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Induction of cell proliferation by cyclosporine A in primary cultures of rat hepatocytes

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Abstract

Cyclosporine A (CsA) has been reported to be able to promote cell proliferation, although the precise mechanism by which CsA stimulates cell growth remains uncertain. In the present study, we examined, in hepatocyte cultures, the effect of CsA on parameters related to the cell cycle as well as the levels of proteins involved in the control and progression of the cycle. Flow cytometry analysis detected an increase in the percentage of cells involved in the S phase of the cycle, which correlated with increases in the levels of cyclins D1 and E (two G_1-progression regulators), as well as in those of PCNA (proliferating cell nuclear antigen), and without modification in p27, an inhibitory protein of CDKs. We also examined in nucleus the levels of nuclear factor κB (a nuclear factor involved in the transcription of the cyclin D1 gene) and found that this transcription factor increased in the presence of CsA. We conclude that the increases in cyclin D1, PCNA, and cyclin E, together with the invariable level of p27, clearly show that CsA induces hepatocytes to proliferate. These results reinforce the idea of the growth-promoting effect of CsA in cultured hepatocytes.

Keywords: Cyclosporine; Hepatocyte; Proliferation; Cyclins; PCNA; p27

1. Introduction

CsA is a potent immunosuppressant widely used to prevent rejections after organ transplantation [1]. In clinical transplantation, it is known that the recipients under immunosuppression are liable to a higher incidence of development of malignant disease [2]. Moreover, the growth of recurrent tumor is markedly accelerated after liver transplantation for hepatic malignancies in recipients treated with CsA immunosuppression [3].

Several investigators have reported that CsA stimulates cell proliferation [4–6] and the regeneration response of hepatocytes in rats and mice after partial hepatectomy [7,8]. Recently, Hojo *et al*. [9] showed that CsA induces cancer progression in different cell lines by a cell-autonomous mechanism (phenotypic changes, including invasiveness of non-transformed cells where CsA-induced TGF-β [transforming growth factor-β] production is involved). However, the precise mechanisms by which CsA stimulates growth of carcinogen-initiated hepatocytes remain uncertain.

Progression around the cell cycle is governed by a family of CDKs and their regulatory subunits the cyclins [10]. As cells enter the cell cycle from G_0, cyclins D and E are synthesized sequentially and both are rate-limiting for S phase entry. A key role of CDKs is to inactivate, by phosphorylation, negative regulators of progression, notably pRb, to permit exit from G_1 and entry into S phase. Cyclins D bind pRb directly, with pRb being the critical substrate of CDK4 and CDK6, although cyclin E/CDK2 also phosphorylate pRb. Phosphorylation of pRb relieves its inhibitory effect on the transactivation function of E2F family transcription factors that are required for S phase. Contributing to the proliferative quiescence is a high level of p27, a cyclin-dependent kinase inhibitor that regulates cell number and size by blocking initiation of a G_1 buildup by binding to

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Abbreviations: CsA, cyclosporine A; CDK, cyclin-dependent kinase; ECL, enhanced chemiluminescence; IκB-α, inhibitory protein kappa B; LDH, lactate dehydrogenase; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; and pRb, retinoblastoma protein.

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G_1-specific CDKs [11]. The disappearance of p27 clears the way for the parade of cyclins and the assembly of the stage-specific CDK2/4–cyclins that come and go to drive the cell through the G_1 buildup into, then through, the S phase.

PCNA is a protein that functions as an auxiliary protein of DNA polymerase-δ [12]. PCNA is expressed beginning in late G_1, with a 2- to 3-fold increase in S and a decrease at the S/G_2 transition during G_2+M. Afterwards, it is degraded with a half-life at 20 hr [13].

A role of the NF-κB gene products has been proposed in cell proliferation, transformation, and tumor development [14,15], since NF-κB controls the expression of a number of growth-promoting cytokines. In fact, a nuclear NF-κB-like DNA-binding activity is induced during G_0/G_1 transition following serum stimulation in mouse fibroblasts and in regenerating liver [16–18]. Moreover, the NF-κB transactivation potential appears to be linked to signaling pathways that control cell cycle progression [19]. Recently, Hinz *et al*. [20] reported that NF-κB transmits growth signals directly to key regulators of the cell cycle, since NF-κB activates transcription of the cyclin D1 promoter primarily through a proximal binding site. The NF-κB binding sites that were identified are required for induction of cyclin D1 transcription.

In order to elucidate a potential proliferative action of CsA *in vitro*, in the present study we examined whether CsA alters the hepatocyte cell cycle, in particular the levels of proteins involved in the control and progression of the cell cycle such as PCNA, p27, cyclin D1, cyclin E, and those related to NF-κB activation and nuclear translocation.

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. Kinesis 50 and Kinesis-PCNA were from Bio-Rad. Anti-p27, anti-cyclin E, anti-cyclin D1, anti-PCNA, anti-NF-κB p65, and anti-IκB-α antibodies were from Santa Cruz Biotechnology. Cyclosporine A was provided by Dr. Armin Wolf, Novartis.

