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Novel mechanism of Vitamin E protection against cyclosporine A cytotoxicity in cultured rat hepatocytes

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Abstract

Cyclosporine A (CsA) is the immunosuppressor most frequently used in transplant surgery and in the treatment of autoimmune diseases. It has been shown that CsA is able to generate reactive oxygen species and lipid peroxidation which are directly involved in the CsA hepatotoxicity. As antioxidant, Vitamin E (VitE) has been used to diminish the toxicity of CsA *in vitro*. Besides its direct action as the classical antioxidant implicated in preventing lipid peroxidation, we decided to investigate the effect of VitE on the endogenous antioxidant defense system, such as Mn and CuZn superoxide dismutase (MnSOD, CuZnSOD) catalase and glutathione peroxidase (GPx) on CsA cytotoxicity in primary cultures of rat hepatocytes. In cells incubated in the presence of CsA, there was an increase in the expression and activity of MnSOD and CuZnSOD but not in that of catalase and GPx. However, when hepatocytes were coincubated with CsA and VitE, an increase in the expression and activity in all antioxidant enzymes (MnSOD, CuZnSOD, catalase and GPx) was observed. In conclusion, we suggest (a) that the imbalance between SOD and catalase/GPx by the effect of CsA is the main mechanism responsible for peroxide accumulation and cell death in hepatocytes, and (b) that the presence of VitE in culture media reduces the oxidative stress through the inhibition of lipid peroxidation, but also through the increase of the expression and activity of catalase and GPx which allows the restoration of SOD and catalase/GPx coordination, indispensable for the correct cell defense against ROS.

Keywords: CsA; MnSOD; CuZnSOD; Catalase; Vitamin E; ROS

1. Introduction

Cyclosporine A (CsA) is the immunosuppressor 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 cell T receptor through the formation of a CsA–cyclophilin complex [3,4]. The enzymes involved in CsA metabolism are hepatic CYP 3A isozymes [5,6] leading to a large number of demethylated and hydroxylated metabolites which still retain the undecapeptide nature of the parent compound [7,8]. 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 the way to counteract the toxicity of this drug [9–11]. Considering its character, most of the studies mentioned above investigated the functional impairment of the liver manifested by parameters of hepatotoxicity.

It has also been shown that CsA is able to generate reactive oxygen species(ROS) and lipid peroxidation [12–15] which are directly involved in the CsA hepatotoxicity. This thesis led investigators to use antioxidants in order to diminish the toxicity of CsA. Among numerous antioxidants checked, VitE was the best which reduced the CsA toxicity [15].

It is widely known that VitE is the main antioxidant that stabilizes cell membranes by interfering with lipid peroxidation [16]. On the basis of this consideration it has been accepted that the mechanism by which VitE exerts its protective effect is mainly due to lipid peroxidation inhibition, and no later studies have investigated other possible pathways involved in VitE cytoprotection.

In this context, we decided to investigate another possible mechanism by which VitE exerts its protection. Thus, we studied the state of enzyme systems implicated in antioxidant defense in cells, such us MnSOD, CuZnSOD, catalase and glutathione peroxidase in primary cultures of

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Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; ROS, reactive oxygen species; CsA, cyclosporine A; VitE, Vitamin E.

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rat hepatocytes incubated in the presence of CsA or CsA plus VitE.

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. VitE succinate was obtained from Sigma. CsA was kindly provided by Dr. Armin Wolf, Novartis. Primary Antibodies were from Calbiochem (CuZnSOD, MnSOD, catalase) and Sigma (β-Actin). Secondary antibodies were from Sigma and Santa Cruz Biotechnology.

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 Institute 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 [17,18] and cell viability, determined by trypan blue exclusion, was always greater than 90%. 2×10^6 freshly isolated hepatocytes 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 penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamicin and 10% fetal calf serum (FCS). 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% FCS containing CsA or CsA and VitE. Hepatocytes were exposed to the drug at a dose range of 0–50 μM for 3, 6, and 22 hr. CsA was dissolved in a stock solution of dimethyl sulfoxide (DMSO) and VitE in ethanol and further diluted in the DMEM medium. DMSO end-concentrations on all plates were 0.2%. VitE end-concentration was 50 μM.

2.4. Measurement of cytotoxicity by LDH leakage

Cytotoxicity was measured using the index of membrane lysis, lactate dehydrogenase (LDH) leakage from damaged hepatocytes [19]. The release of intracellular LDH to the extracellular medium was measured by determining this enzyme activity following Vasault [20], and was expressed as a percentage of total cellular activity.

