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Biochemical Pharmacology 69 (2005) 493–501
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Attenuation of cyclosporine A toxicity by sublethal heat shock Role of catalase

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Received 24 September 2004; accepted 27 October 2004

Abstract

Cyclosporine A (CsA) is the immunosuppressor most frequently used in transplant surgery and in the treatment of autoimmune diseases because of its specific inhibiting effect on signal transduction pathways of cell T receptor. It has been shown that CsA is able to generate reactive oxygen species and lipid peroxidation, which are directly involved in the CsA hepatotoxicity. In the present study, we investigated the effect of a sublethal heat pre-treatment (43°C for 30 min) on the hepatoma cell line HepG2 exposed to cytotoxic concentrations of CsA (10 and 25 μM) for 3 and 24 h. Parameters of cytotoxicity were assayed by measuring LDH (lactate dehydrogenase) leakage into the medium. Peroxide concentration was tested by flow cytometry by measuring the fluorescence intensity of DCF (dichlorofluorescein). Gene expression of catalase was detected by measuring the respective mRNA and proteins, as well as protein level of HSP70. The enzymatic activity of catalase was also determined. Heat pre-treatment significantly reduced CsA cytotoxicity as well as the level of peroxide generation. The protective effect of the previous heat treatment (corroborated by the irreversible catalase inhibitor 3-aminotriazole) against the CsA cytotoxicity was due to an increased expression and activity of catalase that was significantly reduced by the effect of CsA. We conclude that heat pre-treatment strongly protects against CsA injury, and the mechanism of this protection is by means of inducing not only the expression of HSP70 but also the expression and activity of catalase, the main enzyme system involved in H_2O_2 elimination.

Keywords: CsA; Heat shock; Catalase; ROS; HSP70; 3-Aminotriazole; HepG2

1. Introduction

Cyclosporine A (CsA) is the immunosupressor most frequently 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 [3–6]. As the clinical use of CsA presents the inconvenience of its adverse side effects, such as hepatotoxicity (cholestasis and high levels of blood bilirubin) and nephrotoxicity, studies have been directed towards finding a way to counteract the toxicity of this drug [7–9].

The heat shock response is an immediate, transient, and highly conserved cellular mechanism that is induced by different types of physical and chemical agents [10]. The induction of this response consists of the transcriptional activation of specific genes responsible for the synthesis of heat shock proteins (HSP). HSPs, also known as molecular chaperones, help proteins to fold and transport limiting proteotoxicity (aggregation and aberrant folding) during stress situations [11,12].

The expression of HSPs not only induces thermotolerance but also protects against a number of different stress situations, including reactive oxygen species (ROS), tumor necrosis factor-α, ischemia/reperfusion, sepsis, and acute inflammation [13–15]. Several reports have described that the heat shock response is associated with protection against stressors [16], and that the in vitro overexpression of HSP70 protects against H_2O_2 toxicity [17]. One major group of HSPs is the HSP70 family, and the inducible HSP70 (also called HSP72) which likely represents the major protective stress protein [15]. However, the specific molecular

Abbreviations: HSP70, heat shock protein 70; SOD, superoxide dismutase; ROS, reactive oxygen species; CsA, Cyclosporine A; 3-AT, 3-aminotriazole

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doi:10.1016/j.bcp.2004.10.012

mechanism underlying this HSP-mediated protection remains undefined.

CsA is able to generate reactive oxygen species and lipid peroxidation [18–20], which are directly involved in the CsA hepatotoxicity. This ROS production takes place in several places such as mitochondria, and endoplasmic reticulum membranes and through different mechanisms in the cells, such as alterations in the mitochondrial membrane potential ($\Delta\Psi_m$) [21], imbalance between catalase and SODs [20] and also through its biotransformation by cytochrome P450 [22,23]. It has also been shown that CsA, when added to hepatocyte cultures, induced an increase in HSP70 expression and that this increase was parallel to the enhanced ROS production [21].

Previous studies of Kingma et al. [24], in rabbit heart have postulated that increased intracellular activity of catalase could be an important mechanism for hyperthermia-mediated cellular protection. On the other hand, we previously demonstrated that the mechanism of CsA hepatotoxicity is through interfering the expression and activity of catalase [20]. Thus, on the basis of these previous results, in the present study we investigated the effect of a sublethal heat pre-treatment on the hepatoma cell line HepG2 cultures exposed to cytotoxic concentrations of CsA, to study the hepatic mechanism of heat shock protection and to corroborate the proposed mechanism of CsA hepatotoxicity. The relevance of this line as a human hepatic model has been previously evaluated [25]. HepG2 cells synthesize a large number of plasma proteins, and the CYP activities of the different subtypes are close to that found in human liver that are responsible for CsA biotransformation. Other advantages are that HepG2 cells maintain viability for longer periods when compared to fresh isolated rat hepatocytes, and also that HepG2 cells allow researchers to work with human cells instead of rat cells. However, in vitro cytotoxicity studies do not replace the in vivo acute toxicity studies, since in the intact animal many possible toxic responses are screened simultaneously. In spite of these limitations, in vitro tests provide essential information of the mechanisms involved in the intrinsic toxicity of individual cells. Thus, in this in vitro model we study parameters of cytotoxicity, ROS production, enzyme activities, as well as the expression of HSP70 and catalase were determined. The data obtained indicate that in the protective mechanism induced by heat shock the upregulation of HSP70 and catalase are involved and that the enhanced levels of catalase play the important role of being the main mechanism involved in ROS removal.

