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Changes in Antioxidant Defence Systems Induced by Cyclosporine A in Cultures of Hepatocytes from 2- and 12-Month-Old Rats

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ABSTRACT. The *in vitro* effect of cyclosporine A (CsA) was studied in reference to the production of reactive oxygen species (peroxides and superoxide anion) and to cell enzyme-mediated antioxidant defence in hepatocytes isolated from rats aged 2 and 12 months. Primary cultures of hepatocytes were incubated in the presence of concentrations of cyclosporine in the range of 0 to 50 μM for 24 hr, and the release of lactate dehydrogenase into the culture medium was evaluated as a parameter of cytotoxicity and membrane lysis. Peroxides were quantified by using 2′,7′-dichlorodihydrofluorescein diacetate, and superoxide anion levels were evaluated by the fluorescence of dihydroethidium. Enzyme activity and gene expression of catalase and Mn- and Cu,Zn-superoxide dismutase were also assayed. CsA cytotoxicity was significantly higher in hepatocytes from rats aged 12 months when compared to those aged 2 months. Intracellular peroxide content resulted in a dose-dependent increase, while the anion superoxide intracellular level slightly decreased as CsA increased from 0–50 μM. The progressive increase in intracellular peroxides in cell cultures in the range from 0–50 μM CsA was associated with the loss of cell viability and accompanied by significantly higher levels of Mn- and Cu,Zn-superoxide dismutase enzyme activities and mRNAs, and slight increases in catalase activity and mRNA. We conclude that, in primary hepatocyte cultures, the cytotoxicity of CsA was dose-dependent in both age groups and significantly higher in cultures from 12-month-old rats when compared to those from 2-month-old animals. The non-coordinated regulation of the gene expression of antioxidant enzyme systems, i.e. catalase and Mn- and Cu,Zn-superoxide dismutases, evidenced to a greater extent in hepatocytes from the older group of rats, could be one of the mechanisms involved in CsA toxicity. BIOCHEM PHARMACOL **59**;9:1091–1100, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cyclosporine A hepatotoxicity; antioxidant enzymes; reactive oxygen species; age-related effect

CsA§ is a potent immunosuppressive agent used in transplant surgery and in the treatment of autoimmune diseases [1, 2] because of its specific inhibiting effect on signal transduction pathways of T cell receptor through the formation of a cyclosporine A–cyclophilin complex [3, 4]. CsA, bound to its cognate cyclophilin, inhibits calcineurin activity and prevents the dephosphorylation of the nuclear factor of activated T cells, thereby blocking its entry into the nucleus and prohibiting transcriptional activation of the *IL-2* gene. Although the clinical use of CsA is limited by its adverse side effects such as interactions with other drugs, hepatotoxicity, and nephrotoxicity, CsA is the most commonly used immunosuppressive agent in the therapy of transplants [5–7]. It follows, then, that most of the studies mentioned above have investigated the effects of CsA on the immune system. The functional impairment of the liver manifested by parameters of hepatotoxicity has also been frequently studied [8–10]. However, the mechanisms of hepatotoxicity have not yet been clarified, and an effort should be made to understand which of them could be utilized before any attempt is made to counteract some of their undesirable effects.

Acute hepatotoxicity is initiated by a series of events that culminate in the loss of cell viability and tissular damage. Studies by our group and others [11–13] on xenobiotic-induced liver injury models led us to propose that in the sequence of events between the administration of a hepatotoxin and hepatocyte death the following occur: (a) generation of reactive species; (b) glutathione depletion and modification of protein thiol groups; and (c) an increase in cytosolic calcium. It has been shown that CsA is able to generate reactive oxygen species [8, 9] and lipid peroxidation. The enzymes involved in CsA metabolism are hepatic CYP 3A isozymes [14, 15], leading to a large

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§ *Abbreviations:* CsA, cyclosporine A; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; LDH, lactate dehydrogenase; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; and HE, dihydroethidium.

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number of demethylated and hydroxylated metabolites which retain the undecapeptide nature of the parent compound [10, 16]; and its hepatotoxicity is shown by cholestasis and high levels of blood bilirubin. However, the exact nature of liver damage induced by CsA is not fully understood and is still a matter of debate.

Free radicals are intermediary products of biological redox reactions involved in many pathological processes [17]. To eliminate these free radicals, the cell possesses enzymatic defence mechanisms such as antioxidants, scavengers of free radicals, or reductants, all strategically compartmentalized inside the cell. Some of these, such as Mn-dependent SOD, are located inside mitochondria where a great part of oxygen radicals are generated, while others such as Cu,Zn-SOD and catalase are mainly located in the cytosol [18].

