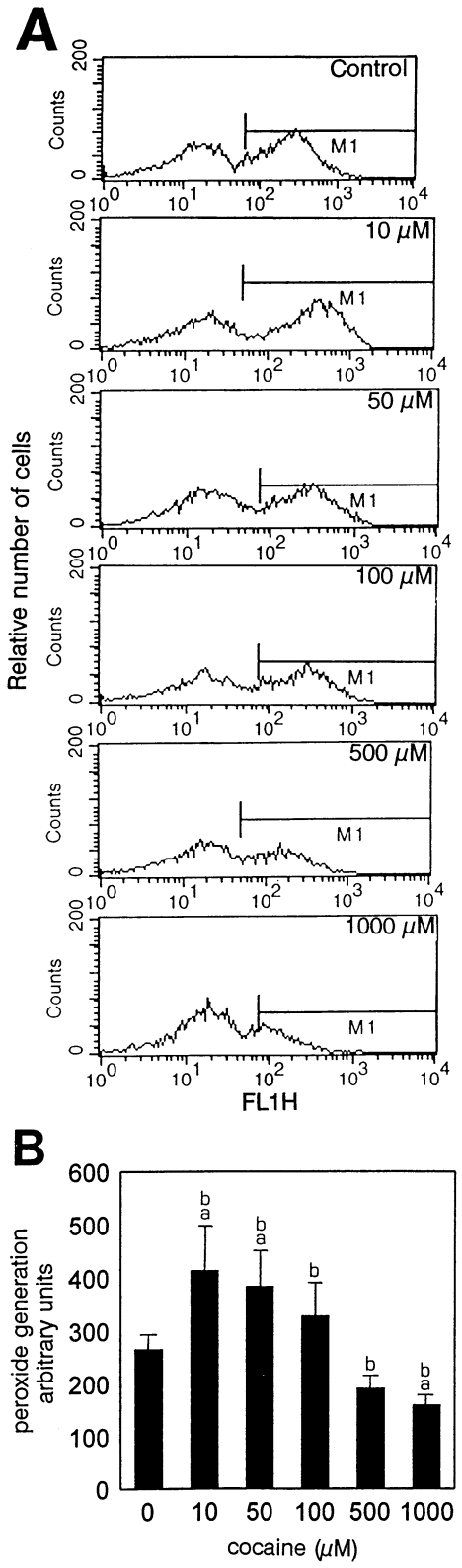

FIG. 2. Intracellular generation of peroxides in cultures of hepatocytes incubated in the presence of cocaine (0–1000 μM). Following incubation with the drug, cultured hepatocytes were detached with tripsine and incubated with 5 μM DCFH-DA in 2 mL PBS for 30 min at 37°. The samples were placed on ice and peroxide production was determined by measuring the DCF fluorescence with FL1-H channel, against the number of cells, by flow cytometry. (A) A representative histogram of the four independent experiments. (B) Results, expressed as arbitrary units, are the means ± SD of experimental observations from four rats. Student's *t*-test was performed for statistical evaluations between all values against control as (a) and Kruskal Wallis test for evaluations between more than two groups (b). *$P < 0.05$ was considered the level of significance.

for 24 hr, and LDH leakage was measured as an index of membrane lysis and cell toxicity [27]. The kinetics of cocaine citotoxicity in hepatocyte cultures were assayed at 2, 10, and 24 hr of incubation in the presence of the concentrations of the drug mentioned above (data not shown). We found that 24 hr of incubation was the best time point to detect significant changes. Figure 1 shows the percentages of LDH release in hepatocyte cultures incubated with increasing concentrations of cocaine. The cytotoxic effect of cocaine was dose-dependent and the lowest concentration of the drug able to cause significant increases in LDH leakage was 250 μM, reflecting a loss of plasma membrane integrity associated with necrosis. Cocaine concentrations 4-fold higher than necessary in PB-pretreated cells were required before loss of viability was observed when hepatocytes were isolated from non-PB-pretreated rats (data not shown).

Because cytotoxic activity of cocaine in liver has been ascribed to ROS production [5, 8, 9, 21], we investigated intracellular generation of peroxides by hepatocytes using the DCFH-DA probe. Figure 2 shows the intracellular concentration of peroxides in cocaine-treated hepatocyte cultures measured by flow cytometry of the fluorescence emitted due to DCFH oxidation. Figure 2A shows the histograms in which fluorescence of DCF, detected with the FL1-H channel, is plotted against the number of cells. |-M1-| defines the peak of peroxides (intense fluorescence). Figure 2B shows the quantification in arbitrary units of the |-M1-| peak of Fig. 2A. These results show that cocaine increased endogenous levels of peroxides at doses between 10–100 μM and that exposure to higher concentrations (500 and 1000 μM) resulted in a decrease in the DCF intensity. These results indicate that the generation of ROS is an immediate response to exposure to cocaine and therefore occurs very early in cocaine-treated hepatocytes.