2. Materials and methods

2.1. Reagents

The human hepatoma cell line HepG2 (ATCC number: HB-8065) was kindly provided by ECVAM (European Centre for the Validation of Alternative Methods). Tissue culture media were from GIBCO^{TM}. Standard analytical-grade laboratory reagents were obtained from Merck and Sigma. 3-Aminotriazole (3-AT) was procured from Sigma. Cyclosporine A was kindly provided by Dr. Armin Wolf, Novartis. Primary antibodies were from Calbiochem (catalase), Santa Cruz (HSP70) and Sigma (β-Actin). Secondary antibodies were from Sigma and Santa Cruz Biotechnology.

2.2. Cell culture

HepG2 cells were routinely grown in culture flasks in MEM supplemented with 10% (v/v) Foetal Calf Serum (FCS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 50 μg/mL gentamicin and 50 μg/mL streptomycin, at pH 7.4. Cells were seeded in multiwell dishes and incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. Cultures were allowed to reach 80–100% confluence before experiments were performed.

2.3. Heat and CsA treatment

Heat treatment was performed by incubating cells at 43 °C (95% air/5% CO_2) for 30 min followed by a recovery period of 2 h at 37 °C. The concentrations of CsA used in the experiments were selected after carrying out a range screening (0–75 μM). Finally, cells were exposed to CsA at concentrations of 0, 10 and 25 μM for 3 and 24 h. These concentrations allowed us to better study the mechanism of heat protection because concentrations higher than 25 μM resulted in high toxicity (over 50% of LDH release at 50 μM), and concentrations lower than 10 μM produced slight toxicity. We chose these two time points (3 and 24 h) because our experience in CsA cytotoxicity indicates that the oxidative stress situation, assayed in a time-course experiment by flow cytometry (DCF fluorescence), always precedes LDH release [21]. CsA was dissolved in a stock solution of dimethyl sulfoxide (DMSO). DMSO end-concentrations on all plates were 0.2%.

2.4. Catalase inhibition in heat-shocked cells treated with CsA

HepG2 cells were incubated with the irreversible catalase inhibitor 3-aminotriazole (20 mM) [26] after heat pre-treatment and before CsA addition.

2.5. Measurement of cytotoxicity by LDH leakage

After the different cell treatments (CsA, heat + CsA, and heat + 3-AT + CsA), cytotoxicity was measured using the index of membrane lysis, lactate dehydrogenase (LDH) leakage from damaged hepatocytes [27]. The release of intracellular LDH to the extracellular medium was measured by determining this enzyme activity following

Vasault [28], and was expressed as a percentage of total cellular activity.

2.6. Determination of intracellular generation of reactive oxygen species

H_2O_2 production was monitored by flow cytometry using DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate). This dye is a stable nonpolar compound that readily diffuses 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 (dichlorofluorescein). Thus, fluorescence intensity is proportional to the amount of peroxides produced by the cells. Following the incubation for 3 and 24 h with CsA, with or without heat treatment, cells were washed with PBS and immediately detached with Trysin/EDTA, then incubated with agitation for 30 min in 2 mL of PBS containing 5 μM DCFH-DA at 37 °C. 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 and emission 525 nm). M1 peak defines cells non-DCF-dyed; M2 peak defines cells DCF-dyed (peak of peroxides).

2.7. Enzyme activity assay

Following the different treatments (CsA, heat + CsA and heat + 3-AT + CsA), cells were washed with PBS in order to eliminate dead cells, collected from culture dishes, resuspended in phosphate buffered saline (PBS) and sonicated on ice. The solution was centrifuged for 15 min at 4 °C in microcentrifuge to eliminate cell debris and the supernatant used for enzyme activity assays. Catalase enzyme activity was spectrophotometrically determined by measuring the decrease in absorbance at 240 nm using hydrogen peroxide as substrate [29]; one unit of catalase is defined as the amount of enzyme that transforms 1 μmol of hydrogen peroxide per minute at 25 °C. Protein estimation was made following Bradford [30] using bovine serum albumin as standard.