In the present study, we investigated, in hepatocytes from rats aged 2 and 12 months, the mechanisms of hepatotoxicity induced by CsA and the relationship between the rate of reactive oxygen species generation and its implication in the endogenous mechanisms of cell antioxidant defence systems. Thus, our first objective was to study the mechanism of CsA toxicity and the second the effect of age on this toxicity. Once it was established that the hepatotoxicity was dose-related, the *in vitro* cytotoxic effect of CsA on primary hepatocyte cultures was investigated in both age groups in the range from 0–50 μM, with special attention paid to the question of whether reactive oxygen species were involved in mediating CsA toxicity. A sequenced chain reaction occurs in which pro- and antioxidants could be involved and which can ultimately lead to substantial cell death. Determining the relationship between the intracellular levels of reactive oxygen species (superoxide anion and peroxides) and the enzyme activities and gene expression of the endogenous antioxidant enzyme systems catalase and Mn- and Cu,Zn-SOD will give us a better understanding of the molecular mechanisms of CsA toxicity and the age-related effects of these mechanisms.

MATERIALS AND METHODS

Reagents

Tissue culture media were from BioWhittaker. Standard analytical grade laboratory reagents were obtained from Merck. (+)-α-Tocopherol acid succinate was from Sigma. 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 and HE were obtained from Molecular Probes. CsA was provided by Dr. Armin Wolf, Novartis.

Animals

Male Wistar rats aged 2 and 12 months, with an average body weight of 180–230 g and 500–580 g, respectively, 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.

Isolation and Culture of Hepatocytes

Hepatocytes were isolated by liver perfusion with collagenase as described elsewhere [11, 19] and cell viability, determined by trypan blue exclusion, was always greater than 90%. Freshly isolated hepatocytes (2×10^6) were seeded into 60 × 15 mm culture dishes (Becton Dickinson) in 3 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented by 100 IU/mL penicillin, 50 μg/mL streptomycin, 50 μg/mL gentamicin, and 10% fetal bovine serum (FBS). After a 3-hr incubation at 37° in a humidified 5% CO_2: 95% air atmosphere, the medium was replaced with fresh medium supplemented by 2% FBS and containing CsA. Hepatocytes were exposed to the drug at a dose range of 0–50 μM for 24 hr. CsA was dissolved in a stock solution of DMSO and further diluted in the DMEM medium. DMSO end concentrations on all plates were 0.2%.

Measurement of Cytotoxicity by LDH Leakage

Cytotoxicity was measured using the index of membrane lysis, i.e. LDH leakage from damaged hepatocytes [20]. The release of LDH into the medium was measured by determining this enzyme activity according to Vasault [21] and was expressed as a percentage of total cellular activity.

Determination of Intracellular Generation of Reactive Oxygen Species

H_2O_2 and $O_2^{\cdot-}$ (superoxide anion) production was monitored by flow cytometry using DCFH-DA and HE, respectively [22, 23]. These dyes are stable non-polar compounds that readily diffuse into cells. Once inside the cells, the acetate groups of DCFH-DA are cleaved from the molecule by intracellular esterases to yield DCFH, which is trapped within the cells. Intracellular H_2O_2 or low-molecular-weight peroxides, in the presence of peroxidases, oxidize DCFH to the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cytosolic HE exhibits blue fluorescence when excited by UV light; however, once this probe is oxidized by superoxide anion to ethidium, it intercalates in the cell's double-strand nucleic acid, staining its nucleus and cytoplasm with a bright red fluorescence which is proportional to the intracellular superoxide anion level. Following a 24-hr incubation with CsA, hepatocytes were washed with PBS and immediately detached with trypsin/EDTA, then incubated by agitation for 30 min in 2 mL of PBS containing 5 μM DCFH-DA and 10 μM HE at 37°. The cells were washed twice with PBS to remove the extracellular DCFH-DA and HE and analyzed on a FACScan flow cytometer (Becton Dickinson) (excitation 488

nm and emission 525 nm for DCFH; excitation 488 nm and emission 605 nm for HE).

Enzyme Activity Assays

Following the 24-hr incubation with CsA, hepatocytes were washed with PBS in order to eliminate dead cells, collected from culture dishes, resuspended in PBS, and sonicated on ice. The solution was centrifuged at 2000 *g* for 15 min at 4° in microcentrifuge to eliminate cell debris and the supernatant was used for enzyme activity assays. Antioxidant enzyme activities were measured as follows: catalase was spectrophotometrically determined by measuring decreased absorbance at 240 nm using H_2O_2 as substrate [24]; superoxide dismutase (Cu,Zn-SOD and Mn-SOD) was measured spectrophotometrically by monitoring the inhibition of the autoxidation of pyrogallol. Sodium cyanide (1 mM) was added to dissect Mn-SOD activity from that of Cu,Zn-SOD [25]. Enzyme activities were expressed as units/mg protein. 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 H_2O_2 per min at 25°. Protein estimation was carried out according to Bradford [26], using BSA as standard.