Figure 3 shows the simultaneous PI- and DCF-associated fluorescence in hepatocytes visualized through the laser scanning confocal microscopy system. The red fluorescence of PI, inside the cells, shows the lack of cytoplasmic membrane integrity and is considered a marker of necrotic cell death observed in hepatocyte cultures incubated in the presence of 500 and 1000 μM cocaine (C and D). However, green fluorescence of DCF (peroxides) decreased in cells incubated with these high doses (500 and 1000 μM) of cocaine when compared to the control (A), the highest intensity being in the green fluorescence observed at 100 μM (B). These results confirm the above-mentioned data obtained both by flow cytometry and by the measurement of LDH leakage.

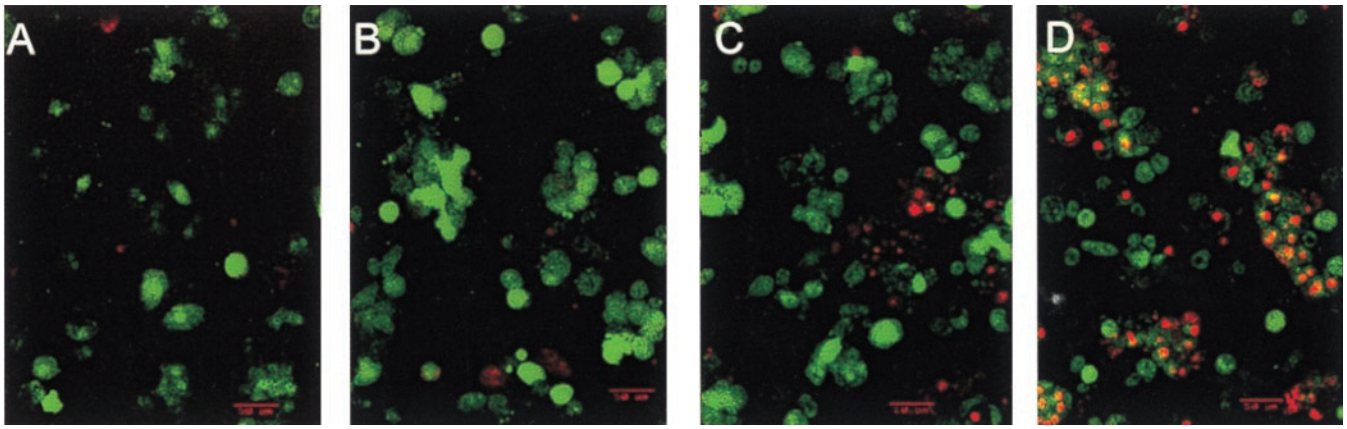

FIG. 3. Confocal imaging of DCF fluorescence in rat hepatocytes exposed to cocaine for 24 hr. Cultured rat hepatocytes were loaded with DCFH-DA and PI fluorochromes. DCF fluorescence (green fluorescence) was an index of oxygen-derived free radical generation and fluorescence of the DNA-intercalating dye PI (red fluorescence) was a marker of membrane barrier dysfunction. (A) control hepatocytes; (B) 100 μM cocaine; (C) 500 μM cocaine; and (D) 1000 μM cocaine (bar = 50 μm).

When exposed to cocaine, a large fraction of cell nuclei appeared to be denser and smaller, or fragmented when observed by light microscopy. Figure 4 (A–C) shows photographs obtained by fluorescence microscopy in which apoptosis was visualized by observing the morphology of hepatocytes incubated for 24 hr in the presence of cocaine (0, 100, and 1000 μM). Staining with the DNA-binding dye Hoechst 33258 showed that in cocaine-treated cells, the chromatin was dispersed into multiple small nuclear fragments, while control cells rarely exhibited an apoptotic morphology.

Condensation and fragmentation of chromatin are associated with lower DNA fluorescence in flow cytometric analysis, which is considered to be a useful tool for quantitative detection of apoptosis [7]. Multiploid DNA histograms and the percentages of apoptotic cells (hypodiploid DNA content) determined by flow cytometry are shown in Fig. 5. Figure 5A shows DNA multiploid histograms in which the fluorescence of PI-stained DNA was detected with FL2-A channel and plotted against the relative number of cells. |-M1-| defines the percentage of hypodiploid cells <2C as an apoptotic index. Quantitative analysis of these cells showed the percentage of hepatocyte population that underwent apoptosis. These values are shown in Fig. 5B. At 24-hr incubation the following results were obtained: apoptosis of cultured hepatocytes was 8.0 ±

