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Relationship between the activation of heat shock factor and the suppression of nuclear factor-κB activity in rat hepatocyte cultures treated with cyclosporine A☆

David Andrés, Carmen Díez-Fernández, Antonio Castrillo, María Cascales*

Instituto de Bioquímica (CSIC-UCM), Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal sn, 28040 Madrid, Spain

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Abstract

We investigated on primary cultures of rat hepatocytes the effect of cyclosporine A (CsA) on the activation of nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and heat shock factor 1 (HSF1), three transcription factors involved in cellular response pathways. Hepatocytes were subjected to a time-course (1, 3, 6, and 22 hr) incubation and CsA treatment in the range 1–50 μM. NF-κB, AP-1, and HSF1 binding activities were established through electrophoretic mobility shift assay. Levels of HSP70 mRNA and protein were measured by Northern and Western blot analysis respectively. In cells incubated for 1 and 3 hr, electrophoretic mobility shift assay experiments showed a dose-dependent increase of the NF-κB binding activity; while following 22 hr of incubation, a suppression of the positive effect of CsA at shorter times was detected. At all periods of incubation assayed, CsA induced the activation of AP-1 which was detected by DNA-binding activity of this transcription factor. A dose-dependent activation of HSF1 was observed at 22 hr of incubation. We conclude that in rat hepatocyte cultures, CsA induces the transcriptional activation of NF-κB, AP-1, and HSF1. However, the time point at which activation of each transcription factor occurs is different. Thus, at 22 hr of incubation, the CsA-induced activation of HSF1 is accompanied by the reduction of the positive effect of CsA on NF-κB activation at earlier time points.

Keywords: CsA; HSP70; HSF1; IκB-α; AP-1; NF-κB

1. Introduction

CsA is the immunosuppressor most frequently used in transplant surgery and in the treatment of autoimmune diseases [1,2] because of its specific inhibitory effect on signal transduction pathways of T cell receptor through the formation of a CsA–cyclophilin complex [3,4]. The clinical use of CsA encompasses serious side effects, among which hepatotoxicity, nephrotoxicity, and hypertension are often encountered. Several authors have proposed that AP-1 activation is involved in some of these side effects, since it has been described that CsA induces the activation of this transcription factor [5–8] involved in many processes like TGFβ expression, oxidative stress, proliferation, apoptosis, antigen binding, inflammation, cellular stress, etc. [9–11].

The HSF is transiently induced by different types of physical and chemical stressors [12]. In mammalian cells, activation of HSF–HSE binding and expression of HSPs can be triggered by a wide variety of toxic conditions including alterations in the intracellular redox environment, exposure to heavy metals or cytotoxic drugs, and virus infection [13]. HSF, when activated and translocated into the nucleus, induces the transcription of specific genes responsible for the synthesis of HSPs. HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage [14].

The inducible activation of NF-κB participates in the regulation of genes involved in inflammation and the immune response, as well as in proliferation, transformation, and tumor development [15–17]. NF-κB is activated by its release from cytoplasmic IκB proteins and subsequently translocates into the nucleus. In this activation process, phosphorylation in specific serine residues of IκB by IκB kinase is required as a specific mechanism that

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* Corresponding author. Tel.: +34-91-5436262; fax: +34-91-5436262/3941782/5447254.

E-mail address: cascales@farm.ucm.es (M. Cascales).

Abbreviations: HS, heat shock; HSP, heat shock protein; HSF, heat shock factor; CsA, Cyclosporine A; AP-1, activator protein-1; NF-κB, nuclear factor kappa B; IκB, inhibitory protein kappa B.

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targets these proteins for ubiquitin conjugation and degradation by the 26S proteasome [18,19]. NF-κB transactivation potential appears to be linked to signaling that controls cell cycle progression [20]. Recent experiments of our group have shown that hepatocyte proliferation induced by CsA is accompanied by an increase of NF-κB level into the nucleus responsible for increasing gene expression of the cyclin D1 which causes cells to enter in S phase and DNA replication [21]. The role of CsA in the activation of NF-κB has been investigated in different cellular systems reporting contradictory results. Meyer *et al.* [22] have reported that CsA is an uncompetitive inhibitor of proteasome activity and prevents NF-κB activation; however, recently Alexanian and Bamburg [23] reported that the activation of NF-κB is enhanced by calcineurin inhibitors. In rat hepatocytes, Kaibori *et al.* [24] demonstrated that CsA does not inhibit the induction of inducible nitric oxide synthase expression during NF-κB activation.

The interaction between these transcription factors has been studied by several authors who have proposed that HS inhibits the activation of NF-κB, since inducers of HS response, such as sodium arsenite [25], nonsteroidal anti-inflammatory drugs [26], or hyperthermia itself [25], mimic the inhibition of NF-κB by prostaglandins [25,27] resulting in the inhibition of inflammation. However, the precise mechanism by which the HS interferes with this activation remains unclear.