2.8. Immunoblotting for detection of HSP70 and catalase proteins

Treated cells were washed once in PBS and lysed in ice-cold buffer containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 and the protease inhibitors phenylmethylsulfonylfluoride (PMSF), aprotinin and leupeptin (Sigma). Protein concentrations were determined using the Bradford reagent (Sigma). Whole-cell lysates were boiled in equal volumes of loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol). Protein levels were then assessed by Western blot analysis. Aliquots of cell lysates containing equal amounts of protein (20 μg) were loaded onto a 12% precast ready gel Tris–HCl (BioRad). Proteins were separated electrophoretically and transferred to PVDF membranes (Hybond-P, Amersham Life Science) using the BioRad Electrophoretic Transfer Cell. For immunoblotting, membranes were blocked with 10% non-fat dried milk in Tween-PBS (TPBS) for 2 h. Primary antibody against catalase (Calbiochem) and HSP70 (Santa Cruz Biotechnology) were applied at dilution of 1:20,000 and 1:2000, respectively, for 24 h at 4 °C. After washing, appropriate secondary antibodies (anti-rabbit and anti-goat IgG-peroxidase conjugated) were applied at a 1:10,000 and 1:20,000 dilution, respectively, for 1 h at 4 °C. Blots were washed, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham) and exposed to autoradiographic film. β-Actin (Sigma) was analyzed and used as loading control.

2.9. RT-PCR

For RT-PCR (reverse transcriptase-polymerase chain reaction), total RNA (1 μg) was subjected to random primed first-strand cDNA synthesis in 40 μL reactions composed of (in mM): 50, Tris–HCl; 75, KCl; 3, $MgCl_2$; 10, DTT (dithiotreitol); 1, dNTPs (deoxynucleotides, each), 50 ng of random hexamer, 0.5 IU/μL Mo-Mu-LV reverse transcriptase (Superscript Pre-Amplification System; Gibco BRL, Life Technologies). The reactions were incubated for 60 min at 42 °C and terminated at 65 °C for 15 min. The first-strand cDNAs were subsequently amplified by PCR; β-actin cDNA was used as an internal control. The sequences of the primers were as follows: Human catalase sense 5′-TTT GGC TAC TTT GAG GTC AC-3′; human catalase antisense 5′-TCC CCA TTT GCA TTA ACC AG-3′; human β-actin sense 5′-ACG GCT CCG GCA TGT GCA AG-3′; Human β-actin antisense 5′-TGA CGA TGC CGT GCT GCA TG-3′. The PCR reaction mixture contained PCR buffer (20 mM Tris–HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 100 μM dNTPs (each), 0.4 μM primers and 0.0025 U/μL Taq polymerase in a final volume of 50 μL. The number of PCR cycles was adjusted to avoid saturation of the amplification system [94 °C for 30 s, at 53 °C for 1 min and 72 °C for 30 s (21 cycles) for catalase and 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s (24 cycles) for β-actin] with a final elongation at 72 °C for 10 min. Amplification products were visualized on 1.8% agarose gels containing ethidium bromide (1 μg/mL): catalase product, 440 bp; β-actin product 196 bp. A 100-bp DNA ladder was used as marker. The products were quantified by laser densitometer.

2.10. Statistical analysis

The results were reported as means ± S.D. of three experimental observations. Concentration course data were

compared by using a Student's *t*-test. (a) Significant differences against the control of each series (''none'', ''heat'' and ''heat + 3-AT''). To compare means of groups, a two-way ANOVA test was used. Pairwise comparisons were conducted employing a Student–Newman–Keuls post hoc test. (b) Significant differences between sample with CsA and sample with heat shock + CsA. (c) Significant differences between sample with heat shock + 3-AT + CsA, and sample with heat shock + CsA.

For all tests, $p < 0.05$ was accepted as significant.

3. Results

3.1. Heat shock effect on LDH release

HepG2 cells were exposed to increasing concentrations of CsA (0, 10 and 25 μM) for 3 and 24 h with or without a previous heat shock (30 min at 43 °C). At the end of the incubation period, lactate dehydrogenase (LDH) leakage was measured as an index of cell toxicity. Fig. 1A shows, as percentages of the total, that at 3 h of incubation, no significant differences were found at any experimental condition. Fig. 1B shows that at 24 h of incubation, the cytotoxic effect of CsA was significant at the concentration of 10 and 25 μM. Those cells that were heat-shocked displayed a minor leakage of LDH with respect to cells without heat pre-treatment.

3.2. Heat shock effect on ROS production

Fig. 2 shows ROS production in cells submitted or not to heat shock and incubated in the presence of CsA for 3 and 24 h. Fig. 2A depicts 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. |-M2-| defines the peak of peroxides (intense fluorescence). Fig. 2B shows the quantification in arbitrary units of the |-M2-| peak of Fig. 2A. At 3 h of incubation no differences were found at any experimental condition; however, at 24 h the levels of peroxides increased progressively with CsA concentration reaching statistical significance against the control at 10 and 25 μM. In cells that underwent a previous heat treatment, the increase in the peroxide level was much lower than that in the heat-untreated cells.

3.3. Catalase activity

Fig. 3(A and B) shows the catalase activity in HepG2 cells cultures with or without a previous heat treatment, following 3 or 24 h of incubation with CsA. It can be observed that heat treatment significantly increased catalase activity. Incubation with CsA produced a slight and progressive decrease in catalase that reached statistical significance at 25 μM in cells without heat treatment.