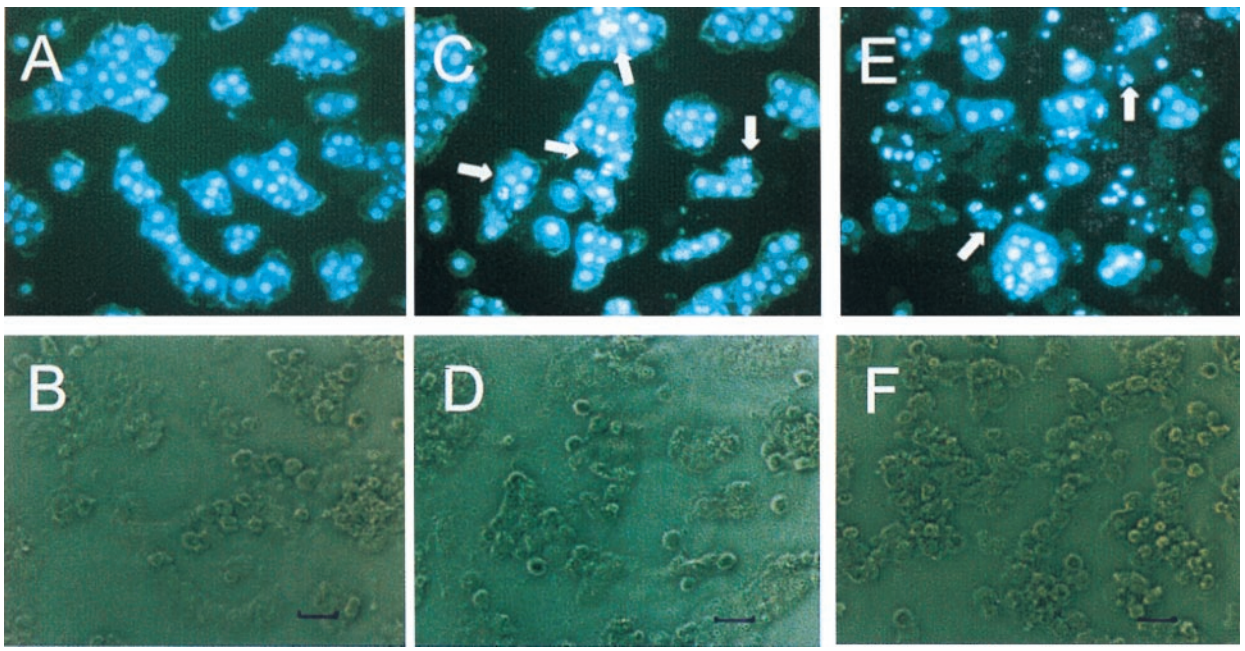

FIG. 4. Morphology of cocaine-treated hepatocytes. (A) and (B) control; (C) and (D) 100 μM cocaine; (E) and (F) 1000 μM cocaine. Photographs on the upper row of the panel show fluorescent nuclei stained with Hoechst 33258, those on the lower row the corresponding phase contrast images. Arrows show hepatocytes with apoptotic morphology in which condensed or fragmented nuclei are clearly observed (bar = 50 μm).