2.2. Animals

Male Wistar rats aged 2 months, with an average body weight of 180–230 g, were used for the cell preparations. All animals received care as outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health. Rats were supplied with food and water *ad lib*. and exposed to a 12-hr light–dark cycle.

2.3. Isolation and culture of hepatocytes

Hepatocytes were isolated by liver perfusion with collagenase as described elsewhere [21,22] 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 Dulbecco's modified Eagle's medium (DMEM), supplemented by 100 IU/mL of penicillin, 50 μg/mL of streptomycin, 50 μg/mL of gentamicin, and 10% fetal bovine serum (FBS). 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% FBS containing CsA. Hepatocytes were exposed to the drug at a dose range of 0–50 μM for 1, 3, 6, and 22 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%.

2.4. Measurement of cytotoxicity by LDH leakage

Extracellular LDH activity (LDH, EC 1.1.1.27) was determined spectrophotometrically, following Vasault [23], as a parameter of plasma membrane damage and an indicator of irreversible cell damage [24]. Enzyme activity was expressed as the extracellular LDH activity percentage of the total activity on the plates. The cellular activity in scraped-off cells was measured after three 5-sec periods of ultrasonification.

2.5. Analysis of cell cycle and detection of PCNA by flow cytometry

For the analysis of DNA content, 1×10^6 cells were stained with propidium iodide (PI) following the multistep procedure of Vindelov *et al*. [25]. The emitted fluorescence of the DNA–PI complex was analyzed in a FACScan flow cytometer (Becton Dickinson) in the FL2-A channel. A double discriminator module was used to distinguish between signals coming from a single nucleus and those products of nuclear aggregation. Data analysis was carried out by means of evaluation of single inputs (10^4 nuclei/assay) and was expressed as percent of DNA distribution in the cell cycle phases G_0/G_1(2C), S_1, G_2+M (4C), S_2, $(G_2+M)_2$(8C), and hypodiploid peak (< 2C). PCNA detection was carried out using the Kinesis-PCNA kit (Bio-Rad) by flow cytometry.

2.6. Immunoblotting for detection of cyclin D1, cyclin E, p27, PCNA, NF-κB p65, and IκB-α proteins

For the preparation of cytosolic and nuclear extracts, protein extracts were prepared following the method of Schreiber *et al*. [26] described previously. All steps of cell fractionation were carried out at 4°. For the 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 (PMSF), 40 μg/mL of aprotinin, and 4 μg/mL of leupeptin.

Protein concentrations were determined using 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. Aliquots containing equal amounts of protein (20 μg) were loaded onto a precast ready gel Tris–HCl (Bio-Rad). Proteins were separated electrophoretically and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Life Science) using the Bio-Rad Electrophoretic Transfer Cell. For immunoblotting, membranes were blocked with 10% non-fat dried milk in T(Tween)PBS for 2 hr. The primary antibodies used were rabbit polyclonal antibodies against cyclin D1 (no. sc-718), PCNA (no. sc-7907), cyclin E (no. sc-481), p27 (no. sc-528), NF-κB p65 (no. sc-109), and IκB-α (no. sc-371) (all from Santa Cruz Biotechnology). After washing, appropriate secondary antibody (anti-rabbit immunoglobulin G (IgG) peroxidase-conjugated from Santa Cruz) was applied for 1 hr. Blots were washed, incubated in commercial ECL reagents (Amersham), and exposed to chemiluminescence film. Quantification of the films was performed by a laser densitometer (Molecular Dynamics).

2.7. Statistical analysis

The results were reported as means ± SD of four experimental observations (four animals). Data were compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ against its corresponding control.

3. Results

3.1. LDH release

Primary hepatocyte cultures were exposed to increasing concentrations of CsA from 0 to 50 μM for 3, 6, and 22 hr, and LDH leakage was measured as an index of cell toxicity. Fig. 1 shows that the cytotoxic effect of CsA was dose- and time-dependent. No cytotoxic effect was observed at 3 and 6 hr, but at 22 hr, 10- and 50-μM CsA concentrations caused significant increases versus control, reflecting a loss of plasma membrane integrity associated with necrosis.

3.2. Analysis of cell cycle and PCNA by flow cytometry

Table 1 shows the percentages of cell cycle populations related to ploidy and DNA content at different times of incubation. At 3 and 6 hr, the percentage of cells in S_1 phase increased in parallel to CsA concentration, reaching, at 50 μM, almost three times (299% and 267%) the control value (3 and 6 hr, respectively). No significant differences were found in diploid, tetraploid, octoploid, hipodiploid, and S_2 populations. At 22 hr, the percentage of cells in S_1 phase did not undergo any variation versus control, but did decrease at 10 and 50 μM versus these concentrations at 3 and 6 hr. The percentage of hypodiploid (< 2C) peak at 22 hr increased significantly from control to 50 μM CsA.