On the basis of this evidence and that of our recent publication describing that CsA induces the HS response in rat hepatocytes [28], in the present study we examine the interactions between HSF1, NF-κB, and AP-1 in primary cultures of rat hepatocytes, in order to elucidate the controversial role of CsA in NF-κB and AP-1 activation. The results obtained will help to understand the mechanisms that are involved in the transcriptional cell response to CsA.

2. Materials and methods

2.1. Reagents

Tissue culture media were from Biowhittaker. Standard analytical grade laboratory reagents were obtained from Merck. Collagenase was from Boehringer. AP-1 oligonucleotide was from Santa Cruz Biotechnology. Anti-HSP70, anti-c-jun, and anti-IκB-α antibodies were from Santa Cruz Biotechnology, and anti-HSF1 was from Alexis Biochemicals. Cyclosporine A was kindly provided by Dr. Armin Wolf, Novartis.

2.2. Animals

Male Wistar rats aged 2 months, with an average body weight of 180–230, 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 Institute of Health. Rats were supplied with food and water *ad libitum* and exposed to a 12-hr light-dark cycle.

2.3. Isolation and culture of hepatocytes

Hepatocytes were isolated by liver perfusion with collagenase as described elsewhere [29,30], and cell viability, determined by trypan blue exclusion, was always greater than 90%. Freshly isolated 2×10^6 hepatocytes were seeded into 60×15 mm culture dishes (Becton–Dickinson) in 3 mL Dulbecco's modified Eagle's medium, supplemented by 100 IU/mL penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamicin, and 10% fetal calf serum. After 3 hr incubation at 37 ° in a humidified 5% CO_2–95% air atmosphere, the medium was replaced with fresh medium supplemented by 2% fetal calf serum containing CsA. Hepatocytes were exposed to the drug at a dose range of 0–50 mM for 1, 3, 6, and 22 hr. CsA was dissolved in a stock solution of dimethyl sulfoxide and further diluted in the Dulbecco's modified Eagle's medium. Dimethyl sulfoxide end-concentrations on all plates were 0.2%.

2.4. RNA extraction and Northern blot analysis of HSP70

Total RNA (4×10^6 cells) was extracted following the guanidinium thiocyanate/phenol reagent method [31]. Twenty milligrams RNA 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 [32]. The relative level of mRNA transcript was determined using HSP70 cDNA probe [33], 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%.

2.5. Electrophoretic mobility shift assays (EMSAs)

The oligonucleotide sequences corresponding to the distal NF-κB binding site 5′-TGCTAGGGGGATTTTCC-CTCTCTCTGT-3′ [34] of the murine iNOS promoter, and the consensus HSF1 binding site 5′-GCCTCGAATGTTC-GCGAAGTTTCG-3′ [35] of the HSP70 promoter were synthesized. AP-1 oligonucleotide, 5′-CGCTTGATGACT-CAGCCGGAA-3′ was from Santa Cruz Biotechnology. Aliquots of 100 ng of this annealed oligonucleotide was end-labeled with Klenow enzyme fragment. A total of

5×10^4 dpm of DNA probe was used for each binding assay of nuclear extracts as follows: 3 μg of protein were incubated for 15 min at 4° with the DNA and 2 μg of poly(dI:dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10 mM Tris–HCl (pH 7.8) in a final volume of 15 μL. The DNA–protein complexes were separated on native 6% polyacrilamide gels in 0.5% Tris–borate–EDTA buffer. Supershift assays were carried out after incubation of the nuclear extracts with 2 μg of Ab (α-p50, α-p65, α-HSF1, and α-jun) for 1 hr at 4°, followed by EMSA. Specificity of the binding was shown by competition with a 50-fold amount of the same unlabeled oligonucleotide, and by the lack of competition with an unrelated oligonucleotide at all times of treatment (not shown).

2.6. Immunoblotting for detection of c-jun, HSP70, HSF1, and IκB-α proteins

2.6.1. Preparation of cytosolic and nuclear extracts

Protein extracts were prepared following the method of Schreiber *et al.* [36] described previously. All steps of cell fractionation were carried out at 4°.

2.6.2. Preparation of whole cell lysates

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, 1 mM dithiothreitol, and the protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride, 40 μg/mL aprotinin, and 4 μg/mL leupeptin.

Protein concentrations were determined by the Bradford reagent (Sigma). Whole cell lysates and cytosolic and nuclear extracts 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 analyzed by Western blot analysis. Aliquots containing equal amounts of protein (20 μg) were loaded onto a precast ready gel Tris–HCl (BioRad). Proteins were separated electrophoretically and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Life Science) using the BioRad Electrophoretic Transfer Cell. For immunoblotting, membranes were blocked with 10% nonfat dried milk in TPBS for 2 hr. The primary antibodies employed were rabbit polyclonal antibodies against HSF1 (Alexis Biochemicals) and IκB-α (sc-371, Santa Cruz Biotechnology Inc.) and goat polyclonal antibodies against c-jun (sc-45-G) and HSP70 (sc-1060, Santa Cruz Biotechnology Inc.). After washing, appropriate secondary antibody (anti-rabbit (sc-2004) or anti-goat (sc-2020) IgG-peroxidase conjugated from Santa Cruz) was applied for 1 hr. Blots were washed, incubated in commercial enhanced chemiluminescence reagents (Amersham) and exposed to chemiluminescence film. Quantification of the films was performed by a laser densitometer (Molecular Dynamics). Ponceau staining was used as an internal standard.