3.4. Western blot and RT-PCR analysis of catalase

Fig. 4 shows the effect of the heat shock on catalase protein levels measured in HepG2 cell cultures incubated for 3 and 24 h at 0, 10 and 25 μM CsA, by immunoblotting assay. A representative Western blot appears in the upper panel of the figure, and the quantification of chemiluminescence signals in the lower panel. It can be observed at both periods of incubation (3 and 24 h) that heat pre-treatment significantly enhanced catalase protein concentration. At 24 h, a very slight and not significant decrease was observed in catalase level in those cells without heat treatment.

In Fig. 5(A and B) the analysis of catalase mRNA by RT-PCR can be seen in HepG2 cells treated with CsA (0, 10 and 25 μM) and submitted or not to a previous heat shock. The PCR product presents a significant increase in heat-shocked cells at both periods of incubation (3 and 24 h). No significant differences were found between cells incubated with CsA and controls at 3 h of incubation. However, at 24 h a significant decrease was observed in non-heat-shocked cells treated with 25 μM CsA.

3.5. Western blot analysis of HSP70

The analysis of HSP70 by immnunoblotting is shown in Fig. 6. A representative Western blot and the quantification

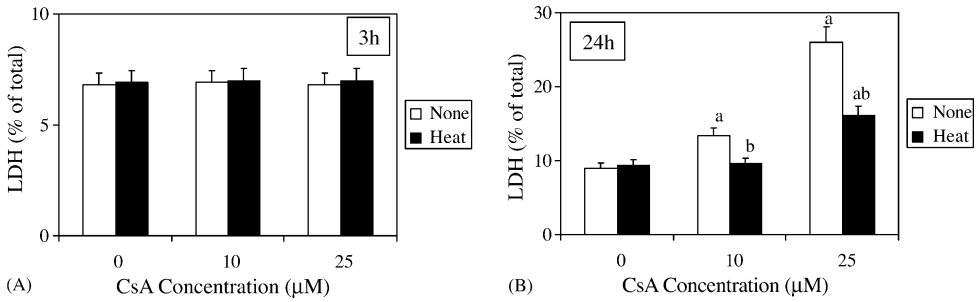

Fig. 1. Effect of heat pre-treatment on cytotoxic effects of CsA (0, 10 and 25 μM) for 3 and 24 h. HepG2 cells were incubated with CsA (0, 10 and 25 μM) for 3 h (A) and 24 h (B) with/without heat pre-treatment (43 °C 30 min). LDH leakage to extracellular medium was measured as a cytotoxicity index. (a) Significant differences against the control of each series (''none'' and ''heat''). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