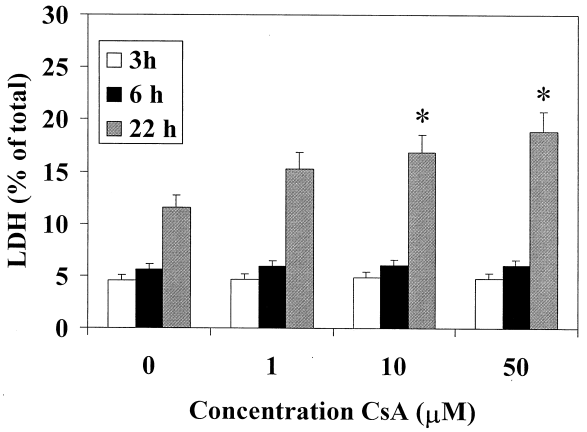

Fig. 1. Time–course of cytotoxic effects of CsA (0–50 μM) in primary cultures of rat hepatocytes. Hepatocytes were incubated with CsA (0–50 μM) for 3, 6, and 22 hr. LDH leakage was measured as a cytotoxicity index. Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

Fig. 2 shows the percentage of diploid cells (2C) at 22 hr that did not express the PCNA (PCNA−), which indicates that cells are in S phase or have been passed through it (since PCNA has a half-life of 20 hr). At the highest concentration of CsA assayed (50 μM), this percentage showed a significant decrease, to 26% of control. Fig. 3 shows, also at 22 hr of incubation, the percentage of hypodiploid cells (< 2C) that expressed PCNA (PCNA+). At 50 μM, a significant increase, versus control, was detected. When comparing the values of hypodiploid cells in Table 1 (5.7 ± 0.43) and the values of hypodiploid PCNA+ cells at 50 μM and 22 hr (Fig. 3), almost 50% of total hypodiploid cells were PCNA+.

3.3. Western blot analysis of PCNA, cyclin D1, IκB-α, NF-κB p65, cyclin E, and p27

Fig. 4 shows the Western blot (Fig. 4A) and the quantification of signals (Fig. 4B) corresponding to PCNA. PCNA is a protein that functions as an auxiliary protein of DNA polymerase-δ and enhances DNA replication. A significant increase can be observed in the level of this protein at 3 and 6 hr of incubation with 50 μM CsA, reaching 373% and 207% versus control, respectively. No significant differences were found at 22 hr of incubation at any CsA concentration.

Table 1
Quantitative analysis of DNA ploidy in hepatocytes incubated with CsA

		Cell cycle population (% of total) 3 hr					
		< 2C	2C	S_1	4C	S_2	8C
Concentration CsA (μM)	0	0.4 ± 0.04	46.6 ± 4.5	1.8 ± 0.20	49.8 ± 5.1	1.1 ± 0.09	0.2 ± 0.10
	1	0.7 ± 0.06	47.6 ± 5.0	1.3 ± 0.11	49.2 ± 4.8	0.8 ± 0.10	0.2 ± 0.09
	10	0.5 ± 0.04	43.9 ± 4.8	3.5 ± 0.33*	50.7 ± 5.2	1.3 ± 0.14	0.2 ± 0.07
	50	0.1 ± 0.01	44.4 ± 4.0	5.4 ± 0.58*	49.2 ± 4.8	0.6 ± 0.08	0.5 ± 0.11
		Cell cycle population (% of total) 6 hr					
		< 2C	2C	S_1	4C	S_2	8C
Concentration CsA (μM)	0	1.3 ± 0.11	47.4 ± 5.1	1.8 ± 0.20	48.5 ± 4.3	0.8 ± 0.06	0.3 ± 0.04
	1	1.2 ± 0.10	48.4 ± 4.6	1.9 ± 0.19	47.5 ± 5.0	0.6 ± 0.07	0.2 ± 0.01
	10	1.4 ± 0.15	47.1 ± 4.9	4.8 ± 0.52*	45.7 ± 4.6	0.5 ± 0.04	0.5 ± 0.03
	50	1.7 ± 0.20	44.7 ± 4.3	4.8 ± 0.50*	47.9 ± 5.1	0.7 ± 0.10	0.3 ± 0.02
		Cell cycle population (% of total) 22 hr					
		< 2C	2C	S_1	4C	S_2	8C
Concentration CsA (μM)	0	2.4 ± 0.26	50.2 ± 5.6	1.6 ± 0.18	43.5 ± 4.9	1.8 ± 0.21	0.5 ± 0.04
	1	2.2 ± 0.19	48.3 ± 5.3	2.1 ± 0.19	46.2 ± 4.3	1.0 ± 0.13	0.2 ± 0.04
	10	2.0 ± 0.23	45.4 ± 4.2	1.5 ± 0.20	49.2 ± 5.2	1.6 ± 0.19	0.3 ± 0.03
	50	5.7 ± 0.43*	48.7 ± 5.2	1.5 ± 0.16	41.8 ± 4.6	1.6 ± 0.17	0.6 ± 0.07

The values are expressed as the percentage of DNA in: hypodiploid population (<2C), G_0/G_1 diploid population (2C), S_1 population (2C → 4C), G_2+M tetraploid population (4C), S_2 population (4C → 8C) and $(G_2+M)_2$ octoploid population (8C). Data are reported as the mean ± SD of four different observations (four animals) and compared by using a Student's *t*-test.