2.7. Statistical analysis

The number of experiments analyzed is indicated in the corresponding figure legend. The results were reported as means ± SD of the number of experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control.

3. Results

3.1. Effects of CsA on NF-κB activation

Electrophoretic mobility shift assay experiments performed with hepatocyte nuclear extracts and a κB-like oligonucleotide (Fig. 1(A)) showed a dose-dependent increase of the NF-κB binding activity in cells treated with CsA for 1 and 3 hr, reaching the highest differences, vs. control, at the concentration of 50 μM CsA in both cases. However, when hepatocytes were incubated for longer periods (22 hr), the binding activity of NF-κB did not increase at any of the concentrations of CsA assayed. Moreover, in 50 μM CsA treated cells a decrease in the activation of NF-κB, when compared to control, was detected at 6 and 22 hr. At 1 and 10 μM CsA and 22 hr of incubation, no activation was induced, since the level of NF-κB binding was similar to the control.

It is well known that NF-κB is sequestered in the cytosol of unstimulated cells through interactions with inhibitory proteins, where IκB-α is the most studied member. IκB-α masks the nuclear localization signal of NF-κB and prevents its nuclear translocation. Signals that induce the activation of NF-κB cause the dissociation and subsequent degradation of IκB-α, allowing free NF-κB to enter the nucleus and induce gene expression. Fig. 1(A) also shows the Western blot analysis of IκB-α in cytosolic extracts. At 1 and 3 hr, the level of cytosolic IκB-α decreased when CsA concentration increased, allowing the translocation of NF-κB into the nucleus. However, when hepatocytes were incubated for 6 and 22 hr, IκB-α degradation was not induced at 10 and 50 μM CsA, and a slight increase of IκB-α level was observed at 22 hr and 50 μM. Fig. 1(B) shows the quantification of the signals from the EMSA and Western blot analysis of NF-κB and IκB-α respectively.

In Fig. 1(C) is shown a gel supershift to identify the subunits involved in the activation of NF-κB by CsA. α-p50 and α-p65 were used in these assays. The α-p50 caused a shift of both, the lower and the upper complexes (p50–p50 homodimer and p50–p65 heterodimer, respectively), but mainly in the lower one. The α-p65 supershifted only the upper complex. When both antibodies were used, the supershift of both complexes was better appreciated.