2.5. Determination of intracellular generation of ROS

H_2O_2 and $O_2^{\bullet -}$ (superoxide anion) production was monitored by flow cytometry using DCFH-DA and HE, respectively. These dyes are stable nonpolar compounds that readily diffuse into cells. Once inside the cells, the acetate groups of DCFH-DA are cleaved from the molecule by intracellular esterases to yield DCFH, which is trapped within the cells. Intracellular H_2O_2 or low-molecular-weight peroxides, in the presence of peroxidases, oxidize DCFH to the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxides produced by the cells. Cytosolic dihydroethidium exhibits blue fluorescence when excited by UV light; however, once this probe is oxidized by superoxide anion to ethidium, it intercalates within the cell's double strand nucleic acid, staining its nucleus and cytoplasm a bright red fluorescence which is proportional to the intracellular superoxide anion level. Following the incubation for 24 hr with CsA, hepatocytes 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 and 10 μM HE at 37°. The cells were washed twice with PBS to remove the extracellular DCFH-DA and HE, followed by analysis on a FACScan flow cytometer (Becton-Dickinson) (excitation 488 nm and emission 525 nm for DCFH; excitation 488 nm and emission 605 nm for HE).

2.6. RNA extraction and Northern blot analysis of catalase, MnSOD, CuZnSOD and GPx

Total RNA (4×10^6 cells) was extracted following the guanidinium thiocyanate/phenol reagent method [21]. About 20 μg 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 [22]. The relative level of mRNA transcript was determined using catalase, CuZnSOD, MnSOD and GPx cDNA probe [23], labeled with a^{32}P-dCTP using a multiprimer DNA-labeling system kit (Amersham Life Science). 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%.

2.7. Enzyme activity assays

Following the incubation for 3, 6 and 22 hr with CsA, hepatocytes were washed with PBS in order to eliminate dead cells, collected from culture dishes, resuspended in phosphate buffer saline (PBS) and sonicated on ice. The

solution was centrifuged for 15 min at 4° in microcentrifuge to eliminate cell debris and the supernatant used for enzyme activity assays. Antioxidant enzyme activities were measured as follows: catalase was spectrophotometrically determined by measuring decreased absorbance at 240 nm using hydrogen peroxide as substrate [24]; superoxide dismutase (CuZnSOD and MnSOD) was measured spectrophotometrically by monitoring the inhibition of the autooxidation of pyrogallol. Sodium cyanide (1 mM) was added to dissect MnSOD activity from that of CuZnSOD [25]. Glutathione peroxidase activity was expressed as nanomole per minute per milligram of protein and determined by the method previously described [26]. SODs and catalase activities were expressed as units per milligram protein. One unit of SOD refers to nanogram of enzyme that produces 50% inhibition in pyrogallol autooxidation. One unit of catalase is defined as the amount of enzyme that transforms 1 mmol of hydrogen peroxide per min at 25°. Protein estimation was made following Bradford [27] using bovine serum albumin as standard.

2.8. Immunoblotting for detection of CuZnSOD, MnSOD 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 TPBS for 2 hr. Primary antibody against catalase, CuZnSOD and MnSOD (Calbiochem) were applied at dilution of 1:20000, 1:1000 and 1:1000, respectively for 24 hr at 4°. After washing, appropriate secondary antibodies (anti-rabbit and anti-sheep IgG-peroxidase conjugated) were applied at a 1:10000 and 1:2000 dilution, respectively for 1 hr at 4°. Blots were washed, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham Life Science) and exposed to autoradiographic film. β-Actin (Sigma) was analyzed and used as loading control.

2.9. Statistical analysis

The results were reported as means ± SD of four experimental observations (four animals). Time course data were compared by using a Student's *t* test. (a) Value against the corresponding control. To compare means of groups treated with/without VitE, a two-way ANOVA test was used. Pairwise comparisons were conducted employing a Student–Newman–Keuls *post hoc* test. (b) Differences due to VitE. For all tests, $P < 0.05$ was accepted as significant.

3. Results

3.1. CsA cytotoxicity. Modulation by VitE

Isolated rat hepatocytes offer a useful system for the study of xenobiotic cytotoxicity. Primary hepatocyte cultures were exposed to increasing concentrations of CsA from 0 to 50 μM for 3, 6 and 24 hr, and LDH leakage was measured as an index of cell toxicity. Fig. 1 shows, as percentages of the total, that the cytotoxic effect of CsA was dose- and time-dependent. The lowest concentrations of the drug able to cause significant increases, vs. control, in LDH leakage at 24 hr of incubation, reflecting a loss of plasma membrane integrity associated with necrosis, were 10 μM with the values 25.5 ± 2.8 vs. 13.2 ± 1.4 ($P < 0.05$). At 50 μM CsA differences vs. control were higher, reaching more than 2-fold (226%) the control value 29.9 ± 3.0 vs. 13.2 ± 1.2 ($P < 0.05$).

Fig. 2 shows the effect of VitE when coincubated with CsA (0–50 μM) at 24 hr. We observe that VitE clearly decreased cell death, and this beneficial effect was significant at doses 10 and 50 μM CsA. Thus, cell death decreased to values of 18.0 ± 1.2 vs. 25.5 ± 2.8 (70%, $P < 0.05$) and 20 ± 2.1 vs. 29.9 ± 3.0 (67%, $P < 0.05$), respectively.