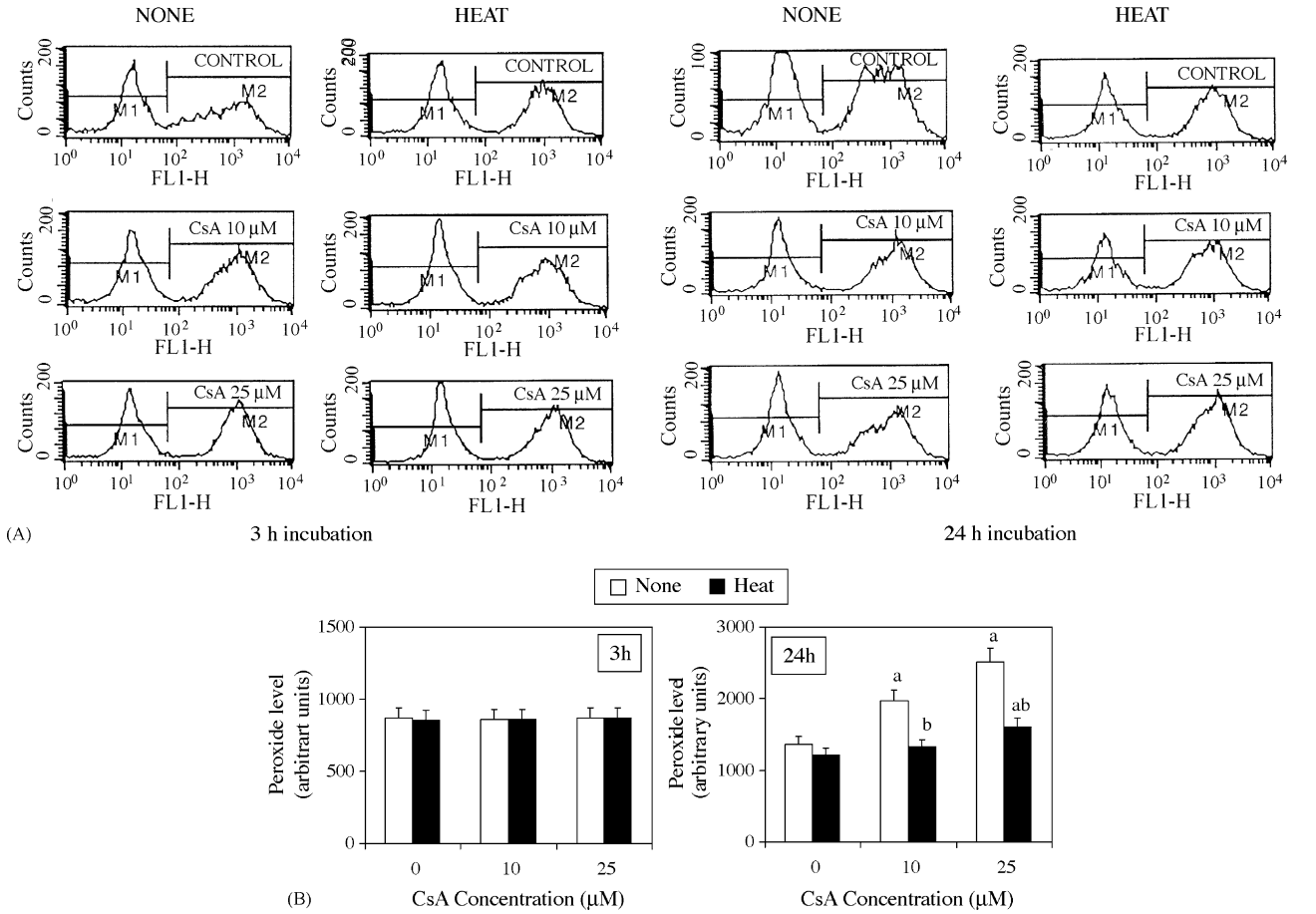

Fig. 2. Effect of heat pre-treatment on the intracellular generation of peroxides in HepG2 cells in the presence of CsA (0, 10 and 25 μM) for 3 and 24 h. Following incubation with CsA, with/without heat pre-treatment, HepG2 cells were detached with trypsine and incubated with 5 μM DCFH-DA in 2 mL PBS for 30 min at 37 °C. (A) Shows representative histograms obtained by flow cytometry. (B) Shows the quantification of histograms in (A) in arbitrary units (fluorescence intensity). Data represent the mean ± S.D. of the average values (mean of M2 peak) of three individual experiments. (a) Significant differences against the control of each series (“none” and “heat”). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

of the signals, at both periods of incubation, appear in the upper and lower panel, respectively. At 3 h of incubation the level of HSP70 was higher at all concentrations of CsA assayed in heat-shocked cells versus non-heated cells. No differences were observed between controls and CsA-treated cells. At 24 h of incubation, the level of HSP70 was also significantly higher in heat-shocked cells. However, at 25 μM CsA there was observed a significant increase of HSP70 level versus control in both, heat-shocked and non heat-shocked cells.

3.6. 3-AT effect on LDH release and catalase activity in heat-shocked cells treated with CsA

Fig. 7 shows the 3-AT effect on LDH release. At 3 h of incubation no significant differences were observed between

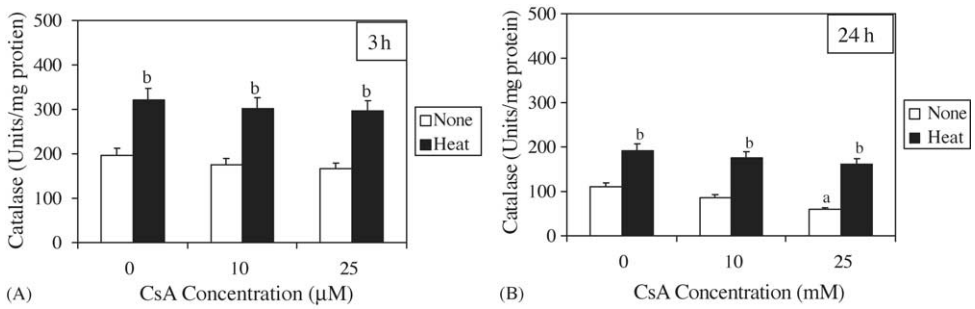

Fig. 3. Effect of heat pre-treatment on catalase activity in HepG2 cells in the presence of CsA (0, 10 and 25 μM) for 3 and 24 h. The activity of catalase was measured as described [29]. One unit of catalase is defined as the amount of enzyme that transforms 1 μmol of hydrogen peroxide per min at 25 °C. Fig. 3A and B show the catalase activity at 3 and 24 h of incubation, respectively. (a) Significant differences against the control of each series (“none” and “heat”). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