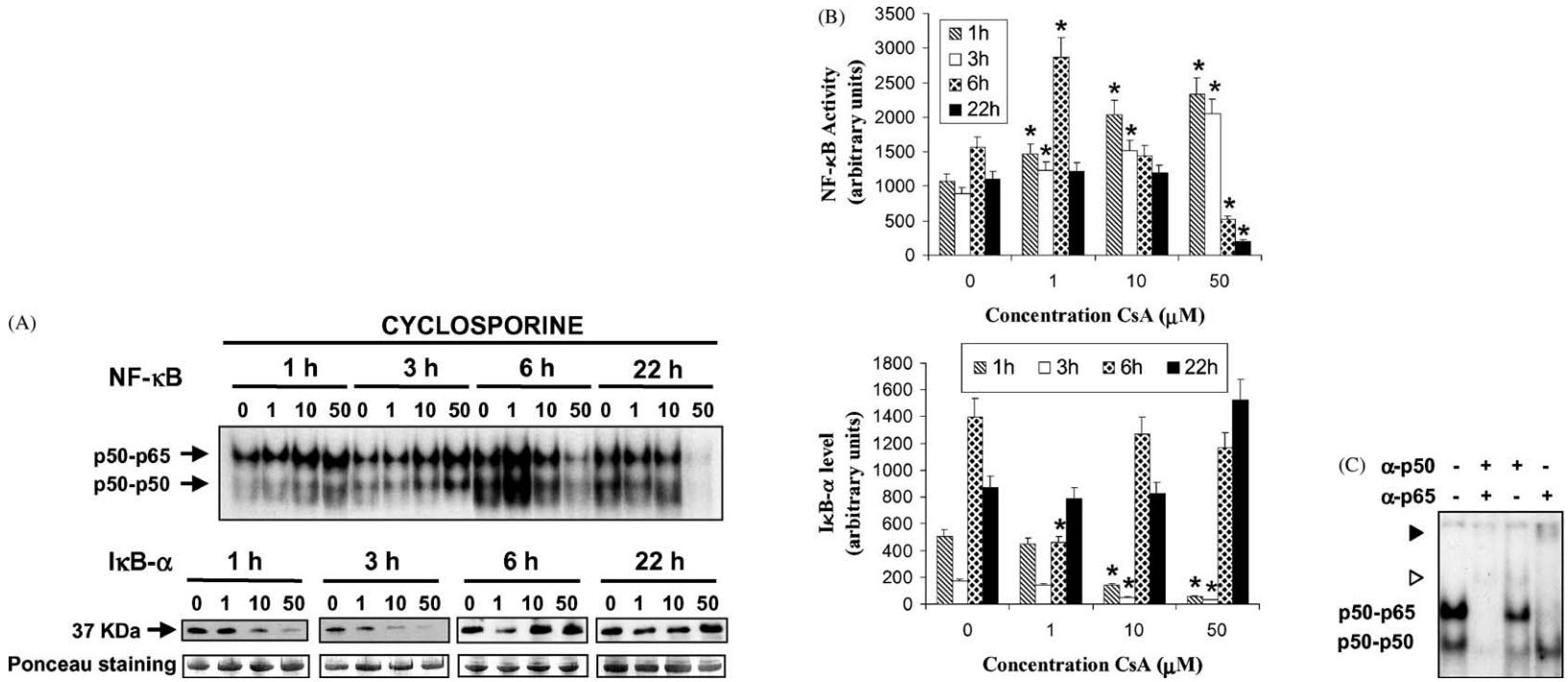

Fig. 1. Time-course of the NF-κB complex DNA binding in rat hepatocytes treated with CsA. (A) Hepatocytes were incubated with 0–50 μM CsA for the indicated periods of time. Nuclear extracts were prepared as described under ''Section 2,'' and the binding of nuclear proteins to the κB motif was determined by EMSA. The corresponding amount of IκBα in cytosolic extracts was determined by Western blot analysis. Ponceau staining was used as an internal standard. (B) The intensity of the bands was determined by laser densitometry, and the corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control. (C) Supershift analysis of nuclear extracts from hepatocytes treated with 50 μM CsA. Antibodies against p50 (α-p50) and p65 (α-p65) subunits were used. The symbols (▶) and (▷) denote supershifted complex after incubation with α-p65 and supershifted complex after incubation with α-p50, respectively.