3.2. Effect of VitE on ROS production

Fig. 3 shows the effect of VitE when coincubated with CsA (0–50 μM) for 24 hr on peroxide generation in

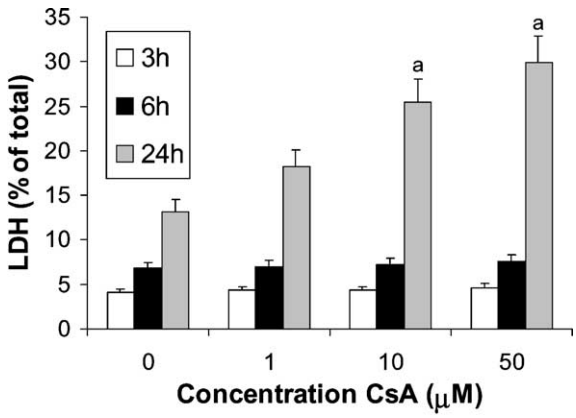

Fig. 1. Time course of cytotoxic effects of CsA (0–50 μM) in primary cultures of rat hepatocytes. Hepatocytes were incubated with CsA (0–50 μM) for 3, 6 and 24 hr. LDH leakage to extracellular medium was measured as a cytotoxicity index. Data are reported as the mean ± SD of four different observations (four animals) and compared by using a Student's *t* test. Differences were considered significant at $P < 0.05$ (a) value against its corresponding control.

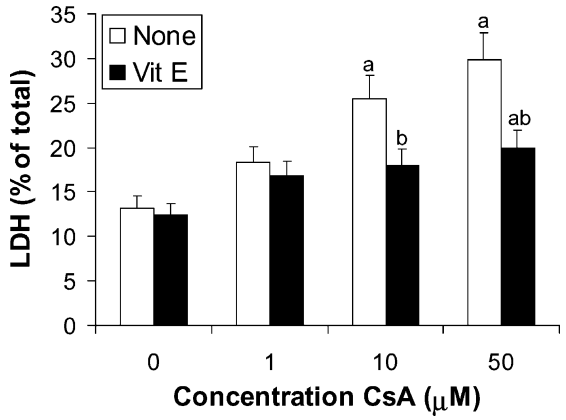

Fig. 2. Effect of VitE on cytotoxic effects of CsA (0–50 μM) for 24 hr. Hepatocytes were incubated with CsA (0–50 μM) in the absence or presence of 50 μM VitE for 24 hr. LDH leakage to extracellular medium was measured as a cytotoxicity index. Data are reported as the mean ± SD of four different observations (four animals) and 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 $P < 0.05$ (a) values against their controls, (b) differences due to VitE.

cultures of rat hepatocytes. The values obtained clearly indicate that VitE decreased the levels of peroxides increased by the effect of CsA. These VitE-dependent changes were significant at CsA 10 and 50 μM with values of 1659 ± 171 vs. 2514 ± 249 (65%, $P < 0.05$) and 2000 ± 189 vs. 3022 ± 299 (66%, $P < 0.05$), respectively.

Fig. 4 shows the effect of VitE on superoxide generation in cultures of rat hepatocytes when incubated with CsA

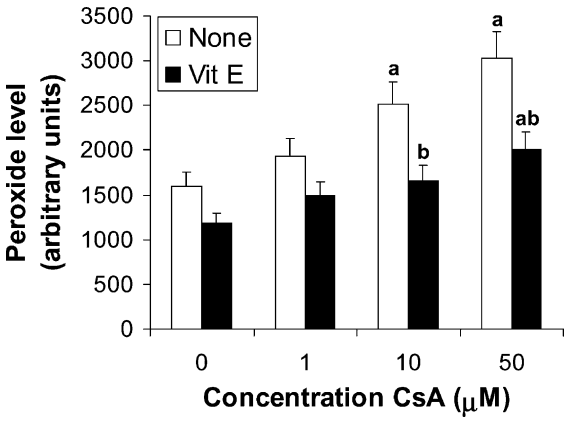

Fig. 3. Effect of VitE on the intracellular generation of peroxides in cultures of rat hepatocytes in the presence of CsA (0–50 μM) for 24 hr. Following incubation with CsA, in absence or presence of 50 μM VitE, hepatocytes were detached with tripsine and incubated with 5 μM DCFH-DA in 2 mL PBS for 30 min at 37°. The samples were placed on ice and peroxides production was determined by flow cytometry. Data are expressed as arbitrary units (fluorescence intensity). Data are reported as the mean ± SD of four different observations (four animals) and 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 $P < 0.05$ (a) values against their controls, (b) differences due to VitE.