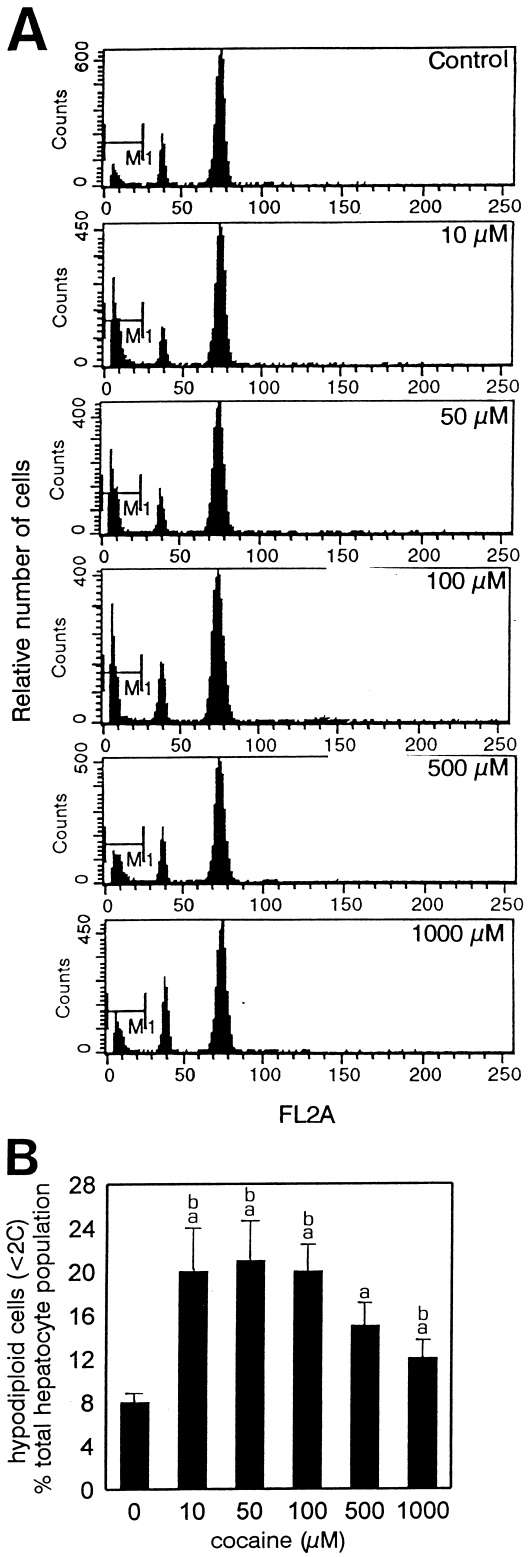

FIG. 5. Multiploid DNA histograms and scatter profiles of cultured hepatocytes treated with cocaine (0–1000 μM) and stained with the DNA-intercalating dye PI [30]. Fluorescence was detected with the FL2-A channel and plotted against the number of cells, while |-M1-| defines the percentage of hypodiploid cells (<2C) as an index of apoptosis. (A) Representative histograms of four independent experiments. (B) The quantification of the hypodiploid <2C peaks. The results, expressed as a percentage of total hepatocyte population, are the means ± SD of experimental observations from four rats. Student's t-test was performed for statistical evaluations between all values against control as (a) and Kruskal Wallis test for evaluations between more than two groups (b). *$P < 0.05$ was considered the level of significance.

0.9% for control cells. Under our experimental conditions, hepatocyte apoptosis increased significantly versus control when incubated in the presence of cocaine at doses between 10–100 μM (19 ± 2.0, 21 ± 2.5, and 20 ± 1.9% for 10, 50, and 100 μM, respectively), significantly diminishing (versus the values obtained at 10–100 μM) at doses of 500 and 1000 μM (14 ± 1.5 and 12 ± 1.4%, respectively).

Figure 6 shows the activities of enzymes involved in the primary antioxidant defense, such as superoxide dismutases and catalase. Mitochondrial SOD (Mn-SOD) activity, expressed as units per mg of protein, decreased progressively according to cocaine concentration, the decrease being significantly different when compared to control at concentrations of cocaine above 100 μM. However, cytosolic SOD (Cu,Zn-SOD) slightly increased and showed significant differences only at 1000 μM (148%, $P < 0.05$ vs control). Catalase activity, expressed as units per mg of protein, showed a decrease detectable from 100 μM.

We used Northern blot hybridization to measure the relative levels of the mRNA transcripts for the genes coding for superoxide dismutases and catalase as a means of studying the effect of increased concentrations of cocaine on their gene expression. Northern blots prepared with RNA extracted from cocaine-treated hepatocytes from PB-pretreated rats were probed with cDNA sequences of catalase, Mn-SOD, and Cu,Zn-SOD [39]. Figure 7 shows representative autoradiographs and their corresponding quantifications by scanning densitometry. After 24 hr of cocaine exposure, catalase and Mn-SOD expression underwent slight increases at 10 μM cocaine (129% and 120%, respectively). However, at 100 μM cocaine sharp decreases in catalase and Mn-SOD mRNAs, to 55% ($P < 0.05$) and 46% ($P < 0.05$) versus control, respectively, were detected. From 100 to 1000 μM cocaine, only slight changes were observed in both enzyme expressions. Cu-Zn SOD expression did not change significantly in the range of all cocaine concentrations assayed.

DISCUSSION

There is increasing evidence that hepatocyte apoptosis may occur in a number of liver diseases [40, 41]. Besides necrosis, apoptosis is considered as a significant pathway of cell clearance under pathological conditions. In histopathological studies of hepatic diseases, including acute and chronic hepatitis, alcohol-induced liver diseases, and primary biliary cirrhosis, it has been reported that apoptosis plays an important role in the course of liver cell death. In a previous publication, we showed that morphological and biochemical characteristics of apoptotic changes were observed in liver of acute-cocaine-treated mice either induced