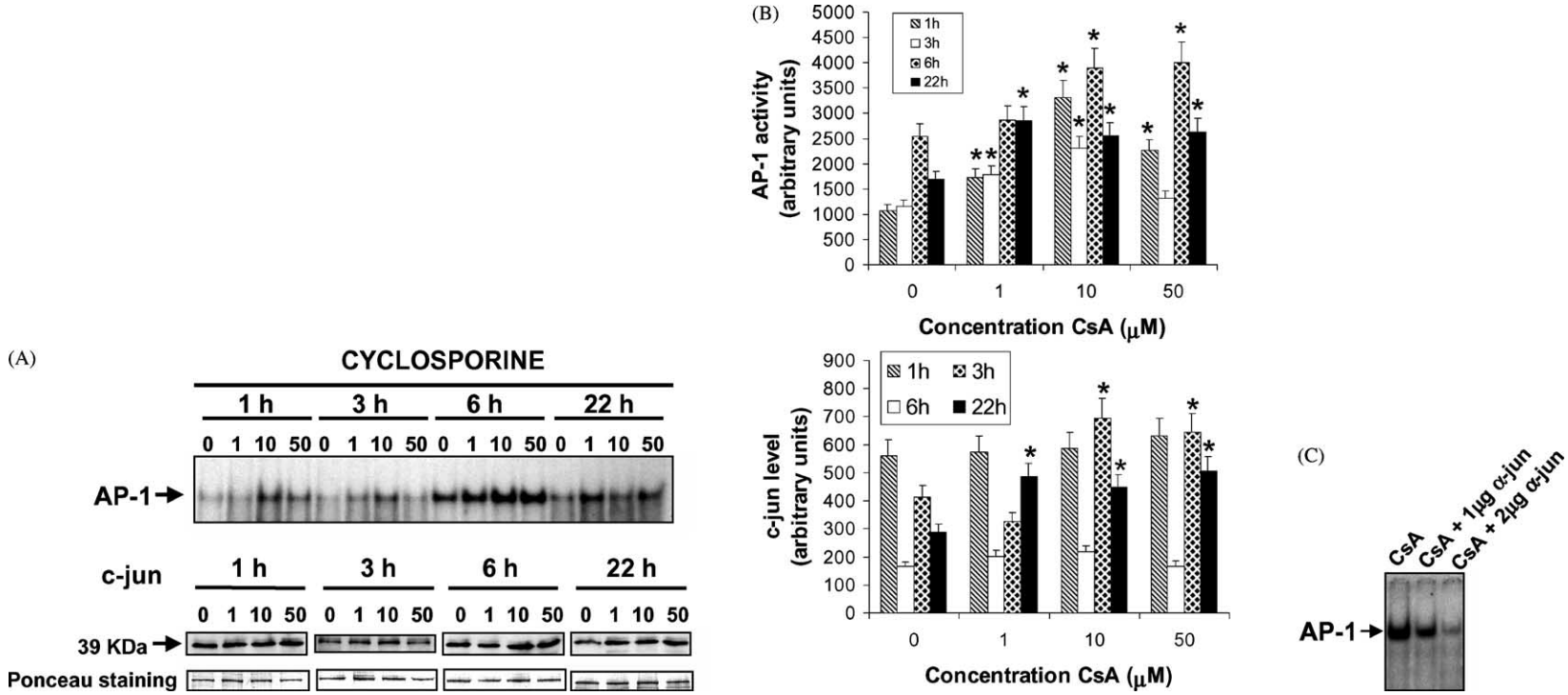

Fig. 2. Time-course of the AP-1 complex DNA binding in rat hepatocytes treated with CsA. (A) Hepatocytes were incubated with 0–50 μM CsA for the indicated periods of time. Nuclear extracts were prepared as described under ''Section 2,'' and the binding of nuclear proteins was analyzed by EMSA using an oligonulceotide including the AP-1 site. The corresponding amount of c-jun in nuclear extracts was determined by Western blot analysis. Ponceau staining was used as an internal standard. (B) The intensity of the bands was determined by laser densitometry, and the corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control. (C) Supershift analysis of nuclear extracts from hepatocytes treated with CsA. Antibody against c-jun (α-jun) was used. α-jun is an antibody directed against the DNA binding domain of the c-jun subunit of AP-1.

3.2. Effects of CsA on AP-1 activation

Transcription factor AP-1 is an ubiquitous regulatory protein complex that interacts with AP-1 binding sites of target genes. AP-1 is composed of protein products of members of *fos* and *jun* proto-oncogene families, which form homodimeric (jun–jun) or heterodimeric (fos–jun) complexes. In Fig. 2(A) are shown the electrophoretic mobility shift assay of AP-1 and the Western blot analysis of c-jun from nuclear extracts of hepatocytes treated with CsA at different times and concentrations and incubation periods. It can be observed that CsA induced the activation of AP-1 at all periods of incubation maintaining the binding activity of the transcription factor from 1 to 22 hr. However this increase in AP-1 binding activity was not dose-dependent. Western blot analysis indicates that c-jun levels did not undergo significant variations in hepatocytes incubated with CsA for 1 and 3 hr. However, significant increases in c-jun levels were observed in those hepatocytes treated with CsA for 6 and 22 hr.

In Fig. 2(C) is shown the supershift assay for AP-1. The antibody used was α-jun that is an antibody directed against the DNA binding domain of the c-jun subunit of AP-1. The addition of this antibody resulted in a marked reduction of the binding in the EMSA.

3.3. Effects of CsA on heat shock response

To assess the effects of CsA on HSF1, electrophoretic mobility shift assay was performed with HSF1 oligonucleotide and hepatocyte nuclear extracts. In Fig 3(A) are shown the dose and time responses of CsA-induced activation of HSF1 in hepatocytes. A dose-dependent activation of HSF1 was observed between 1 and 50 μM of CsA at 22 hr of incubation. However, when hepatocytes were incubated for 6 hr, a slight but not significant increase in HSF1–HSE binding at 50 μM CsA was observed. No significant variations were detected at 3 hr of treatment at any of the concentrations of CsA assayed.

To check and confirm the factor responsible for the HSE-binding activity, nuclear extracts from hepatocytes treated with 50 μM CsA at 22 hr were subjected to supershift assay with antibody targeted against HSF1 (α-HSF1). The gel supershift data indicates the activation of HSF1 in CsA-treated cells (Fig. 3(C)). This was confirmed by Western blot analysis of nuclear extracts using α-HSF1 (Fig. 3(B)).

Once the activation of HSF1 in the presence of CsA was confirmed, Northern blot analysis of HSP70 was performed as described in Section 2. Fig. 4 shows the time-course of HSP70 mRNA level measured in cell cultures incubated for 3, 6, and 22 hr at 0, 1, 10, and 50 μM CsA, by Northern blot analysis. The increase in HSP70 mRNA level was appreciated at 22 hr of incubation with 10 and 50 μM of CsA, while no differences were detected in those hepatocytes treated for 3 and 6 hr with CsA. These results confirm those related to HSF1 activation, so that the HSF1 binding rise was followed by the increase of HSP70 mRNA.