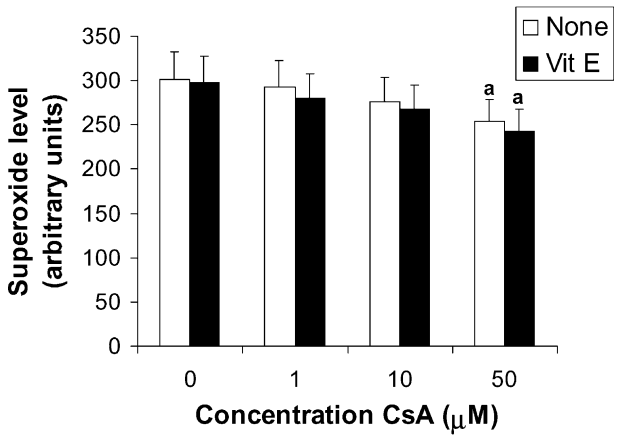

Fig. 4. Effect of VitE on the intracellular generation of superoxide in cultures of rat hepatocytes in the presence of CsA (0–50 μM) for 24 hr. Following incubation with CsA, in absence or presence of 50 μM VitE, hepatocytes were detached with tripsine and incubated with 10 μM HE in 2 mL PBS for 30 min at 37°. The samples were placed on ice and peroxides production was determined by flow cytometry. Data are expressed as arbitrary units (fluorescence intensity). Data are reported as the mean ± SD of four different observations (four animals) and 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 $P < 0.05$ (a) values against their controls, (b) differences due to VitE.

(0–50 μM) for 24 hr. It can be observed that superoxide level slightly decreased when CsA concentration increased. Differences were significant at 50 μM CsA in cultures incubated with or without VitE. However, no significant differences were observed between the cultures treated with or without VitE.

3.3. mRNA level of antioxidant enzymatic systems. Effect of VitE

Fig. 5 shows the Northern blot analysis (Fig. 5A) and the quantification by laser densitometry (Fig. 5B) of endogenous antioxidant systems (CuZnSOD, MnSOD, catalase and GPx) at 3, 6 and 22 hr of incubation with CsA in absence or presence of VitE 50 μM. We observe that CuZnSOD mRNA did not undergo significant variations at 3 and 6 hr of incubation when incubated in the absence of VitE. However, at 22 hr of treatment the level of CuZnSOD transcript increased significantly with 10 and 50 μM CsA reaching more than 3-fold (310%) the control value (480 ± 42 vs. 155 ± 16, $P < 0.05$). When hepatocytes were coincubated in the presence of CsA and VitE the CuZnSOD mRNA level showed a progressive increase that reached at 22 hr more than 4-fold (410%) the control value (1034 ± 99 vs. 252 ± 27, $P < 0.05$) at 50 μM CsA, so the increase obtained in the presence of the antioxidant was higher than in its absence (410% vs. 310%, respectively).

MnSOD mRNA level underwent a dose and time dependent increase that reached significant values at 50 μM CsA