* Differences were considered significant at a value of $P < 0.05$ against its corresponding control.

In Fig. 5 is shown the immunoblotting of cyclin D1 (Fig. 5A) and its quantification (Fig. 5B). Cyclin D1 forms complexes with CDK4 and CDK6 that are implicated in the phosphorylation of pRb. Large differences were found in cyclin D1 levels when hepatocytes were incubated for 3 hr with 10 and 50 μM CsA (240% and 439% increase versus control, respectively) and for 6 hr with 10 μM CsA (an increase of 275% versus control value).

Fig. 6 shows a representative Western blot (Fig. 6A) corresponding to IκB-α and the quantification of signals (Fig. 6B). IκB-α is an inhibitory protein that blocks the translocation of NF-κB from the cytosol into the nucleus. The disappearance of IκB-α in cytosol by proteolysis allows the translocation of NF-κB into the nucleus. In Fig. 6, decreases in the IκB-α level can be observed at 1 and 3 hr of incubation with CsA, reaching significant differences at

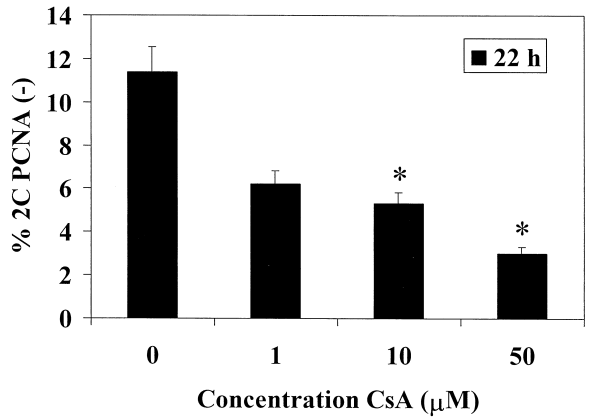

Fig. 2. Percentage of diploid cells that did not express PCNA (2C PCNA−). Following incubation with CsA for 22 hr, DNA content and PCNA detection were carried out by flow cytometry using the Kinesis 50 and Kinesis-PCNA kits (Bio-Rad). Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

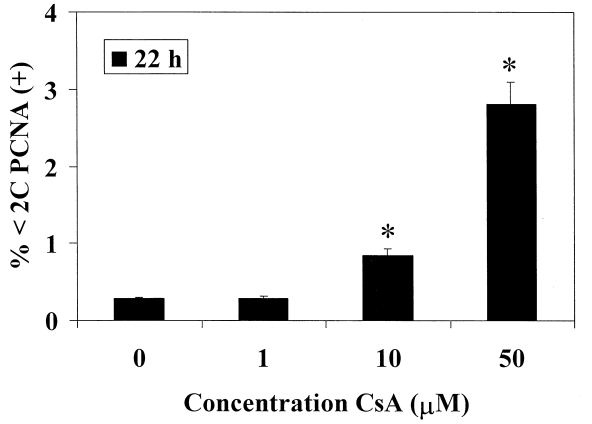

Fig. 3. Percentage of apoptotic cells that expressed PCNA (< 2C PCNA+). Following incubation with CsA for 22 hr, DNA content and PCNA detection were carried out by flow cytometry using the Kinesis 50 and Kinesis-PCNA kits (Bio-Rad). Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