After treatment with CsA, hepatocytes were lysed and whole cell extracts were analyzed by Western blot analysis

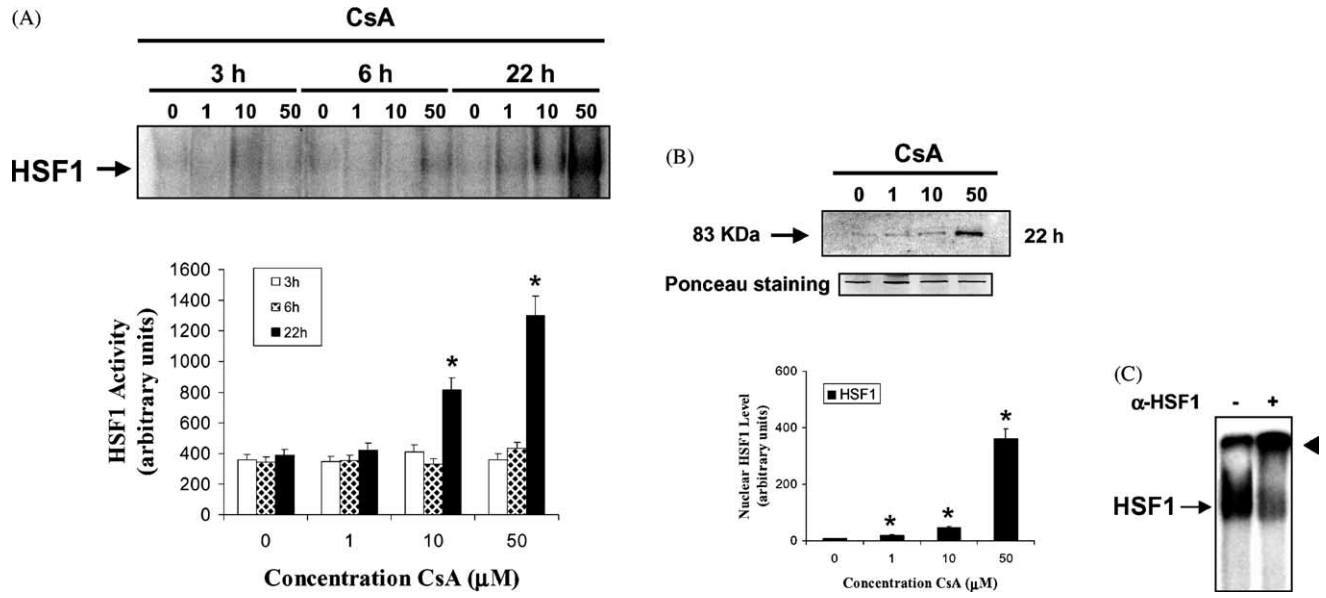

Fig. 3. Time-course of the HSF1 complex DNA binding in rat hepatocytes treated with CsA. (A) Hepatocytes were incubated with 0–50 μM CsA for the indicated periods of time. Nuclear extracts were prepared as described under "Section 2," and the binding of nuclear proteins to the HSE motif of the HSP70 promoter was determined by EMSA. (B) The corresponding amount of HSF1 in nuclear extracts was determined by Western blot analysis. Ponceau staining was used as an internal standard. The intensity of the bands was determined by laser densitometry, and the corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (*) $P < 0.05$ values against their control. (C) Supershift analysis of nuclear extracts from hepatocytes treated with CsA. Antibody against HSF1 (α-HSF1) was used. The symbol (◀) denotes supershifted complex after incubation with α-HSF1.

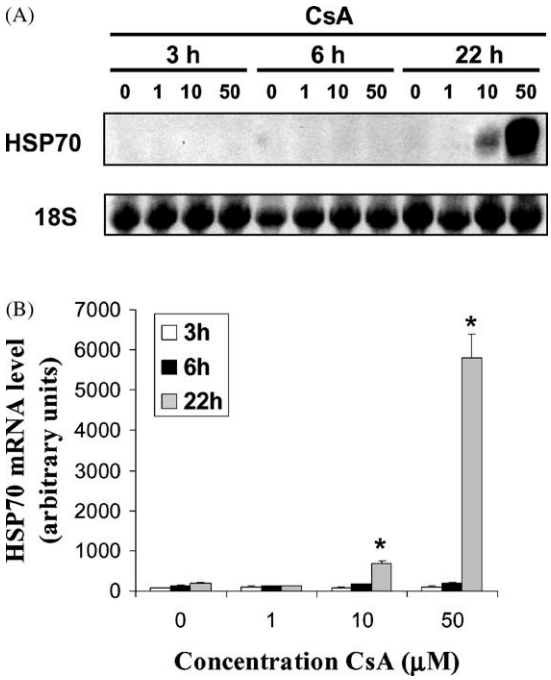

Fig. 4. Effect of CsA on gene expression of HSP70 in primary cultures of rat hepatocytes. Northern blot analysis of HSP70 mRNA. RNA was isolated and analyzed by Northern blotting using radiolabeled HSP70 cDNA. Panel (A) shows representative Northern blots with 18S rRNA probe for RNA normalization. Panel (B) shows the quantification in arbitrary units after correction with 18S rRNA. The intensity of the bands was determined by laser densitometry, and the corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control.