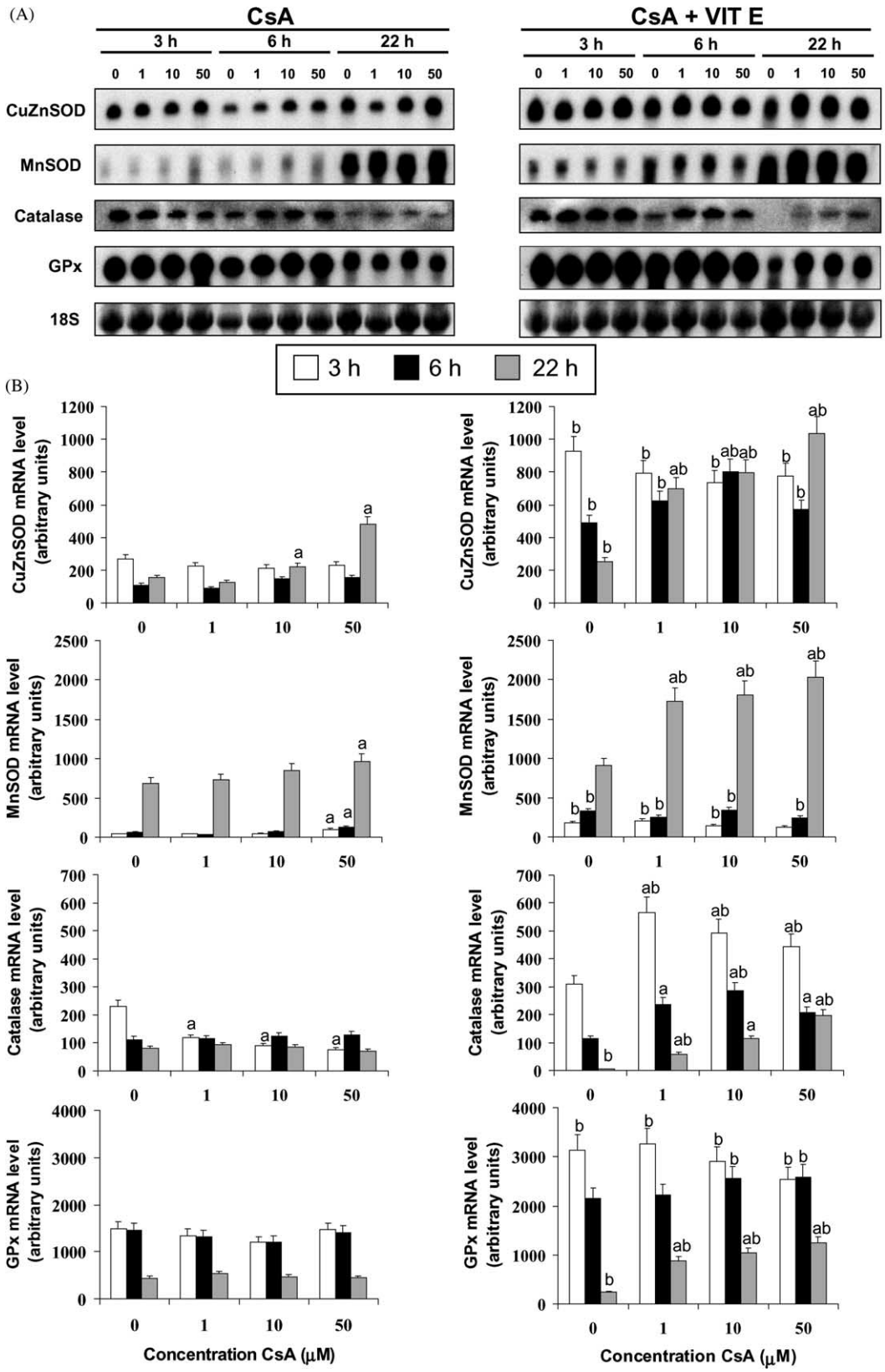

Fig. 5. Effect of CsA and VitE on gene expression of CuZnSOD, MnSOD, catalase and GPx in primary cultures of rat hepatocytes. Northern blot analysis of GPx, catalase, MnSOD and CuZnSOD mRNAs. RNA was isolated and analyzed by Northern blotting using radiolabeled GPx, catalase, MnSOD and CuZnSOD cDNAs. Panel A shows representative Northern blots with 18S rRNA probe for RNA normalization and panel B shows the quantification in arbitrary units after correction with 18S rRNA. The values are reported as the mean ± SD of four different observations (four animals) and 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 $P < 0.05$ (a) values against their respective time controls, (b) differences against the respective experiment without VitE.