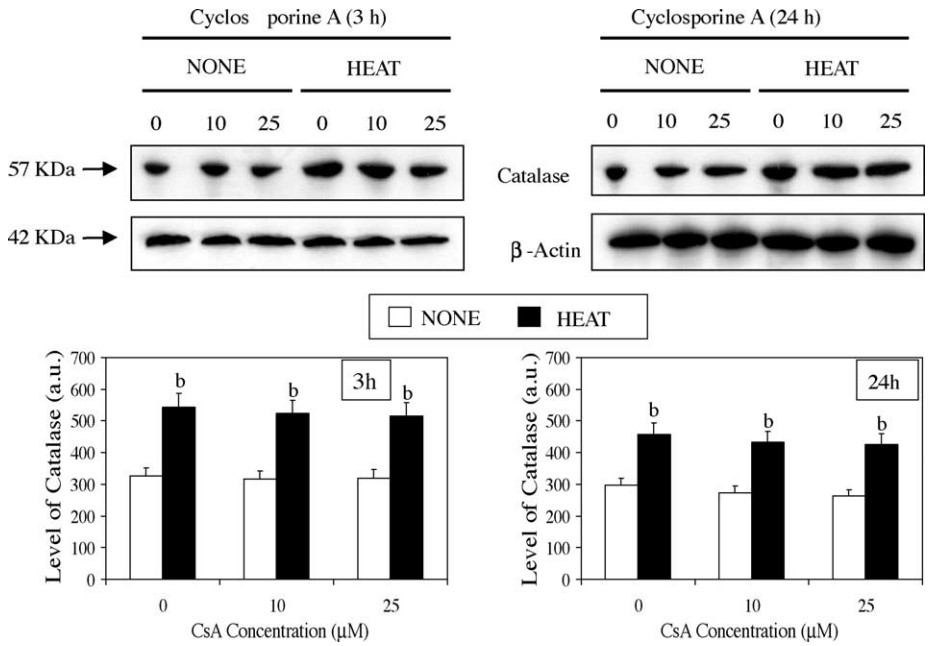

Fig. 4. Western blot analysis of catalase in HepG2 cells incubated with CsA (0, 10 and 25 μM) for 3 and 24 h with/without heat pre-treatment. Protein levels were assayed by Western blot analysis. In the upper panel can be observed the signals after ECL detection. The lower panel shows the quantification of chemiluminescence signals by laser densitometry expressed as arbitrary units. (a) Significant differences against the control of each series (''none'' and ''heat''). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

cells treated with HS and those treated with HS + 3-AT. At 24 h of incubation the LDH release in 3-AT treated cells was significantly higher with respect to heat-shocked cells without 3-AT treatment, and very similar to LDH release in the series of cells incubated with CsA alone.

The 3-AT effect on catalase activity is shown in Fig. 8. A marked significant inhibition of catalase activity in heat-shocked cells treated with 3-AT at 3 and 24 h of CsA incubation can be clearly observed.

4. Discussion

The heat shock response is an immediate and transient cellular process induced by different types of physical and chemical agents [31]. It has been demonstrated that HSP protects the cells against the toxic effects of oxidative stress and H_2O_2 toxicity [17] and that the induction of HSP70 produces tolerance to CsA toxicity [32]. CsA is able to generate ROS and lipid peroxidation, which are

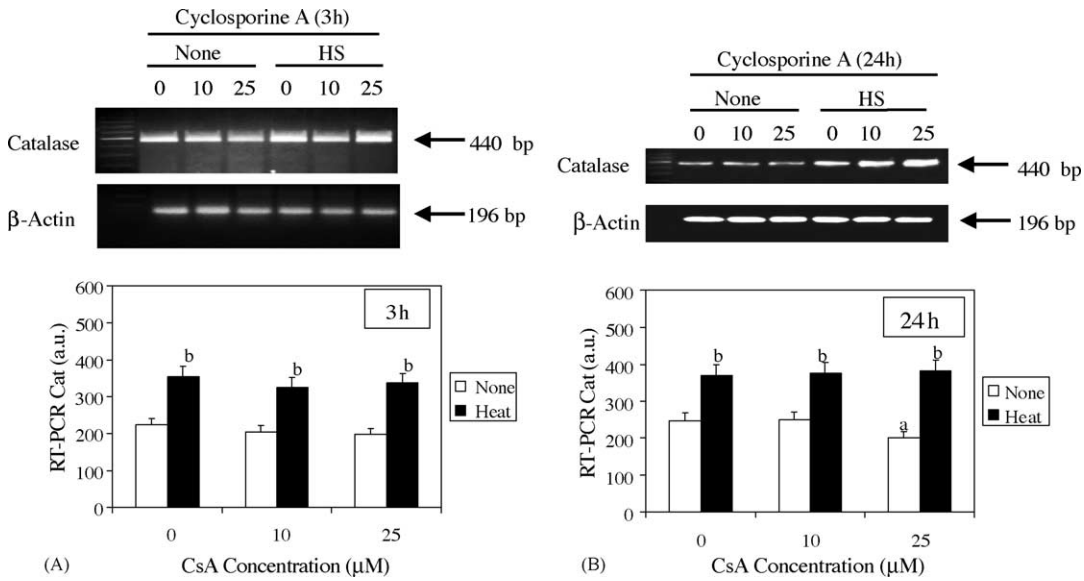

Fig. 5. RT-PCR quantification of catalase mRNA in HepG2 cells incubated with CsA (0, 10 and 25 μM) for 3 and 24 h with/without heat pre-treatment. (A and B) show a representative RT-PCR and the quantification of the signals at 3 and 24 h of CsA incubation, respectively. Catalase product 440 bp. β-Actin product 196 bp. (a) Significant differences against the control of each series (''none'' and ''heat''). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