RNA Extraction and Northern Blot Analysis of Mn-SOD, Cu,Zn-SOD, and Catalase

Total RNA (4×10^6 cells) was extracted following the guanidinium thiocyanate/phenol reagent method [27]. RNA (20 μg) was submitted to Northern blot analysis being electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to Gene Screen™ membranes, and cross-linked to membranes with UV light. Hybridization was carried out as described by Amasino [28]. The relative levels of various mRNA transcripts were determined using catalase, Mn-SOD, and Cu,Zn-SOD cDNA probes [29], labeled with [α-^{32}P]dCTP using the multiprimer DNA-labeling system kit (Amersham). Quantification of the films was performed by a laser densitometer (Molecular Dynamics) using hybridization with an 18S ribosomal RNA probe as internal standard. The variability in the measurement of the fold increase in mRNA, after quantification by scanning densitometry from the filters, was not greater than 15%.

Statistical Analysis

A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of CsA (control). (b) refers to age differences between both age groups.

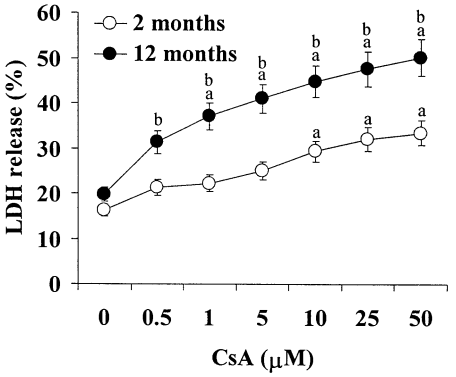


FIG. 1. Cytotoxic effects of CsA in cultured hepatocytes derived from 2- and 12-month-old rats. Hepatocytes were exposed to increasing concentrations of CsA (0–50 μM) for 24 hr, and LDH leakage was measured as a cytotoxicity index. A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of Csa (control). (b) refers to age differences between both age groups.

RESULTS

Isolated rat hepatocytes were exposed to increasing concentrations of CsA from 0 to 50 μM for 24 hr, and LDH leakage was measured as an index of cell toxicity. The kinetics of CsA cytotoxicity in hepatocyte cultures were assayed at 3, 12, and 24 hr of incubation in the presence of the concentrations of the drug mentioned above (data not shown), and we found that 24 hr of incubation was the best time point to detect significant changes. As seen in Fig. 1, the cytotoxic effect of CsA was dose-dependent, and the lowest concentrations of the drug able to cause significant increases in LDH leakage, reflecting a loss of plasma membrane integrity, were 1 μM in the 12-month-old animals and 10 μM in 2-month-old rats. Cytotoxicity was significantly higher in 12-month-old (mature adult) rats when compared to those aged 2 months (young) at concentrations above 1 μM ($P < 0.05$). At 50 μM CsA, LDH release was 50% of the total in 12-month-old rats and 33% in 2-month-old animals. In order to demonstrate whether oxidative stress was involved in CsA cytotoxicity, a parallel incubation was performed in which 50 μM vitamin E succinate was co-incubated with CsA. Significant decreases in LDH leakage were found (data not shown).

Because the cytotoxic activity of CsA in liver has been ascribed to the production of reactive oxygen species [8, 9, 30, 31], we investigated the generation of peroxides by

hepatocytes incubated for 24 hr with CsA in the range from 0–50 μM using the DCFH-DA probe. Figure 2 shows the intracellular concentration of peroxides in CsA-treated hepatocyte cultures from 2- and 12-month-old rats, measured by the fluorescence emitted due to DCFH oxidation. Figure 2A shows the histograms obtained by flow cytometry in which the fluorescence of DCF, detected with the FL1-H channel, is plotted against the relative 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. In 2-month-old rats, the intensity of |-M1-| group fluorescence over the CsA range (0–50 μM) varied progressively from 1100 to 2100 (190%, $P < 0.05$). In hepatocytes obtained from 12-month-old rats, the results showed the same pattern of change, but the differences were more pronounced, i.e. from 1350 to 3600 (267%, $P < 0.05$). The differences between the age groups were significant at 25 and 50 μM CsA (158% and 171%, respectively; $P < 0.05$).