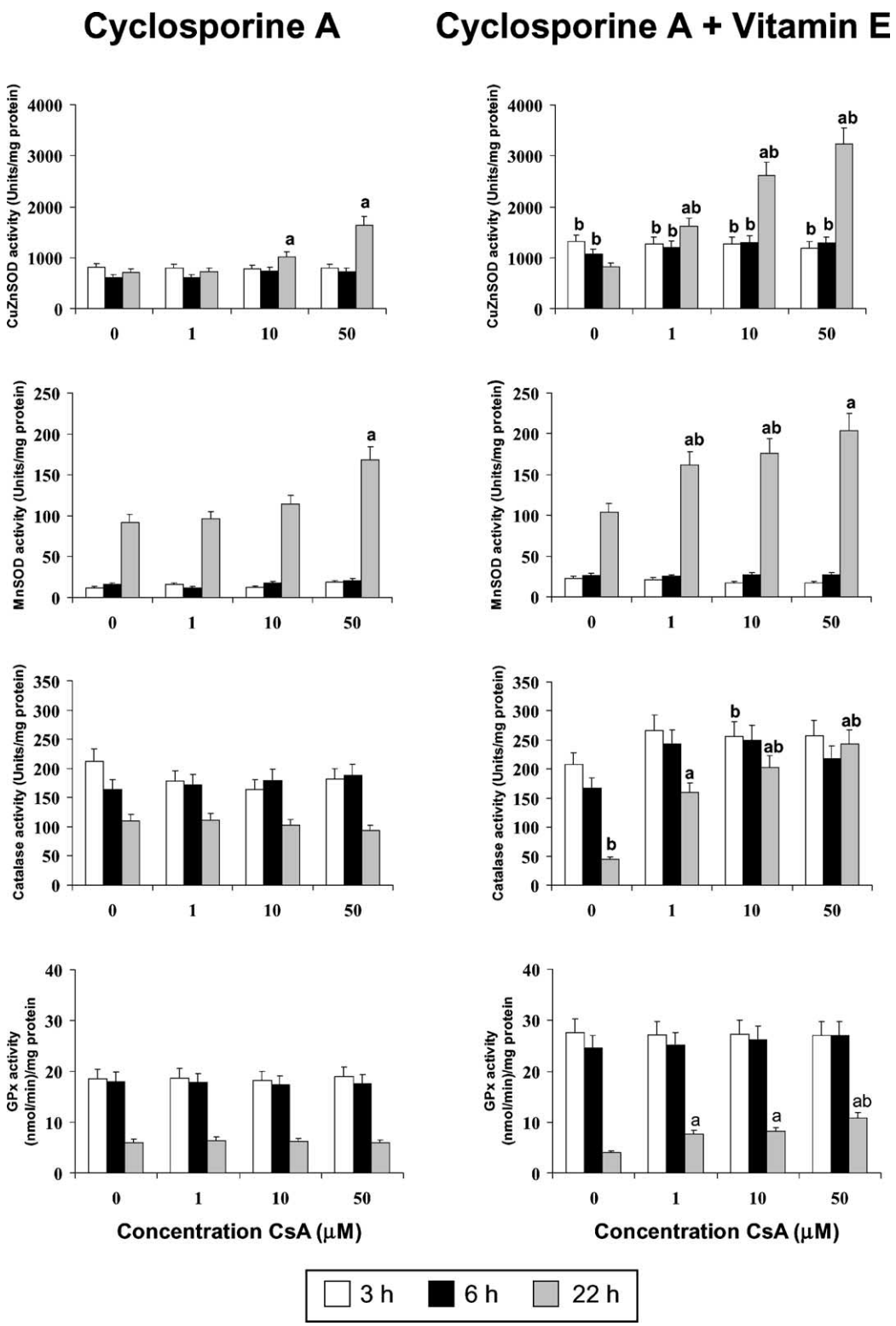

Fig. 6. Effect of CsA and VitE on GPx, catalase, MnSOD and CuZnSOD activities in cultured rat hepatocytes. The activities of GPx, catalase, CuZnSOD and MnSOD were measured as described [24–26]. GPx expressed as nanomole per minute per milligram protein. One unit of SOD refers to nanogram of enzyme that produces 50% inhibition in pyrogallol autooxidation. One unit of catalase is defined as the amount of enzyme that transforms 1 mmol of hydrogen peroxide per min at 25°. The values are reported as the mean ± SD of four different observations (four animals) and 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 $P < 0.05$ (a) values against their respective time controls, (b) differences against the respective experiment without VitE.