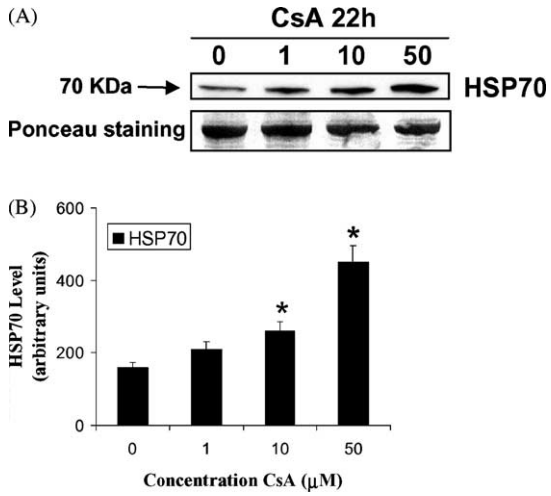

Fig. 5. HSP70 protein level in rat hepatocytes incubated with CsA. Following incubation with CsA for 22 hr, HSP70 protein levels were assayed by Western blot analysis. In panel (A), the signals after enhanced chemiluminescence detection can be observed. Ponceau staining was used as an internal standard. Panel (B) shows the quantification of chemiluminescence signals in panel (A) by laser densitometry expressed as arbitrary units. The corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control.

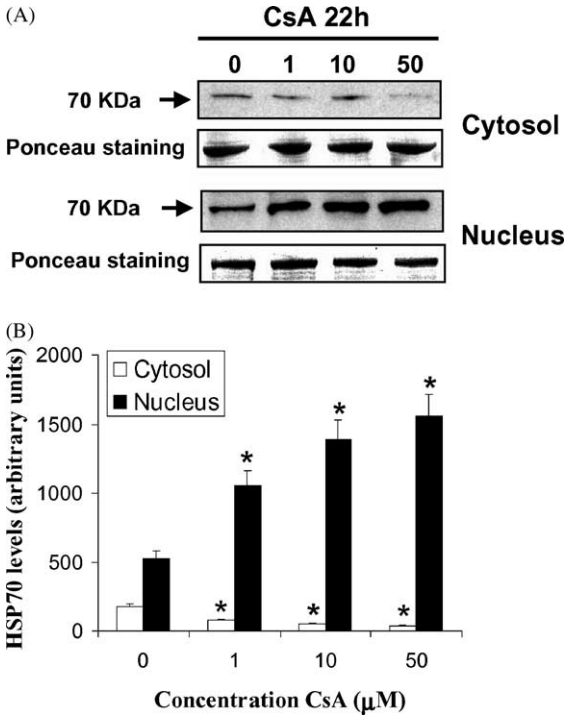

Fig. 6. Cytosolic and nuclear HSP70 protein level in rat hepatocytes incubated with CsA. Following incubation with CsA for 22 hr, HSP70 protein levels in cytosol and nucleus were assayed by Western blot analysis. In panel (A), signals after enhanced chemiluminescence detection can be observed. Ponceau staining was used as an internal standard. Panel (B) shows the quantification of chemiluminescence signals in panel (A) by laser densitometry expressed as arbitrary units. The corresponding values are expressed as means ± SD of three experiments. Data were compared by using a two-way ANOVA test. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. Differences were considered significant at (∗) $P < 0.05$ values against their control.

using anti-HSP70 antibody (Fig. 5). This assay was performed only at 22 hr since it was the incubation period where HSF1 activity and HSP70 mRNA level underwent the highest differences vs. control. In consonance with the results obtained by EMSA and Northern blot, the HSP70 protein level experimented a significant rise when cells were incubated for 22 hr with 10 and 50 μM CsA. Finally, in the experiments represented in Fig. 6, the translocation of HSP70 from cytosol into the nucleus at 22 hr was tested by Western blot analysis. These results showed that HSP70 translocated into the nucleus in a dose-dependent manner reaching at 50 μM CsA the highest level in the nucleus and the lowest level in the cytosol.

4. Discussion

For the last few years, CsA, as the main immunosuppressive drug used in clinic, has been the subject of intense study to elucidate the mechanisms involved in its toxicity and in its multiple pharmacological effects. Investigations of our group have been focused on the mechanisms implicated in CsA toxicity at hepatic level, reporting an impor-

tant role for oxidative stress in this toxicity [37,38]. Recently, we have reported that the increased peroxide levels are responsible for the CsA-induced HSP70 induction in cultured rat hepatocytes [28], and that CsA exerts a proliferative effect where NF-κB activation seems to be involved [21]. Nowadays, there are controversial studies about the role of CsA in the activation of NF-κB, since it depends on the cellular system and the experimental conditions [22–24]. Thus, in an effort to elucidate this controversy we have studied the role of CsA on the time-course activation of HSF1, NF-κB, and AP-1, three important transcription factors involved in stress situations.

Previous studies have reported interactions between the transcription factors HSF1 and NF-κB, which play opposite roles in cytoprotection and cell injury [25,39–42]. However, several authors have established the existence of a nexus between both transcription factors so that a single stimulus promotes the activation of both [43,44]. It is known that oxidative stress is a situation capable of inducing the activation of transcription factors such as NF-κB [45–48], HSF1 [49,50], and AP-1 [51,52]. Moreover, it has even been proposed that NF-κB could act as an oxidative stress sensor [53], and that antioxidants exert inhibitory effect on NF-κB activation [54].