Figure 3 shows the presence of intracellular superoxide anion in cultures of hepatocytes obtained from 2- and 12-month-old rats. Following incubation with CsA (0–50 μM) for 24 hr, a further incubation in the presence of HE was carried out. This dye, once oxidized to ethidium, intercalated within the cell double-strand nucleic acid, staining the nucleus and cytoplasm with a bright red fluorescence at 605 nm. Figure 3A shows the histograms obtained by flow cytometry, in which the red fluorescence measured in the FL3-H channel was plotted against the relative number of cells. Figure 3B shows the quantification in arbitrary units of the |-M1-| peak in the histograms of Fig. 3A. In hepatocytes from 2-month-old rats, the fluorescence slightly decreased (from 300 to 250, i.e. 83%) as CsA increased from 0 to 50 μM. In hepatocytes from 12-month-old rats, parallel changes were obtained, i.e. from 399 to 291 (73%). The differences between the age groups were

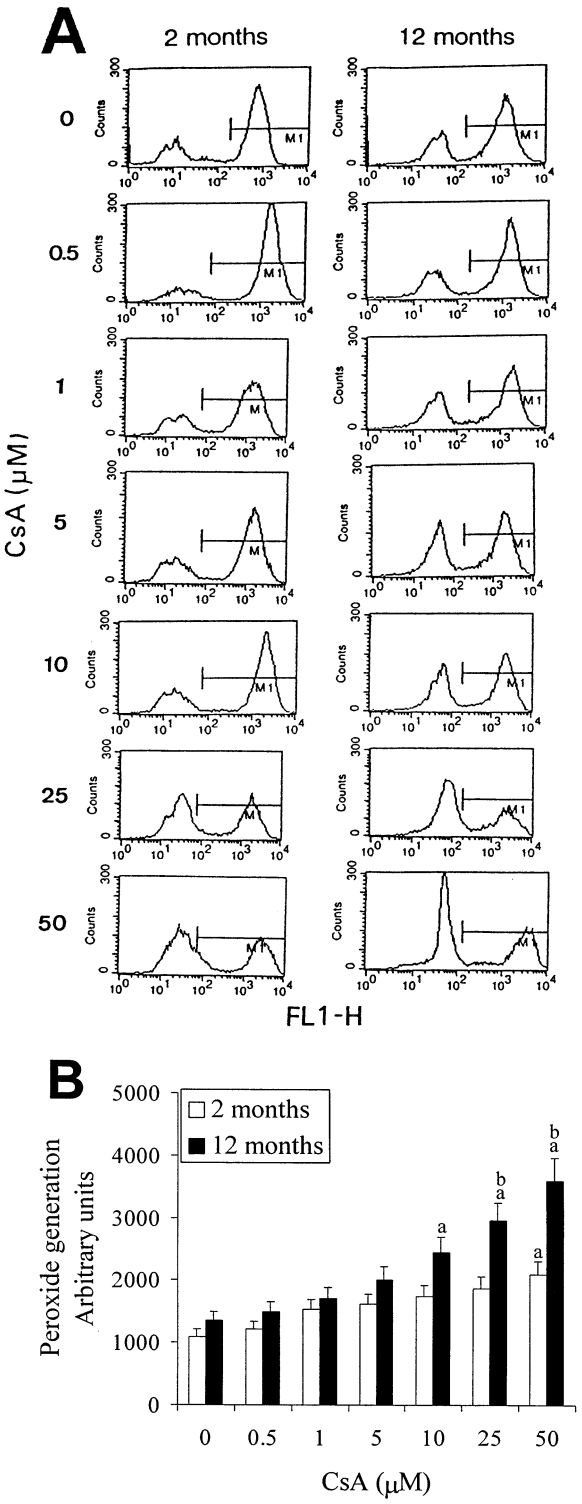
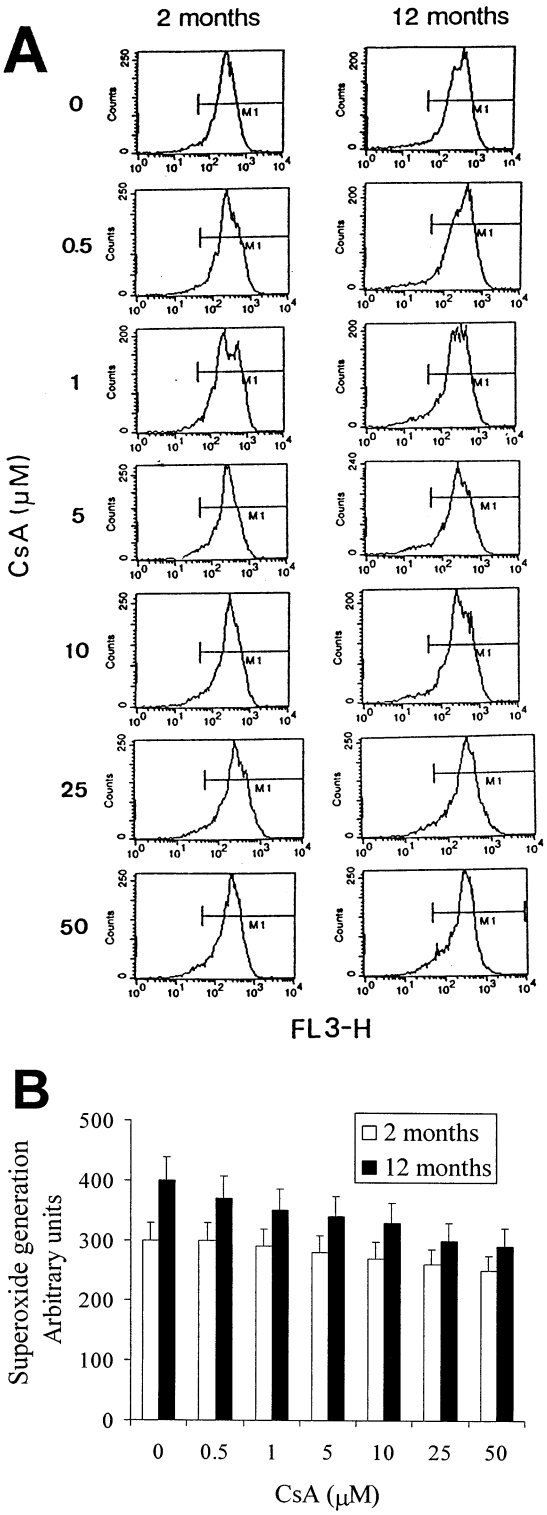