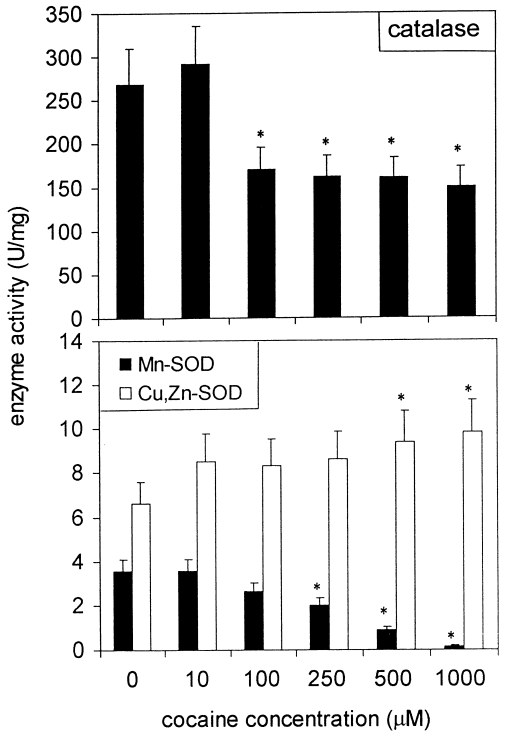

FIG. 6. Catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1) activities in hepatocyte cultures incubated in the presence of cocaine (0–1000 μM). Hepatocytes were collected from culture dishes, resuspended in PBS, and sonicated on ice. The solution was centrifuged at maximum speed for 15 min at 4° in microcentrifuge and the supernatant used for enzyme activity assays. The activities of catalase, Cu,Zn-SOD, and Mn-SOD were measured as previously described [32]. One unit of SOD refers to ng of enzyme that produces 50% inhibition in pyrogallol autoxidation. One unit of catalase is defined as the amount of enzyme that transforms 1 μmol of hydrogen peroxide per min at 25°. The values are the means ± SD of four different observations. *P < 0.05 versus control.

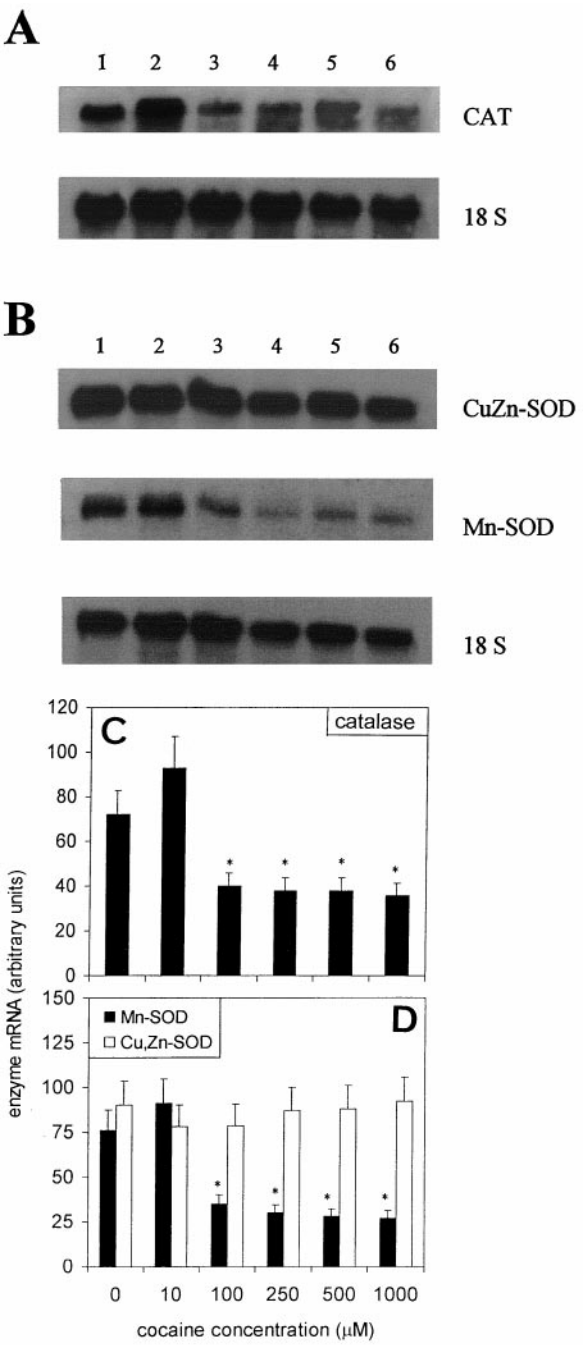

FIG. 7. Northern blot analysis of catalase, Mn-SOD, and Cu,Zn-SOD mRNAs. RNA was isolated and analyzed by Northern blotting using radiolabeled catalase, Mn-SOD, and Cu,Zn-SOD cDNAs. Panels A and B show representative Northern blots with 18S rRNA probe for RNA normalization and panels C and D show the quantification after correction with 18S rRNA obtained by laser densitometry. Lanes 1, 2, 3, 4, 5, and 6 refer to 0, 10, 100, 250, 500, and 1000 μM, respectively.

or not with PB [7]. However, from *in vitro* studies performed to date, no data have been reported which demonstrate the degree of involvement of apoptotic cell death in cocaine-induced cytotoxicity, the relationship between gene expression of enzymes primarily involved in the cell defense against pro-oxidants, or the generation of ROS through the oxidative metabolism of cocaine.