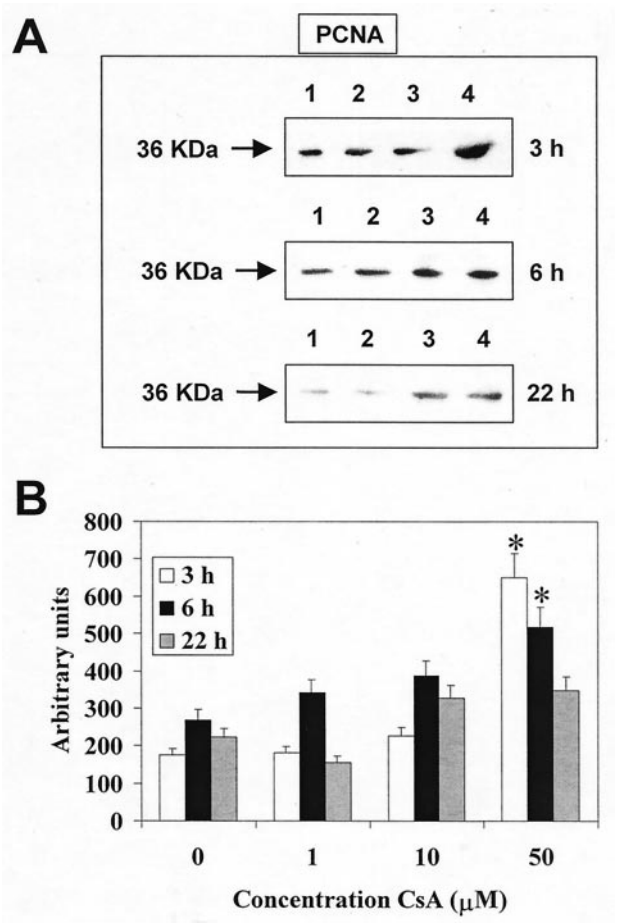

Fig. 4. Time–course of PCNA levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated (3, 6, and 22 hr), PCNA protein levels were analyzed in whole cell lysates by Western blot. (a) The signals after ECL detection are shown (lanes 1–4 refer to 0, 1, 10, and 50 μM CsA, respectively). (b) This panel shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

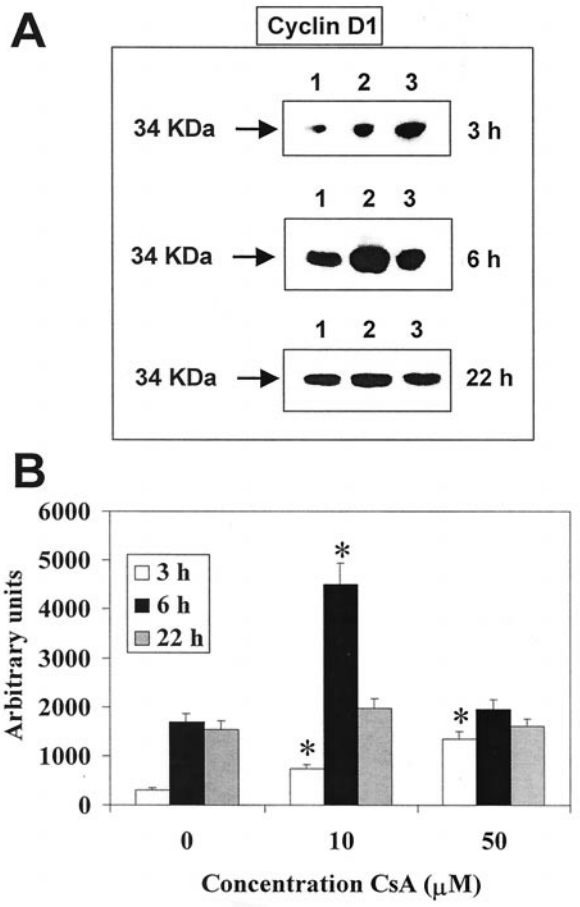

Fig. 5. Time–course of cyclin D1 levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated, i.e. 3, 6, and 22 hr, cyclin D1 protein levels were analyzed in whole cell lysates by Western blot. (a) The signals after ECL detection are presented (lanes 1, 2, and 3 refer to 0, 10, and 50 μM CsA, respectively). Fig. 5b shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

the concentrations of 10 and 50 μM at both times of incubation.

NF-κB is involved in the activation of the transcription of the cyclin D1 promoter. The levels of NF-κB p65 (the subunit that contains the transactivation domain) in the nucleus were determined by Western blot analysis and are shown in Fig. 7A. The quantification of signals is shown in Fig. 7B, as well as the augmentation of NF-κB p65 at 1 and 3 hr of incubation with all concentrations of CsA. These increases were significant at all concentrations assayed and demonstrate the translocation of NF-κB into the nucleus.

Cyclin E plays a key role in activating the G_1/S transition. Cyclin E is periodically expressed at the end of G_1 and forms complexes preferentially with CDK2. Cyclin E/CDK2 complexes are involved in maintaining the phosphorylation state of pRb. Fig. 8 shows a representative Western blot (Fig. 8A) and the quantification of signals (Fig. 8B) corresponding to cyclin E. Significant increases (445% and 212% versus its corresponding control) were found at 3 and 6 hr, respectively, of incubation with 10 μM CsA. At these incubation times (3 and 6 hr) and with 50 μM, the increases reached 330% and 300% versus control, respectively. No significant changes were observed at 22 hr of incubation.

p27 is a cyclin-dependent kinase inhibitor that regulates cell number and size by blocking initiation of a G_1 buildup by binding to G_1-specific cyclin-dependent protein kinases. Fig. 9 shows the immunoblotting detection (Fig. 9A) and the signal quantification (Fig. 9B) of p27. No significant changes were observed at 3 hr of incubation. At 6 hr, only the concentration of 10 μM showed an increase of 160% versus control. However, at 22 hr, the levels of p27 rose to 182% and 170% versus control with 10 and 50 μM, respectively.