FIG. 2. Intracellular generation of peroxides in cultures in hepatocytes from 2- and 12-month-old rats incubated in the presence of CsA (0–50 μM) for 24 hr. Following incubation with the drug, cultured hepatocytes were detached with trypsin and incubated with 5 μM DCFH-DA in 2 mL PBS for 30 min at 37°. The samples were placed on ice and peroxide production was determined by flow cytometry. Figure 2A shows a representative histogram in which the fluorescence (FL1-H) is plotted against the number of cells. Figure 2B shows the quantification of |-M1-| peaks, expressed as arbitrary units, referred to fluorescence intensity (488 excitation and 525 nm emission). A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of Csa (control). (b) refers to age differences between both age groups.



not significant, but the values of superoxide anion in the older group were higher than in the younger group.

Figure 4 shows the effect of CsA on the activities of enzymes involved in the antioxidant defence systems, such as SOD and catalase, in cultures of hepatocytes obtained from 2- and 12-month-old rats. At the end of the 24-hr incubation period with CsA (0–50 μM), mitochondrial SOD (Mn-SOD) activity, expressed as units per mg of protein, increased progressively according to the CsA concentration (25 and 50 μM) in both age groups, the increase being significantly different when compared to control. Furthermore, cytosolic SOD (Cu,Zn-SOD) increased progressively parallel to the CsA concentration, showing significant differences versus untreated control at concentrations of 25 and 50 μM. Although the CsA-induced changes both in Mn-SOD and Cu,Zn-SOD followed the same profile in both age groups, the initial values of both enzyme activities were higher in hepatocytes from 12-month-old rats. Thus, Cu,Zn-SOD activities were 1356 ± 101 and 694 ± 70 U/mg protein (195%; $P < 0.05$) in 12- and 2-month-old rats, respectively, and those of Mn-SOD were 126 ± 10 and 94 ± 8.0 U/mg protein (134%) in hepatocytes from 12- and 2-month-old rats, respectively. Catalase activity, expressed as units per mg of protein, showed a slight and non-significant increase in both animal age groups.

Northern blot hybridization was used to measure the relative levels of the mRNA transcripts for the genes encoding for Mn,- and Cu,Zn-SOD and catalase as a means of studying the effect of increasing concentrations of CsA on their gene expression. Northern blots prepared with RNA extracted from CsA-treated hepatocytes from 2- and 12-month-old rats were probed with cDNA sequences of catalase, Mn-SOD, and Cu,Zn-SOD. Figure 5 shows representative autoradiographs (Fig. 5A) and their corresponding quantifications (Fig. 5B). After 24 hr of incubation with concentrations of CsA in the range of 0–50 μM, catalase

FIG. 3. Effect of CsA on intracellular generation of superoxide anion in primary cultures of hepatocytes from 2- and 12-month-old rats. Following incubation with CsA, cultured hepatocytes were detached with trypsin and incubated with 10 μM HE in 2 mL of PBS for 30 min at 37°. The samples were placed on ice and superoxide production was determined by flow cytometry. Figure 3A shows the histograms (representative of four independent experiments) in which the fluorescence (FL3-H) was plotted against the relative number of cells. Figure 3B shows the quantification, in arbitrary units, of the |-M1-| peaks. A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of Csa (control). (b) refers to age differences between both age groups.