ROS have been reported to be intermediates in signal transduction during apoptosis. ROS show some of the characteristics of second messengers, and there is good evidence that they can promote transcription factor activity and regulate the expression of a variety of genes. Therefore, oxidative stress contributes to the cell death program by signaling [24]. It is well known that PB induces the activity of cytochrome P-450 microsomal monooxygenase isozymes responsible for cocaine oxidative metabolism [6, 7, 10, 11], and in the present paper we use this property to better study cocaine cytotoxicity. Although previous studies have already demonstrated that oxidative damage plays a role in the cytotoxicity of cocaine [9–11], there is as yet no definitive and quantitative evidence showing the extent of ROS production in hepatocytes by the effect of this drug. By using the probe DCFH-DA and confocal microscopy and flow cytometry techniques, we found increases in DCF fluorescence, indicating overproduction of peroxides when rat hepatocyte cultures were incubated in the presence of low concentrations of cocaine. The peak of emitted DCF fluorescence occurred at doses lower than 100

μM cocaine; a decrease in cellular fluorescence was detected at higher cocaine concentrations parallel to membrane lysis, observed by LDH leakage and PI staining in confocal studies. These results provide evidence that cocaine *in vitro* induces intracellular generation of peroxides, which could be involved in the initiation of cytotoxicity.

In the present report, our results demonstrate that cocaine leads to cell death in rat hepatocyte cultures by inducing both apoptosis and necrosis. These two modes of cell death can occur simultaneously in liver damage induced by hepatotoxic substances [19, 42]. In cultured hepatocyte apoptosis initiated at early time points, cells, when exposed to stressful concentrations, may lose membrane permeability before the apoptosis program is finished. However, the distinction between apoptosis or necrosis presents some difficulty *in vitro*, since in our experiments, because of the lack of scavenging cells that phagocyte apoptotic bodies, cells in culture can undergo secondary necrosis releasing their content into the surrounding space [43]. Thus, we obtain evidence for apoptotic-like features by applying techniques such as analyzing apoptotic nuclei stained with Hoechst 33258 dye by fluorescence microscopy and by measuring DNA apoptotic damage quantifying the hypodiploid peak in multiploid DNA histograms. Although necrosis was documented by the leakage of LDH, as a marker of membrane lysis and cytotoxicity associated with cell death [27], the above-mentioned secondary necrosis of apoptotic cells *in vitro* leads to an interference between the two manners of cell death. The results obtained in the present study, together with those in a previous report [7], strongly suggest that apoptosis plays an important role in the toxicity of cocaine in hepatocytes, since at doses of cocaine between 10–100 μM, evident morphological changes were detected that simultaneously occurred with or preceded the development of the loss of membrane integrity and cytotoxicity. Moreover, the extent of apoptosis was parallel to the peroxide production in cell cultures. Furthermore, our results are consistent with other observations that the exposure of cells to low levels of oxidants induces cell death mainly by apoptosis rather than by necrosis [22].

Cell integrity is affected by oxidative stress when the production of active oxidants overwhelms antioxidant defense mechanisms [13, 44]. The sequenced elimination of superoxide and H_2O_2 by the coordinated action of superoxide dismutases and catalase prevents the formation of the highly reactive hydroxyl radical. It has been described that catalase, in response to oxidative stress, is regulated both at transcriptional and translational levels [45]. Our experimental studies have been conducted to ascertain the effects of cocaine administration on enzyme activity and gene expression of those hepatic antioxidant enzymes closely related to superoxide and peroxide generation, such as SOD and catalase. Exposure of cell cultures to doses of cocaine of 500 and 1000 μM was associated with sharp decreases in catalase and Mn-SOD enzyme activities and mRNAs. The lack of apparent changes in Cu,Zn-SOD by the effect of cocaine may be due to the fact that cytosolic SOD appears to be constitutively expressed while mitochondrial SOD (Mn-SOD) is a highly inducible enzyme [46].

It has been reported that generation of ROS and depletion of cellular antioxidants play a role in cocaine-induced liver injury [8–10], but not much information is available concerning the expression of genes involved in primary cellular protection against ROS in hepatocyte cultures incubated in the presence of cocaine. Our results suggest that oxidative stress induced by cocaine may occur because of the down-regulation of primary antioxidant defense genes. The progressive decline over a period of 24 hr of incubation with cocaine in the transcript concentration for catalase and Mn-SOD, as drug concentration increased, brought about an increase in the oxidant redox state of the cells, contributing to the molecular mechanisms of cell injury and death.

From these results, we conclude that in hepatocyte cultures there seems to be a relationship between the generation of peroxides induced by cocaine metabolism, the expression of cell antioxidant enzyme systems, and the modulation of cell death. Furthermore, our data are consistent with the hypothesis that oxidative stress participates in the signaling pathways responsible for inducing cell death.