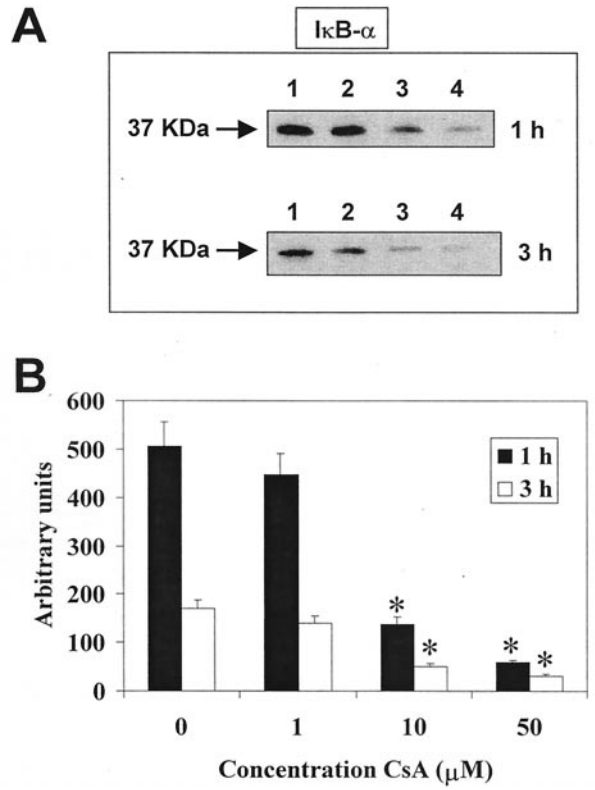

Fig. 6. Time–course of IκB-α levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated, i.e. 1 and 3 hr, IκB-α protein levels were analyzed in cytosolic extracts by Western blot. In Fig. 6a can be observed the signals after ECL detection (lanes 1, 2, 3, and 4 refer to 0, 1, 10, and 50 μM CsA, respectively). (b) This panel shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

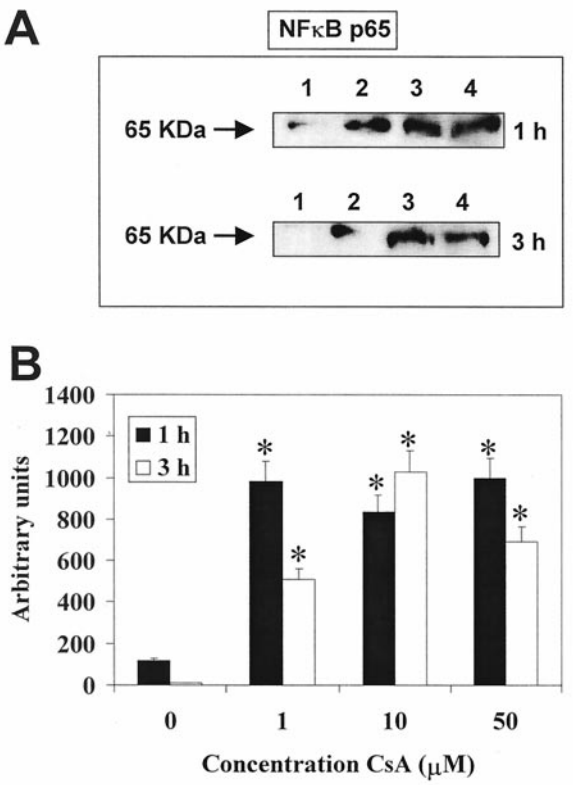

Fig. 7. Time–course of NF-κB p65 levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated, i.e. 1 and 3 hr, NF-κB p65 protein levels were analyzed in nuclear extracts by Western blot. (a) The signals after ECL detection are shown (lanes 1, 2, 3, and 4 refer to 0, 1, 10, and 50 μM CsA, respectively). (b) This panel shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means ± SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

4. Discussion

Several previous studies have shown that CsA stimulates cell proliferation [4–6] and have described the regeneration response of hepatocytes in rats and mice after partial hepatectomy [7,8]. It has also been reported that liver growth is stimulated by intraportal infusion of CsA in dogs [27]. However, the precise mechanisms by which CsA stimulates growth of hepatocytes are still unclear. The results of the present study provide evidence that CsA *in vitro* induced an increase in the percentage of cells in S_1 phase at 3 and 6 hr of incubation. However, this percentage returned to normal values when hepatocytes were incubated with CsA for 22 hr. In order to interpret these results, we decided to determine the levels of proteins involved in the control of cell cycle. We focused our study mainly on the control at G_1/S_1 transition, where proteins such as cyclin D1, cyclin E, and p27 are implicated. We also checked the level of PCNA, an important protein involved in DNA replication, since it is a subunit of DNA polimerase-δ and contributes to the enhancement of DNA replication.

Our data showed that CsA markedly increased the levels of PCNA at 3 and 6 hr of incubation, while at 22 hr the PCNA levels changed only slightly. However, at 22 hr, the percentage of cells that expressed PCNA augmented with the increase in CsA concentration. This is because PCNA has a half-life at 20 hr, which allows the detection of PCNA in cells that have passed through the S phase, and not only in cells that are in S phase. Thus, CsA induces PCNA expression in hepatocytes, indicating that these cells are in late G_1, in S phase, or have passed through it.