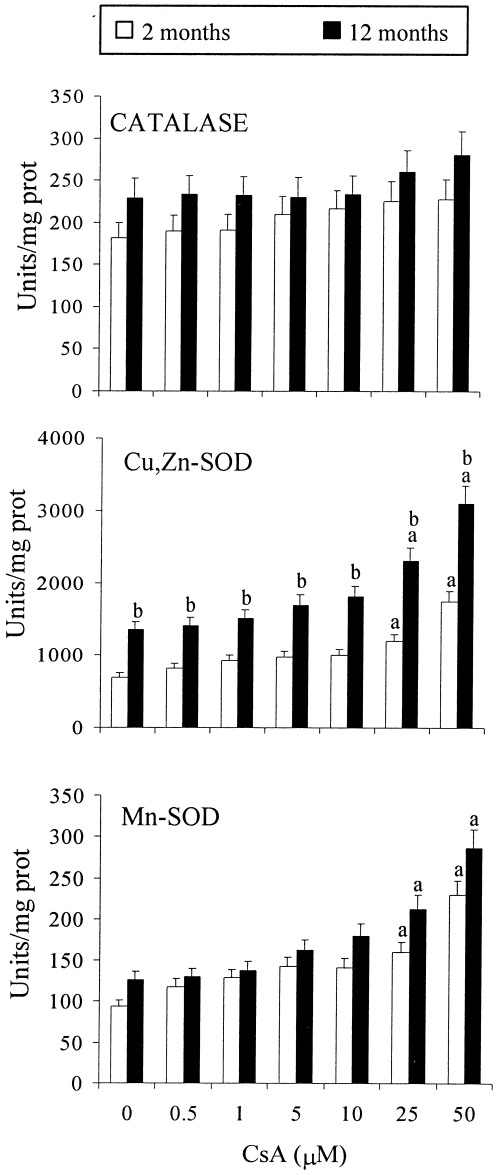


FIG. 4. Effects of CsA on catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1) activities in cultured hepatocytes obtained from 2- and 12-month-old rats. The activities of catalase, Cu,Zn-SOD, and Mn-SOD were measured as described [24,25]. One unit of SOD refers to ng of enzyme that produces 50% inhibition in pyrogallol autoxidation. One unit of catalase is defined as the amount of enzyme that transforms 1 μmol of H_2O_2 per min at 25°. A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of Csa (control). (b) refers to age differences between both age groups.

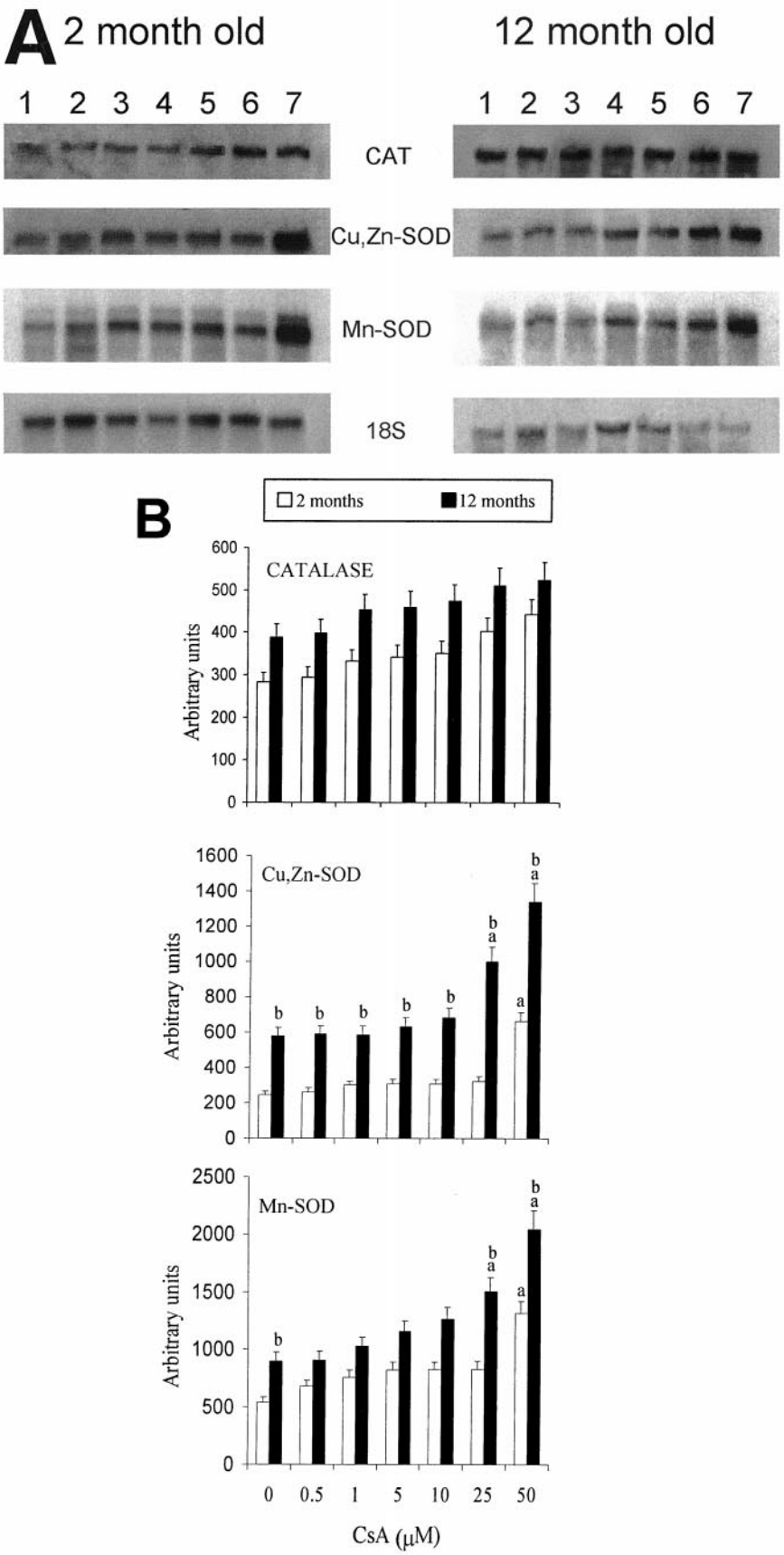
and Mn- and Cu,Zn-SOD gene expression underwent increases in the two age groups studied. However, the increases in the mRNA of Mn- and Cu,Zn-SOD were statistically significant versus control ($P < 0.05$) at 25- and 50-μM CsA concentrations in 12-month-old rats and at 50 μM in 2-month-old rats, while the catalase mRNA increase was not significant at any of the concentrations studied. These results agree with those corresponding to the enzyme activities of all these antioxidant systems and clearly show that the differences between them are not coordinated and that peroxides can accumulate.