We thank Dr J. L. Tilly for providing us with the catalase, Cu,Zn-SOD, and Mn-SOD cDNA probes, Dolores Velasco for her technical assistance, Dr Díaz-Llanos for his advice in statistics, and Professor Erik Lundin for his help in the preparation of the manuscript. This study was supported by a grant from DGICYT (Dirección General de Investigación Científica y Técnica) PM 96/0010 and FIS (Fondo de Investigación Sanitaria) 99/1045.

References

1. Kanel GC, Cassidy W, Shuster L and Reynolds TB, Cocaine-induced liver cell injury: Comparison of morphological features in man and in experimental models. *Hepatology* **11:** 646–651, 1990.
2. Perino LE, Warren GH and Levine JS, Cocaine-induced hepatotoxicity in humans. *Gastroenterology* **93:** 176–180, 1987.
3. Roth L, Harbison RD, James RC, Tobin T and Roberts SM, Cocaine hepatotoxicity: Influence of hepatic enzyme inducing and inhibiting agents on the site of necrosis. *Hepatology* **15:** 934–940, 1992.
4. Kloss MW, Rosen GM and Raukman EJ, Cocaine-mediated hepatotoxicity: A critical review. *Biochem Pharmacol* **33:** 169–173, 1984.
5. Boelsterli UA and Göldlin C, Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. *Arch Toxicol* **65:** 351–360, 1991.
6. Powell CJ, Charles SJ and Mullervy J, Cocaine hepatotoxicity: A study on the pathogenesis of periportal necrosis. *Int J Exp Pathol* **75:** 415–424, 1994.
7. Cascales M, Alvarez A, Gascó P, Fernández-Simón L and Boscá L, Cocaine-induced liver injury in mice elicits specific changes in DNA ploidy and induces programmed cell death of hepatocytes. *Hepatology* **20:** 992–1001, 1994.
8. Bouis D and Boelsterli UA, Modulation of cocaine metabolism in primary rat hepatocyte cultures. Effect of irreversible binding and protein biosynthesis. *Toxicol Appl Pharmacol* **104:** 429–439, 1990.