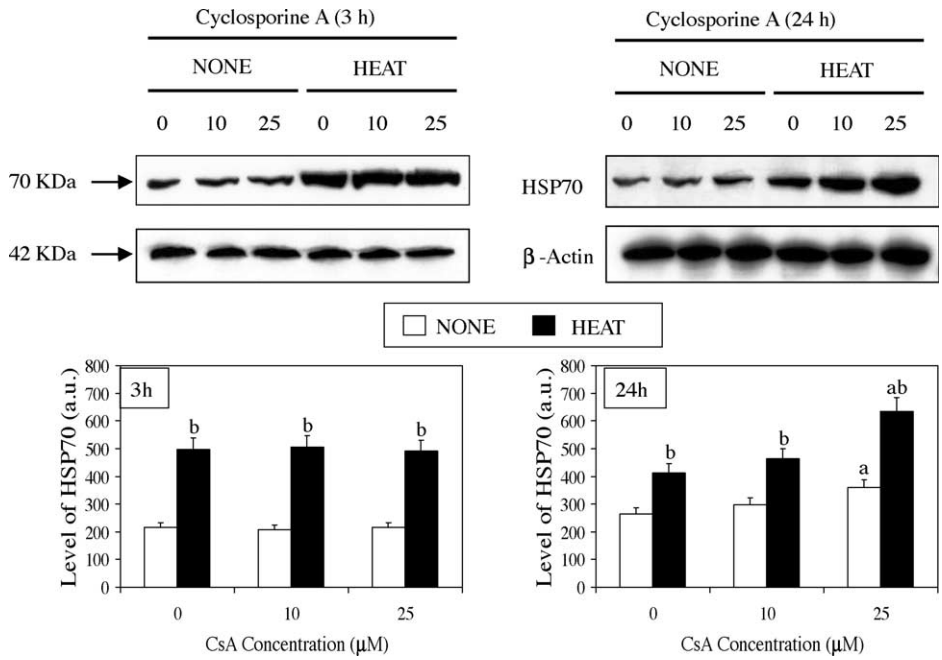

Fig. 6. Western blot analysis of HSP70 in HepG2 cells incubated with CsA (0, 10 and 25 μM) for 3 and 24 h with/without heat pre-treatment. Protein levels were assayed by Western blot analysis. In the upper panel can be observed the signals after ECL detection. The lower panel shows the quantification of chemiluminescence signals by laser densitometry expressed as arbitrary units. (a) Significant differences against the control of each series (''none'' and ''heat''). (b) Significant differences between sample with CsA and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

directly involved in CsA hepatotoxicity [20]. In previous studies of our group it has been reported that CsA induces the expression of HSP parallel to the increase in peroxide concentration [21], which led us to suggest that the peroxides are mainly responsible for inducing HSP70 expression. Moreover, CsA produced an imbalance between superoxide dismutase and catalase, which is considered to be the main mechanism responsible for peroxide accumulation and cell death [20]. These recent data demonstrating the mechanism involved in oxidative stress induced by CsA were reinforced with the restorative effect of Vitamin E on this imbalance [33].

On the basis of these data, we decided to investigate the effect of a previous sublethal heat treatment on CsA-induced cytotoxicity on HepG2 cell line. The present data demonstrate that a mild heat treatment to cell cultures previous to exposure to different concentrations of CsA for 24 h clearly diminished the cytotoxicity of this drug. The diminished cytotoxicity was parallel to the lower levels of peroxides and to a higher extent in catalase expression and activity. Superoxide dismutase activity did not show any significant changes (data not shown). In recent studies we demonstrated in cultured hepatocytes that heat pre-treatment increased catalase expression and activity while minor or no differences were found in SOD, which is in agreement with the present results in HepG2 cells [34]. To corroborate the role of catalase in the protective effect of heat shock against CsA toxicity, heat-shocked cells were

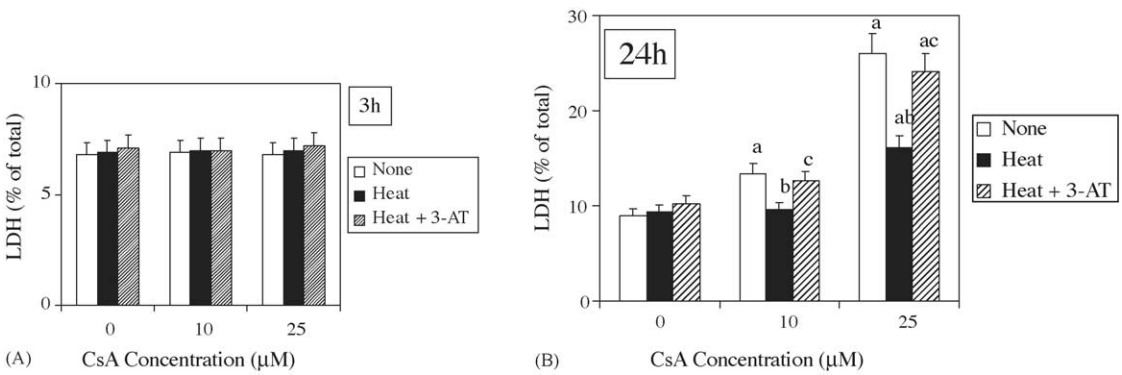

Fig. 7. Effect of 3-AT on LDH release in heat-shocked cells treated with CsA. HepG2 cells were subjected to different treatments (CsA, Heat + CsA and Heat + 3-AT + CsA) for 3 h (A) and 24 h (B). LDH leakage to extracellular medium was measured as a cytotoxicity index. (a) Significant differences against the control of each series (''none'', ''heat'' and ''heat + 3-AT''). (b) Significant differences between sample with CsA and sample with heat shock + CsA. (c) Significant differences between sample with heat shock + 3-AT + CsA, and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