DISCUSSION

Although previous studies have already demonstrated that oxidative damage plays a role in the cytotoxicity of CsA [8, 9], there is as yet no definitive evidence showing how the rate of reactive oxygen species, produced by this drug, affects the endogenous antioxidant mechanisms in hepatocytes of rats aged 2 and 12 months. By using the fluorescence probes DCFH and HE and flow cytometry techniques, we found increases in DCF fluorescence, indicating overproduction of peroxides and slight decreases in the intracellular levels of superoxide when rat hepatocyte cultures were incubated for 24 hr in the presence of CsA. The increase in emitted DCF fluorescence occurred progressively as CsA increased, reaching a peak at 50 μM CsA of 190% and 267% of the initial figures for hepatocytes from 2- and 12-month-old rats, respectively, which was in agreement with the cytotoxicity and the membrane lysis observed by LDH leakage. These results provide evidence that intracellular generation of peroxides could be involved in the initiation of CsA cytotoxicity.

Cell integrity is affected by oxidative stress when the production of active oxidants overwhelms antioxidant defence mechanisms [17, 18]. The sequenced elimination of superoxide and hydrogen peroxide by the action of SOD and catalase prevents the formation of the highly reactive hydroxyl radical. Our experimental studies have been conducted to ascertain the effects of CsA on the enzyme activity and gene expression of those hepatic antioxidant enzymes closely related to superoxide and peroxide generation, namely superoxide dismutase and catalase. It has

FIG. 5. Effect of CsA on gene expression of catalase and Mn- and Cu, Zn-SOD in primary cultures of hepatocytes obtained from 2- and 12-month-old rats. Northern blot analysis of catalase, Mn-SOD, and Cu,Zn-SOD Mn-SOD, and Cu,Zn-SOD cDMAs. Panel A shows representative Northern blots with 18S rRNA probe normalization (lanes 1–7 refer to 0, 0.5, 1, 5, 10, 25, and 50 μM CsA, respectively) and panel B shows the quantification in arbitrary units after correction with 18S rRNA. A two-way ANOVA test analysis of variance was used to test the response between the control and CsA-treated hepatocytes within each group. When significant changes were found between treatments, a Student–Newman–Keuls test was used to compare the means in each group. All data were reported as means ± SD of 4 different observations. In all cases, the criterion for statistical significance was $P < 0.05$. (a) refers to the differences between the values obtained in the presence of CsA as opposed to in the absence of Csa (control). (b) refers to age differences between both age groups.


A
2 month old
12 month old
1 2 3 4 5 6 7
1 2 3 4 5 6 7
CAT
Cu,Zn-SOD
Mn-SOD
18S
B
2 months
12 months
CATALASE
Arbitrary units
Cu,Zn-SOD
Mn-SOD
0 0.5 1 5 10 25 50
CsA (μM)

been described that the generation of reactive oxygen species and the depletion of cellular antioxidants play a role in drug-induced liver injury [8, 9], but little information is available on the expression of genes involved in primary cellular protection against reactive oxygen species in hepatocyte cultures incubated in the presence of CsA. Our results suggest that oxidative stress induced by CsA may occur because of the non-coordinated induction of primary antioxidant defence genes. The slight increase, over a 24-hr incubation with CsA, in the enzyme activity and transcript levels for catalase, together with the significant increase in those of both mitochondrial and cytosolic SOD as drug concentration increased, brought about an increase in the oxidant redox state detected by significant enhancements in the levels of peroxides inside the cells. A failure in the removal of H_2O_2 produces deleterious generation of hydroxyl radical by SOD [32], contributing to the molecular mechanisms of cell injury and death. In recent studies by our group on other hepatotoxins considered as non-genotoxic carcinogens, i.e. phenobarbital and clofibrate [33], we obtained significantly higher enhancements in catalase activity and mRNA, in agreement with previous reports that catalase is strongly regulated both at transcriptional and translational levels in response to oxidative stress, [34]. Our results suggest that other mechanisms, in which a failure occurs in the mechanisms of catalase inducibility, could be involved in CsA hepatotoxicity.