9. Jover R, Ponsoda X, Gómez-Lechón MJ and Castell JV, Cocaine hepatotoxicity: Two different toxicity mechanisms for phenobarbital-induced and non-induced rat hepatocytes. *Biochem Pharmacol* **46:** 1967–1974, 1993.
10. Göldlin CR and Boelsterli UA, Reactive oxygen species and non-peroxidative mechanisms of cocaine-induced cytotoxicity in rat hepatocyte cultures. *Toxicology* **69:** 79–91, 1991.
11. Boelsterli UA, Wolf A and Göldlin C, Oxygen free radical production mediated by cocaine and its ethanol-derived metabolite, cocaethylene, in rat hepatocytes. *Hepatology* **18:** 1154–1161, 1993.
12. Masini A, Gallesi D, Giovannini F, Trenti T and Ceccarelli D, Membrane potential of hepatic mitochondria after acute cocaine administration in rats. The role of mitochondrial reduced glutathione. *Hepatology* **25:** 385–390, 1997.
13. Halliwell B, Gutteridge JMC and Cross CE, Free radicals, antioxidants, and human disease. Where are we now. *J Lab Clin Med* **119:** 598–620, 1992.
14. Sies H, Strategies of antioxidant defense. *Eur J Biochem* **215:** 213–219, 1993.
15. Buttke TM and Sandstrom PA, Oxidative stress as mediators of apoptosis. *Immunol Today* **15:** 7–10, 1994.
16. Briehl MM, Cotgreave IA and Powis G, Downregulation of the antioxidant defence during glucocorticoid-mediated apoptosis. *Cell Death Differ* **2:** 41–46, 1995.
17. Ellis RE, Yuan J and Horvitz HR, Mechanisms and function of cell death. *Annu Rev Cell Biol* **7:** 663–698, 1991.
18. Raffray M and Cohen GM, Apoptosis and necrosis in toxicology. A continuum or distinct modes of cell death? *Pharmacol Ther* **75:** 153–178, 1997.
19. Leist M and Nicotera P, Breakthroughs and views. The shape of cell death. *Biochem Biophys Res Commun* **236:** 1–9, 1997.
20. Wu YB, Shen ML, Gu GG, Anderson KM and Ou DW, The effects of cocaine injections on mouse thymocyte population. *Proc Soc Exp Biol Med* **214:** 173–179, 1997.
21. Nassogne MC, Louahed J, Evrard P and Courtoy PJ, Cocaine induces apoptosis in cortical neurons of fetal mice. *J Neurochem* **68:** 2442–2450, 1997.
22. Gardner AM, Xu F-H, Fady C, Jacoby FJ, Duffey DC, Tu Y and Lichtenstein A, Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. *Free Rad Biol Med* **22:** 73–83, 1997.
23. Nobel CSI, Kimland M, Lind B, Orrenius S and Slater AFG, Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. *J Biol Chem* **270:** 26202–26208, 1995.
24. Powis G, Briehl M and Oblong J, Redox signaling and the control of cell growth and death. *Pharmacol Ther* **68:** 149–173, 1995.
25. Demple B and Amabile-Cuevas CF, Redox redux: The control of oxidative stress responses. *Cell* **67:** 837–839, 1991.
26. Díez-Fernández C, Boscá L, Fernández-Simón L, Alvarez A and Cascales M, Relationship between genomic DNA ploidy and parameters of liver damage during necrosis and regeneration induced by thioacetamide. *Hepatology* **18:** 912–918, 1993.
27. Welder AA and Acosta D, Enzyme leakage as an indicator of cytotoxicity in cultured cells. In: *In Vitro Toxicity Indicators. Methods in Toxicology* (Eds. Tyson CA and Frazier JM), pp. 46–49. Academic Press, San Diego, 1994.
28. Vasault A, Lactate dehydrogenase. UV methods with pyruvate and NADH. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), Vol. III, pp. 118–133. Verlag Chemie, Weinheim, 1987.
29. Bass D, Parce JW, Dechatelet LR, Szejda P, Seeds MC and Thomas M, Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J Immunol* **130:** 1910–1917, 1983.
30. Díez-Fernández C, Sanz N, Alvarez AM, Wolf A and Cascales M, The effect of non-genotoxic carcinogens, phenobarbital and clofibrate on the relationship between reactive oxygen species, antioxidant enzyme expression and apoptosis. *Carcinogenesis* **19:** 1715–1722, 1998.
31. Vindelow LL, Christensen IJ and Nissen NI, A detergent trypsin method for the preparation of nuclei by flow cytometry. *Cytometry* **3:** 323–327, 1993.
32. Sanz N, Díez-Fernández C, Alvarez A and Cascales M, Age-dependent modifications in rat hepatocyte antioxidant defense systems. *J Hepatol* **27:** 525–534, 1997.
33. Aebi H, Hydrogen-peroxide: hydrogen-peroxide oxidoreductase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), Vol. III, pp. 273–282. Verlag Chemie, Weinheim, 1987.
34. Del Maestro RF, McDonald W and Anderson R, Superoxide dismutase, catalase and glutathione peroxidase in experimental and human brain tumors. In: *Oxy Radicals and Their Scavenger Systems* (Eds. Greenwald R and Cohen G), pp. 28–33. Elsevier, New York, 1983.
35. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
36. Chomczynsky P and Sacchi N, Single-step method of RMNA isolation by acid guanidinium thiocianate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
37. Díez-Fernández C, Sanz N and Cascales M, Changes in glucose-6-phosphate dehydrogenase and malic enzyme gene expression in acute hepatic injury induced by thioacetamide. *Biochem Pharmacol* **51:** 1159–1163, 1996.
38. Díez-Fernández C, Sanz N, Wolf A and Cascales M, Effect of phorbol ester (PMA) on antioxidant enzyme expression in TGF-β1-induced apoptosis in primary cultures of hepatocytes. *Biofactors* **8:** 65–71, 1998.
39. Tilly JL and Tilly KI, Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* **136:** 242–252, 1995.
40. Patel T and Gores GJ, Apoptosis and hepatobiliary disease. *Hepatology* **21:** 1725–1741, 1995.
41. Kurose I, Higuchi H, Miura S, Saito H, Watanabe N, Hokari R, Hirokawa M, Takaishi M, Zeki S, Nakamura T, Ebinuma H, Kato S and Ishii H, Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication. *Hepatology* **25:** 368–378, 1997.
42. Leist M, Gantner F, Naumann H, Bluethmann H, Vogt K, Brigelius-Flohe R, Nicotera P, Volk HD and Wendel A, Tumor necrosis factor-induced apoptosis during the poisoning of mice with hepatotoxins. *Gastroenterology* **112:** 923–934, 1997.
43. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P and Lipton SA, Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* **92:** 7162–7166, 1995.
44. Yu BP, Cellular defenses against damage from reactive oxygen species. *Physiol Rev* **74:** 139–162, 1994.
45. Tacchini L, Pogliani G, Radice L, Bernelli-Zazzera A and Cairo G, Post-translational control of increased hepatic catalase gene expression in response to oxidative stress. *Redox Rep* **2:** 273–278, 1996.
46. White CW, Nguyen DH, Suzuki K, Taniguchi N, Rusakow LS, Avraham KB and Groner Y, Expression of manganese superoxide dismutase is not altered in transgenic mice with elevated level of copper-zinc superoxide dismutase. *Free Radic Biol Med* **15:** 629–636, 1993.