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Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells

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1 Cyclosporine A (CsA) increases eNOS mRNA expression in bovine cultured aortic endothelial cells (BAEC). As some effects of CsA may be mediated by reactive oxygen species (ROS), present experiments were devoted to test the hypothesis that the CsA-induced eNOS up-regulation could be dependent on an increased synthesis of ROS.

2 CsA induced a dose-dependent increase of ROS synthesis, with the two fluorescent probes used, DHR123 (CsA 1 μM: $305 \pm 7\%$ over control) and H_2DCFDA (CsA 1 μM: $178 \pm 6\%$ over control).

3 Two ROS generating systems, xanthine plus xanthine oxidase (XXO) and glucose oxidase (GO), increased the expression of eNOS mRNA in BAEC, an effect which was maximal after 8 h of incubation (XXO: $168 \pm 21\%$ of control values. GO: $208 \pm 18\%$ of control values). The ROS-dependent increased eNOS mRNA expression was followed by an increase in eNOS activity.

4 The effect of CsA on eNOS mRNA expression was abrogated by catalase, and superoxide dismutase (SOD). In contrast, the antioxidant PDTC augmented eNOS mRNA expression, both in basal conditions and in the presence of CsA.

5 The potential participation of the transcription factor AP-1 was explored. Electrophoretic mobility shift assays were consistent with an increase in AP-1 DNA-binding activity in BAEC treated with CsA or glucose oxidase.

6 The present results support a role for ROS, particularly superoxide anion and hydrogen peroxide, as mediators of the CsA-induced eNOS mRNA up-regulation. Furthermore, they situate ROS as potential regulators of gene expression in endothelial cells, both in physiological and pathophysiological situations.

Keywords: Endothelial cells; cyclosporine A; reactive oxygen species; superoxide anion; hydrogen peroxide; nitric oxide synthase; AP-1

Introduction

Since its introduction as an immunosuppressor, cyclosporine A (CsA) is claimed to be a very effective drug, especially in the field of transplantation. Among its side effects, one of the most well known is damage to the vascular endothelium (Perico *et al*., 1986; Zoja *et al*., 1986) associated in some cases with hypertension and/or microangiopathic haemolytic anaemia. Although hypertension seems to be related to perturbations in the balance of vasoconstrictors/vasodilators within the wall vessel (Bosaller *et al*., 1989; Cairns *et al*., 1989; Bunchman & Brookshire, 1991; Takeda *et al*., 1993), little is known about the molecular changes in signal transduction and gene expression induced by CsA with the endothelium.

In lymphocytes, CsA and another immunosuppressor, FK506, inhibit T-cell activation by blocking the critical step between the formation of the antigen-T cell receptor complex and subsequent gene expression. CsA interacts with an ubiquitously distributed family of proteins named cyclophilins, the final target of the immunosuppressant-cyclophilin complex being calcineurin, a serine-threonine specifc protein phosphatase (Bierer *et al*., 1993). Calcineurin is activated after an increase in intracellular calcium and acts by dephosphorylating the cytoplasmic subunit of a transcription factor, nuclear factor of activated T-cells (NF-AT) which is crucial in promoting transcription of T-cell genes participating in the immune response (Jain *et al*., 1993). CsA, thus is immunosuppressive by inhibiting calcineurin-dependent NF-AT dephosphorylation.

However, other effects of the drug, mainly those implied in the development of cell toxicity, do not seem to depend on this established pathway. In this sense, it has been proposed that reactive oxygen species (ROS) could be one of the mediators involved in the toxic actions of the drug (Stamler *et al*., 1992; Ahmed *et al*., 1993; Wang & Salahudeen, 1995), and, hence, the treatment with antioxidants improves acute CsA nephrotoxicity (Paller, 1985). On the other hand, ROS have been proposed as biological mediators of cell signals (Lander, 1997). Thus, Sundaresan *et al*. (1995) demonstrated that PDGF-induced cell proliferation was dependent on the synthesis of H_2O_2 and the ability of these ROS to activate some transcription factors, as nuclear factor-κB (NF-κB) or AP-1, has been clearly demonstrated (Schreck *et al*., 1991; Meyer *et al*., 1993; Sen & Packer, 1996).

In a previous study we tested the hypothesis that the CsA-dependent increased blood pressure could be mediated by a decreased nitric oxide synthesis in vascular beds. In contrast with the expected results, we found that CsA was able to augment endothelial constitutive nitric oxide synthase (eNOS) activity in bovine aortic endothelial cells (BAEC), and that this increase was associated with parallel changes in the levels of

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the steady-state expression of the eNOS mRNA (López-Ongil *et al*., 1996). However, the cellular events responsible for the CsA-induced changes in eNOS expression were not analysed in the previous work. Present experiments were designed to test the hypothesis that ROS could be involved in the CsA-dependent eNOS mRNA expression up-regulation. For this purpose, we evaluated the ability of CsA to stimulate ROS synthesis in BAEC. In addition, the effects of ROS on eNOS mRNA expression and activity were tested, and the CsA-dependent up-regulation of the eNOS mRNA expression was studied in presence of antioxidant enzymes. We have found that CsA is able to generate ROS in BAEC. In addition, we demonstrated that CsA-mediated increases in the levels of the eNOS transcript are recapitulated by the generation of ROS and inhibited by superoxide dismutase (SOD) and catalase. Finally, we propose that the transcription factor AP-1, could represent a link between the CsA-induced generation of ROS and the effects on gene expression, including those observed on eNOS.