When hepatocytes are incubated in the presence of CsA, the production of reactive oxygen species is markedly increased, not only because of CsA metabolism, but also through the blocking of the "permeability transition pore" [35], which involves accumulation of mitochondrial Ca^{2+} and the uncoupling of oxidative phosphorylation [36]. In our experiments, there is an apparent lack of parallelism between the intracellular concentration of superoxide anion and peroxides since, in the range of CsA studied, superoxide anion slightly decreased while peroxides significantly accumulated. The discrepancy between the intracellular concentration of these two oxygen derivatives is, however, in agreement with the activities of the enzyme systems responsible for their elimination. Thus, CsA, through its metabolism [37, 38] and action [36], generates important amounts of reactive oxygen species which induce a significant increase in the gene expression and enzyme activity of both cytosolic and mitochondrial SOD, which are responsible for superoxide anion elimination.

The concerted action of SOD and catalase is the main cell defence mechanism against oxygen toxicity. Superoxide anion is generated in aerobic organisms either spontaneously or as a result of pathological situations such as biotransformation of drugs. SOD is the enzyme that converts $O_2^{\cdot-}$ (superoxide anion) to H_2O_2 and catalase is the enzyme that eliminates H_2O_2. It has been described that a failure in the removal of H_2O_2 produces hydroxyl radical catalyzed by SOD [32] and that high levels of peroxides inhibit catalase activity [39]. In our results, as the activities of both forms of SOD increased markedly and the extent of these enhancements was not accompanied by parallel increases in catalase activity and gene expression, we suggest that the cell defence systems against oxygen toxicity are imbalanced, in a dose-dependent manner, in rat hepatocytes incubated in the presence of CsA.

Imbalance between SOD and CAT activities might be the result of increased H_2O_2 formation due to SOD gene expression and activity induction together with the absence of significant changes in CAT gene expression and activity. The enhancement of SOD gene expression and activity should be regarded as an adaptive response against oxidative stress. Moreover, this induction reinforces the data of the enhanced rate of ROS generation, although it is not positive against oxidative attack if not accompanied by a concomitant change in catalase. Our results suggest that the non-coordinated induction of primary antioxidant defence genes (SOD and CAT) explains the enhancement of cytotoxicity, predominantly in the older group of rats. However, this imbalance between the gene expression and enzyme activity of SOD when compared to CAT cannot explain the increased formation of ROS in mitochondria and microsomes, which is due to the action of CsA as uncoupler and inhibitor of the mitochondrial electron transport system and CsA metabolism by cytochrome P-450 3A.

During its life period, the organism undergoes important changes, and in the development of animals, the production of oxygen radicals occurs as a consequence of the enhancement of endogenous metabolic processes. As the expression of hepatic antioxidant systems increases during rat development [13, 40, 41], in the present study we have recorded age-dependent changes in antioxidant enzyme systems in cultures of hepatocytes from 2- and 12-month-old rats, incubated in the presence of CsA. Initial enzyme activities and mRNA values in catalase and both SODs were higher in hepatocytes from 12-month-old rats as compared to those of animals aged 2 months. However, following CsA incubation, the most significant age-dependent changes were recorded in the constitutively expressed cytosolic Cu,Zn-SOD activity and mRNA and in the highly inducible mitochondrial Mn-SOD mRNA [42], while the changes in catalase activity and mRNA were not significant. These results led us to propose that as age increases the imbalance between SOD and catalase, induced *in vitro* by CsA, becomes greater, which is in agreement with the extent of cell lysis and the levels of intracellular peroxides.

From these results, we can conclude the following: (i) the accumulation of intracellular peroxides plays an important role in CsA cytotoxicity in primary cultures of rat hepatocytes; (ii) the imbalance between the enzyme activity and gene expression of SOD and catalase by the effect of CsA could be considered as the main mechanism responsible for peroxide accumulation; and (iii) the dose-dependent changes in cell lysis and death, intracellular concentration of peroxides, and antioxidant endogenous defence enzymes, induced *in vitro* by CsA, were significantly more pronounced in the older group of rats, which clearly demonstrates the influence of age on the toxicity of this drug.

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