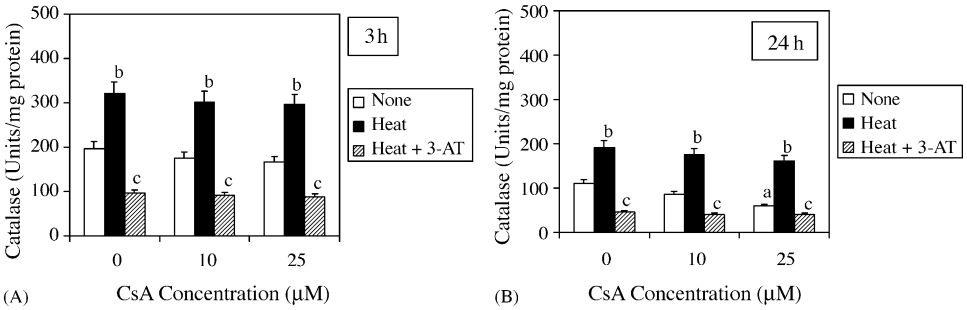

Fig. 8. Effect of 3-AT on catalase activity in heat-shocked cells treated with CsA. The activity of catalase was measured as described [29] after the different treatments (CsA, Heat + CsA and Heat + 3-AT + CsA) for 3 h (A) and 24 h (B). One unit of catalase is defined as the amount of enzyme that transforms 1 μmol of hydrogen peroxide per min at 25 °C. (a) Significant differences against the control of each series (“none”, “heat” and “heat + 3-AT”). (b) Significant differences between sample with CsA and sample with heat shock + CsA. (c) Significant differences between sample with heat shock + 3-AT + CsA, and sample with heat shock + CsA. For all tests, $p < 0.05$ was accepted as significant.

incubated with the irreversible catalase inhibitor 3-AT. As demonstrated previously, this inhibitor does not affect HSP70 expression (no induction and no inhibition) [35] and it is not toxic for control cells without heat shock [36]. The results show that 3-AT abrogates the protective effect of heat pre-treatment so that catalase has a decisive role in the protection of HepG2 cells by heat shock. These findings are in agreement with those obtained by other authors in different experimental models [24].

The protective effect of a previous heat treatment on CsA cytotoxicity in LLC-PK1 cells was described by several authors [32,37], but the precise mechanism of this protection remained unclear. Our present results demonstrate that the way by which heat effect protects against CsA cytotoxicity was by upregulating the expression and activity of the catalase antioxidant system, which was significantly decreased due to the effect of CsA. The interference of CsA with catalase gene expression and activity [20] might represent a way by which CsA causes ROS toxicity. Accordingly, the previous mild heat shock allowed cells to adapt to conditions of increased oxidative stress, fortifying the antioxidant defences of the cell against the overproduction of ROS imposed by CsA. Nevertheless, we cannot dismiss the possibility of a direct protective action of HSPs on cell organelles protecting them from the ROS production induced by CsA. Thus, at present, we are not able to know the exact contribution of each HSP70 and catalase protective effect on CsA-induced injury. Several authors have reported that hyperthermic treatment is associated with enhanced content of the antioxidant enzyme catalase, and obviously with an increase in HSP levels [24,38,39]. So, it is possible that the expression of catalase and HSP70 might be connected mechanistically through a common signalling pathway. Further experiments have to be performed in order to postulate a certain mechanism.

From the present results, we conclude that heat pre-treatment protects against CsA injury, and the mechanism of this protection is through inducing not only the expression of HSP70 but also the expression and activity of catalase, the main enzyme system involved in H_2O_2 elimination. Moreover, these results permit us to corroborate the previously proposed mechanism of CsA hepatotoxicity. So, in the field of transplant surgery, the reduction of CsA toxicity in vitro that we obtained by increasing the level of HSP70 and the activity and expression of catalase could provide the opportunity to diminish the organ rejection rate through the attenuation of CsA adverse effects permitting patients to properly continue their immunosuppressive therapy. These findings also open a possible new pathway to diminish the adverse effects of other drugs used in therapeutics.

Acknowledgements

The authors would like to thank Professor Erik Lundin for his help in the preparation of the manuscript. We are also grateful to Dr. Armin Wolf from Novartis Ltd (Basel, Switzerland) for the generous gift of CsA. This study was supported by FIS (Fondo de Investigación Sanitaria) 99/1045, CAM (Comunidad Autónoma de Madrid) 08.8/0016.1/1998 and by a postdoctoral grant of CAM.

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