Cyclin D1 is involved in the regulation of several cyclin-dependent kinases: CDK4 and CDK6, when activated, phosphorylate the product of *Rb* gene. Phosphorylation of pRb leads to the release of E2Fs, a family of factors required to transactivate the transcription of genes whose products are necessary to promote DNA synthesis. In our study, we observed that CsA, when added to hepatocyte cultures incubated for short periods (3 and 6 hrs), produced a marked increase in the levels of cyclin D1, while no changes were detected at 22 hr of incubation. It has been proposed that the enhancement in cyclin D1 expression is sufficient to pro-

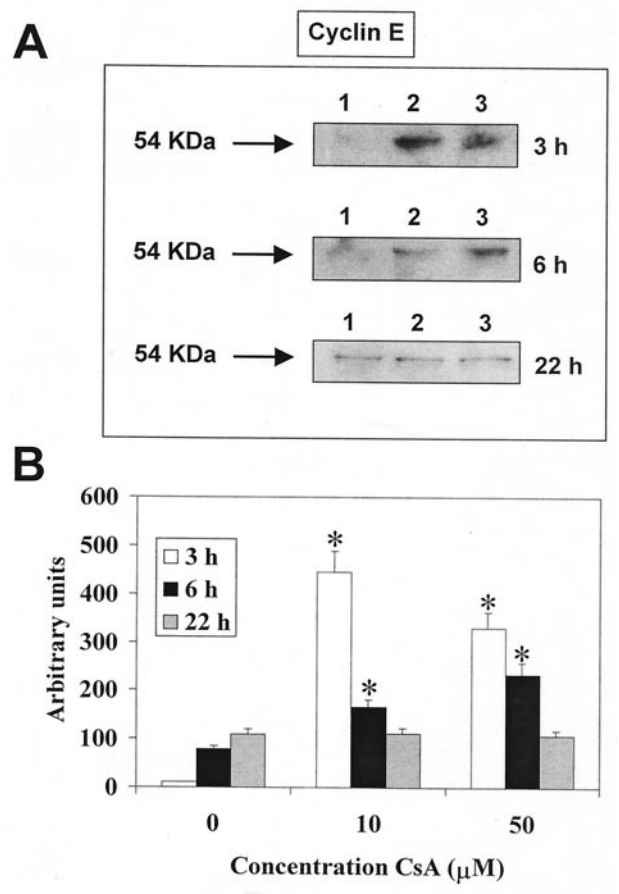

Fig. 8. Time–course of cyclin E levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated, i.e. 3, 6, and 22 hr, cyclin E protein levels were analyzed in nuclear extracts by Western blot. (a) One can observe the signals after ECL detection (lanes 1, 2, and 3 refer to 0, 10, and 50 μM CsA, respectively). Panel b shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means $\pm$ SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

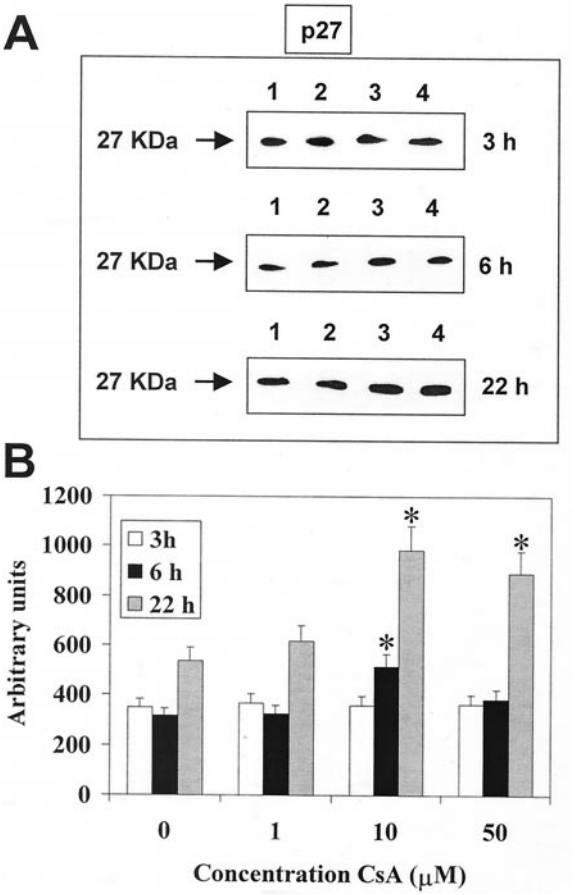

Fig. 9. Time–course of p27 levels in cultures of rat hepatocytes incubated with CsA. Following incubation with CsA at the times indicated, i.e. 3, 6, and 22 hr, p27 protein levels were analyzed in whole cell lysates by Western blot. Panel a shows the signals after ECL detection (lanes 1, 2, 3, and 4 refer to 0, 1, 10, and 50 μM CsA, respectively). Panel b shows the quantification of chemiluminescence signals in (a) by laser densitometry expressed as arbitrary units. Data are reported as the means $\pm$ SD of four different observations (four animals) and compared by using a Student's *t*-test. Differences were considered significant at a value of $P < 0.05$ (*) against its corresponding control.