On the basis of these evidences, in the present study we try to find a link between the different changes induced by CsA in the expression/activity of the transcription factors assayed.

Our results show that, in primary cultures of rat hepatocytes, CsA induced the activation and translocation of NF-κB into the nucleus with the resulting increase in gene expression. Previous studies of other authors [38] show that CsA induces reactive oxygen species production at 2 hr of incubation, thus, it might seem that the activation of NF-κB was owing to the oxidative stress situation generated by CsA, but in our previous studies [28] we proved that the increase in peroxide levels begins at 18 hr of incubation, reaching its maximum at 24 hr. The discrepancy between our and their results [38] can be explained by the different experimental procedures used. Thus, our results show that NF-κB activation shown at 1 and 3 hr of incubation can not be the result of the oxidative stress, and it seems that the proliferative effect of CsA [55] is responsible for NF-κB activation at these times [21]. Moreover, when the stress situation is maximum (around 22 hr with 50 μM CsA) [28], the positive effect of CsA on NF-κB activation disappears. However, at these periods of incubation, when the oxidative stress is maximum [28], the stress response takes place, indicating that the reactive oxygen species are involved in HSF1 activation. Thus, we propose that the activation of HSF1 induced by CsA should be linked to the suppression of CsA-induced activation of NF-κB. These data agree with other studies in different cellular systems and different stimuli [25,41], which explain the apparent and existing contradiction about the role of CsA in the activation or inhibition of NF-κB; thus, the initial activation of NF-κB is suppressed when HSF1 is activated. Although the precise mechanism by which the heat shock response interferes with NF-κB activation is unknown, it has been hypothesized that, due to their chaperone function, HSPs could bind to either the NF-κB subunits, to one or more of the kinases involved in IκB phosphorylation, or to IκB itself, thus blocking its degradation and the NF-κB translocation into the nucleus [39]. In order to clarify the role of reactive oxygen species in NF-κB activation we performed experiments with Vitamin E and no changes in NF-κB activity were detected at 1 and 3 hr of incubation with CsA (data not shown).

These theories and the present data about HSP70 translocation allow us to propose that HSP70 could bind to NF-κB in the nucleus preventing NF-κB binding to DNA. Nevertheless, the cytosolic IκBα levels are increased, so it is also possible that the interference with NF-κB activation takes place upstream (inhibition of IκB kinases, inhibition of IκBα degradation by proteosome, or IκBα induction [40]). Moreover, the interference of HSF1 activity with NF-κB binding activity suggests a link between the induction of heat shock response and the suppression of NF-κB-DNA binding *via* induction of IκB, a putative novel HSP, which can be the nexus between the heat shock response and the inflammatory response [40].

The relatively high NF-κB binding activity in control cultures at short incubation times is possibly due to the classic hepatocyte isolation procedure that induces a rapid activation of NF-κB [56]; and the NF-κB activity, shown in control cultures at 22 hr, could be the result of the persistent activation of this transcription factor that takes place in cultured rat hepatic cells [57].

Another transcription factor that can be activated in stress situations and by CsA is the AP-1 [5]. AP-1 is involved in TGFβ1 expression, the cytokine responsible for some of the adverse effects of CsA [10,11,55]. Our present results show that CsA rapidly induces the activation of AP-1; and in contrast to NF-κB and HSF1, this activity remains increased from 1–22 hr of incubation. In previous studies we have demonstrated that CsA induces hepatocyte proliferation at short incubation periods (3 and 6 hr) where NF-κB activation is involved [21]. At these time points of incubation, CsA exerted a positive effect on NF-κB transcriptional activity; however, at 22 hr, hepatocytes undergo a cell cycle arrest accompanied by a suppression of NF-κB activity. With the results shown in this and in our previous studies, we propose that NF-κB inhibition and the consequent cell cycle arrest are due to the activation of HSF1. As we have shown, HSF1 inhibits the activation of NF-κB, but it does not interfere with the AP-1 activation. Moreover, in agreement with our results, several studies have established that heat shock provokes JNK activation [58], c-jun expression [59], and the activation of AP-1 [60].

In conclusion, in primary cultures of rat hepatocytes, CsA induces the activation of NF-κB, AP-1, and HSF1, but

in a different time-course. Thus, at shorter incubation periods NF-κB is activated; at 22 hr HSF1 is activated and NF-κB is suppressed; while AP-1 activation is maintained at all periods assayed. It is suggested that the activation of AP-1 and HSF1 at 22 hr is due to the oxidative stress generated by CsA, while NF-κB activation at shorter times is possibly due to the growth-promoting effect of CsA. These data open a new pathway to a better understanding of the multiple effects of CsA, since NF-κB, AP-1, and HSF1 control a great variety of processes in the cell.

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