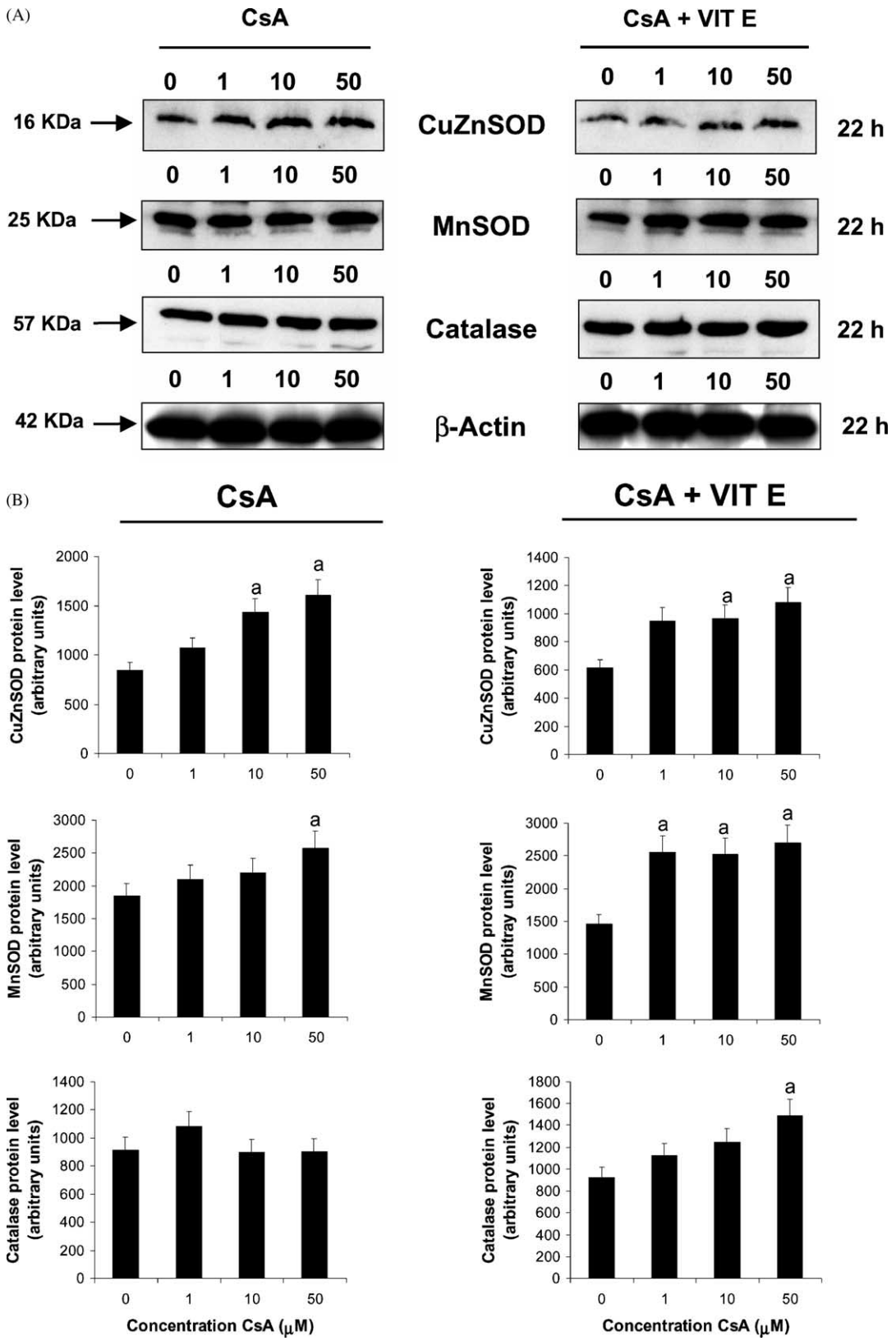

Fig. 7. CuZnSOD, MnSOD, Catalase and GPx levels in cultures of rat hepatocytes incubated with CsA in absence or presence of VitE. Protein levels were assayed by Western blot analysis. In panel A can be observed the signals after ECL detection. Panel B Shows the quantification of chemiluminescence signals in panel A by laser densitometry expressed as arbitrary units. 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 $P < 0.05$ (a) value against its corresponding control.

at all periods of incubation assayed. When VitE was added to culture media, no significant changes were detected at 3 and 6 hr of incubation at any concentration of CsA assayed; however, at 22 hr, increases in MnSOD mRNA were detected in the range 1–50 μM CsA.

In the case of catalase, mRNA level underwent significant and dose-dependent progressive decrease at 3 hr when cells were incubated with CsA. No significant changes were detected at 6 and 22 hr against the controls. In control cells there was a time-dependent decrease in catalase mRNA. When CsA-treated cells were coincubated with VitE, catalase mRNA underwent a significant increase at 3 and 6 hr reaching the higher differences at 1 μM at 3 hr (566 ± 59 vs. 309 ± 28, $P < 0.05$) and at 10 μM CsA at 6 hr (287 ± 27 vs. 114 ± 12, $P < 0.05$). Equally, at 22 hr of incubation, there were significant increases from 1 to 50 μM CsA where the difference was higher (198 ± 17 vs. 5 ± 1.3, $P < 0.05$). It was also observed that the catalase transcript level in control cells progressively diminished when the period of incubation increased.

When GPx mRNA levels were assayed, no differences were obtained against controls in cultures incubated with CsA alone. However, when hepatocytes were coincubated in presence of VitE, GPx transcript level significantly increased at 22 hr of incubation in a dose-dependent manner.

When the levels of antioxidant enzyme mRNAs were compared in cells treated with CsA alone and in those coincubated with VitE, mRNA levels were significantly higher, in almost every condition assayed, in those cultures treated with the antioxidant.

3.4. Enzymatic activity and protein level of endogenous antioxidant systems

Fig. 6 shows the enzymatic activity of CuZnSOD, MnSOD, catalase and GPx in CsA-treated hepatocytes incubated with or without VitE. Basically, the results showed that the activity of these enzymes followed a similar pattern of variations compared to those shown by mRNA level. Thus, the antioxidant enzymatic activity in those cells treated with CsA and VitE were higher than that obtained from CsA-treated cells.

Fig. 7 shows a representative Western blot analysis of CuZnSOD, MnSOD and catalase at 22 hr of incubation with CsA in the absence or presence of VitE. Comparing these results with those obtained by Northern blot, the protein level of the antioxidant enzymes assayed followed the same pattern of changes as those showed by mRNA level at 22 hr of treatment. In this case however, we could not compare the magnitude of the values obtained in cells treated with CsA and those obtained in cells coincubated with CsA and VitE, because these experiments were carried out on different days and in different membranes, thus we could not utilize the two-way ANOVA test to compare the data.