mote progression of hepatocytes through the G_1 restriction point [28]. The increase in cyclin D1 observed in our experiments when hepatocytes were incubated with CsA for short time periods could explain the increase in the percentage of cells in S phase. Recently, Hinz *et al*. [20] reported that NF-κB transmits growth signals directly to key regulators of the cell cycle and that it activates the transcription of the cyclin D1 promoter primarily through a proximal binding site. The NF-κB binding sites which were identified are required for induction of cyclin D1 transcription. Thus, we decided to analyze, via Western blot, the levels of NF-κB in the nucleus, as well as the levels of IκB-α (inhibitory protein of NF-κB) in the cytosol. Our data revealed that incubation with CsA for 1 and 3 hr produced an IκB-α disappearance in the cytosol of hepatocytes, which would permit NF-κB to translocate to the nucleus. This translocation to the nucleus was also demonstrated in our experiments, since we showed that the levels of p65 (the NF-κB subunit that contains the transactivation domain) clearly increased inside the nucleus.

p27 is involved in the control of the restriction point and belongs to a family of CDK inhibitors. In quiescent cells, the levels of p27 are high, but once these cells enter the division cycle, p27 decreases sharply. However, it has been described that several kinds of cancer exhibit paradoxically high levels of p27 co-existing with high levels of cyclin D1, resulting in a high proliferating state [29]. Thus, it would be the balance of the two opposing signals, p27 and cyclins/CDKs, rather than the absolute level of the individual signals, which determines the proliferative status of the cell. If the levels of cyclin D1 are very high, p27 will not be able to counteract the activity of the CDK4/CDK6–cyclin D1 complex efficiently, thereby permitting the progress of the cell through the cycle. In our case, when hepatocytes were incubated for 3 and 6 hr, CsA apparently did not affect the levels of p27 (although these levels did increase at 6 hr and

10 μM CsA). In contrast, cyclin D1 levels increased markedly, indicating a noticeable superexpression of this protein that induced the cells to enter S phase. At 22 hr of incubation, the data obtained showed an increase in p27, while cyclin D1 decreased (when compared to the values obtained at 3 and 6 hr), producing an arrest in DNA replication. These data agree with the percentage of cell population in phase S that was significantly lowered at 22 hr of incubation when compared to cells incubated for 3 and 6 hr.

Cyclin E is periodically expressed at the end of G_1 and forms complexes preferentially with CDK2. While cyclins D are involved in establishing contacts between extracellular signals and cell cycle machinery, cyclin E seems to play a key role in activating G_1/S transition. Cyclin E/CDK2 complexes are involved in maintaining the phosphorylation state of pRb, this being initially triggered by CDK4 and CDK6/cyclin D complexes. Following 3 and 6 hr of hepatocyte incubation with CsA, cyclin E levels showed a marked increase which, together with that of PCNA and cyclin D1, permits the cell to enter DNA synthesis. However, at 22 hr of incubation, the levels of cyclin E were unchanged when compared to control. These data, together with the decrease in cyclin D1 and PCNA and the enhanced levels of p27, are likely to be responsible for the cell cycle arrest at 22 hr of incubation.

Moreover, the data indicating the arrest of cells at 22 hr of incubation with CsA may be explained by the oxidative stress produced by the effect of CsA in the culture following 22-hr incubation [30,31], which can damage the DNA molecule. The arrest in the progression of the cell cycle is a consequence of DNA damage, permitting the repair of DNA injury (through the increase in p53 and p21). It has recently been shown that CsA augments the levels of p21 and p53 [32,33], which could explain the cell cycle arrest at 22 hr. If the DNA damage cannot be repaired, p53 triggers the signaling pathways that lead to apoptotic cell death. We have also determined that apoptotic cell death increased in samples of hepatocytes incubated for 22 hr in the presence of CsA. At 50 μM CsA, 50% of apoptotic cells were PCNA+, indicating that the majority of these cells could be derived from cells either involved in S phase or cells that have recently passed through DNA synthesis. There is direct evidence that when oxidative DNA damage results in proliferating cells, they are more prone to death.

Finally, the increases in cyclin D1, PCNA, and cyclin E, together with the invariable level of p27 at 3 and 6 hr of incubation with CsA, clearly show that CsA induces hepatocytes to proliferate. These results reinforce the idea of the growth-promoting effect of CsA on cultured hepatocytes, an effect mediated by increases in cyclins D1 and E. However, at 22 hr of incubation, the oxidative stress induced by CsA could induce an increase in p27 and decrease in cyclin D1, PCNA, and cyclin E, inducing hepatocytes to exit S phase, or not to enter it.

We conclude that the results of the present study represent a good start to better understand the growth-promoting effect of CsA. Further experiments should be performed to establish at which level (e.g. transcription, translation, etc.) these proteins are affected by CsA.

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