4. Discussion

CsA is the drug most frequently used in transplant surgery because of its potent immunosuppressive action [28]. However, its clinical use is accompanied by adverse side effects such as hypertension, neurotoxicity, nefrotoxicity, and hepatotoxicity [10,11,29]. Previous studies of our group established that ROS production and an oxidative stress situation are involved in CsA hepatotoxicity [14,15]. This conclusion led us to use antioxidants in order to diminish the toxicity of CsA. Among the many antioxidants, we chose three (VitE, N-acetylcystein and deferoxamine) and we measured their protective effect through LDH release assay (data not shown). VitE was selected because it was the most effective antioxidant. We also checked that VitE was able to diminish the ROS production induced by CsA, which, to a lesser extent led to death by necrosis. It is widely known that VitE is the main intramembrane antioxidant that stabilizes cell membranes by interfering with lipid peroxidation [16]; and on the basis of this consideration, it has been accepted that the mechanism by which VitE exerts its protective effect is mainly due to lipid peroxidation inhibition. However, no more thorough works have been developed to reveal other possible mechanisms by which VitE exerts this protection in oxidative stress situations. Therefore, we studied the expression of the antioxidant enzymatic system CuZnSOD, MnSOD, catalase and GPx, the level of protein and the enzymatic activity in hepatocytes treated with CsA in absence or presence of VitE.

None of the antioxidant enzymes above mentioned has by itself a central role in cell defense against ROS; the coordination of all of them is necessary to exert an efficient protection. Indeed, the concerted action of SOD and catalase is the main cell defense mechanism against oxygen toxicity. Superoxide anion is generated in aerobic organisms either spontaneously or as a result of pathological situations, such as biotransformation of drugs. SOD is the enzyme that converts $O_2^{\bullet -}$ (superoxide anion) to H_2O_2 and catalase is the enzyme that eliminates H_2O_2. It has been described that a failure in the removal of H_2O_2 produces hydroxyl radical catalyzed by SOD [30], and that high levels of peroxides inhibit catalase activity [31].

Our results show that in hepatocytes incubated for 22 hr in presence of CsA, the gene expression, protein level and enzymatic activity of CuZnSOD underwent an increase as a response to the oxidative stress generated by CsA. An important conclusion that can be obtained from these results, is that the basal mRNA levels of CuZnSOD in control cells diminished when time of incubation increased, which is in agreement with the results previously described by other authors [32]. Nevertheless, cultured hepatocytes maintained the ability of response against oxidative stress for periods as long as 22 hr of incubation.

MnSOD is the isoform of SOD located in mitochondria where the reduction of oxygen generating large amounts of $O_2^{\bullet -}$ takes place. Our results show that in an oxidative stress situation MnSOD is induced earlier than CuZnSOD. Perhaps milder oxidative stress situations are able to activate MnSOD, but not CuZnSOD. It is known that CsA produces alterations in mitochondria when CsA blocks the permeability transition pore [33], originating an increase in mitochondrial Ca^{2+} concentration and alterations in mitochondrial electron transport chain. These events cause the oxidative phosphorylation uncoupling [34–36] and the subsequent increase in ROS production. In this way, the earlier MnSOD activation could be explain, since the oxidative stress situation begins at mitochondrial level where MnSOD is located. Nevertheless, the ROS generation is not exclusively at mitochondrial level, since CsA is metabolized by cytochrome P-450 3A that also generates ROS [37,38].

As described previously, the concerted action between SOD and catalase is very important to get an efficient protection against ROS. When hepatocytes were incubated with CsA, catalase mRNA level did not increase. In previous studies [14] we demonstrated that CsA increased peroxide levels, while levels of superoxide radical diminished. The decrease of superoxide radical is explained through the activation of both SOD, while the peroxide increase is due to the inactivation or inhibition of catalase, the main enzyme responsible for the elimination of H_2O_2. Moreover, the other enzyme responsible for the protection against H_2O_2 and other peroxides, GPx, did not increase its gene expression in cells treated with CsA when the oxidative stress takes place.

When hepatocytes were coincubated with CsA and VitE, the pattern of response of CuZnSOD and MnSOD in these cells was similar to that obtained from hepatocytes treated only with CsA. However, activation of these enzymes was detected at lower concentrations of CsA, indicating that VitE facilitates the response of these enzymes in stress situations and maintains the levels of mRNA higher than in cells treated with CsA alone. However, the most outstanding event that took place in hepatocytes incubated with VitE was the correct response of catalase and GPx that increased mRNA level, protein level and enzymatic activity in response to the oxidative stress, which permitted cells to eliminate efficiently the peroxides generated. Thus, the presence of VitE in culture media, restores the concerted action between SOD and catalase and GPx that avoids the accumulation of H_2O_2.

In conclusion, we suggest that: (a) the imbalance between SOD and catalase/GPx by the effect of CsA is the main mechanism responsible for peroxide accumulation and cell death in cultured hepatocytes and (b) the presence of VitE in culture media reduces the oxidative stress through the inhibition of lipid peroxidation (a widely known mechanism), but also through the increase of the expression and activity of catalase and GPx which allows the restoration of SOD and catalase/GPx coordination, indispensable for the correct cell defense against ROS.

Acknowledgments

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