Methods

Cell culture

BAEC were isolated by use of previously described methods (Lamas *et al*., 1991; López-Ongil *et al*., 1996). BAEC were grown in gelatine-coated culture plates at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Individual clones were established and subcloned to obtain pure cell populations. Clones were characterized by their typical cobblestone appearance, by the presence of factor VIII-related antigen and by the uniform uptake of fluorescent acetylated low-density lipoprotein, as described by Marsden *et al*. (1990). Cells were fed every 2 days with RPMI 1640 medium supplemented with 15% bovine calf serum (BCS), 100 u ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Cells were passaged every 5–7 days with trypsin-EDTA. Studies were routinely performed on confluent monolayers at passages 2–6, made quiescent by serum deprivation. Toxicity was evaluated at any experimental condition by the trypan blue dye exclusion method.

Measurement of ROS synthesis

ROS synthesis in BAEC cells was measured by flow cytometry, by using modifications of techniques previously described (Bass *et al*., 1983; Pérez de Lema *et al*., 1997). Briefly, endothelial cells (2×10^6 cells in RPMI) loaded with 2 μM DHR123 or 5 μM H_2DCFDA were treated with CsA or vehicle (0.01% ethanol) at the indicated concentrations and times. During the cellular production of ROS, the intracellular DHR123 is irreversibly converted to the green fluorescent compound rhodamine 123 (R123). R123 is membrane-impermeable and accumulates in the cells. H_2DCFDA is first desacetylated to the non membrane-permeable H_2DCFDA and then oxidized, emitting a fluorescent signal. Flow cytometry analysis was carried out with an EPICS XL cytometer (Coulter Corp. FL, U.S.A.) equipped with a ion argon laser, using an excitation wavelength of 488 nm. Fluorescence emission signals were collected with a 525 nm band pass filter. On each sample, specific fluorescence from 10,000 cells was estimated, after selection on gated population of biparametric FS, SS dot plot. Fluorescence signals were collected with logarithmic amplifiers.

RNA isolation and Northern analysis

In order to evaluate changes in the expression of the eNOS mRNA, two groups of experiments were performed. In the first group, cells were incubated with two ROS-generating systems, xanthine plus xanthine oxidase (XXO: xanthine, 100 μM, xanthine oxidase, 1 mu ml^{-1}) or glucose oxidase (GO, 1 mu ml), under different experimental conditions at 37°C, and studies were performed after the incubation periods had finished. In the second group of experiments, cells were incubated with 1 μM CsA, in the presence of antioxidants including superoxide dismutase (SOD, 100 u ml^{-1}), catalase (CAT, 80 u ml^{-1}), and pirrolidine dithiocarbamate (PDTC, 100 μM). Adequate controls were used in every case, including cells incubated with ethanol (final concentration 0.01%), the vehicle of CsA. The specific conditions of each incubation are detailed in the legends to the figures.

Total cellular RNA was isolated by the guanidinium lysis method, as described by Chomczynski & Sacchi (1987). For Northern analysis, 10 μg/lane of total RNA were subjected to electrophoresis in 1% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters, as described by Lamas *et al*. (1992b). Membranes were hybridized with a ^{32}P-labelled bovine eNOS cDNA (Lamas *et al*., 1992a) and washed at final stringency conditions of 0.2 × SSC, 0.1% SDS at 42°C for 30 min and then exposed to X-OMAT film for 24–48 h. Thereafter, the filters were stripped by boiling in 0.1% SDS solution, and reprobed with a ^{32}P-labelled GAPDH cDNA. The densitometric analysis of the films was performed with an Apple scanner and appropriate software. Levels of eNOS mRNA were normalized by using GAPDH expression within the same lane.

Measurement of eNOS activity

The ability of ROS to modify the eNOS activity was measured by analysing the conversion rate of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, in cells treated with XXO or GO for different times. The assay was performed as described previously with L-[^{3}H]-arginine purified by anion-exchange chromatography (Ishii *et al*., 1989; Lamas *et al*., 1991). In brief, at the end of each experimental period, cells were washed twice with Hank's balanced salt solution and maintained for 3 h in medium containing purified L-[^{3}H]-arginine (6×10^5 c.p.m./well) at 37°C. The reaction was stopped by addition of ice-cold 15% trichloroacetic acid. Samples were extracted five times with 2 ml of water-saturated ether, vacuum lyophilized, and resuspended in 2 ml of Buffer A (1 mM EDTA, 20 mM HEPES pH 5.5) and applied to 2 ml wet bed volumes of Dowex AG 50W-X8 (Li^+ form), followed by 2 ml of water. L-[^{3}H]-citruline was quantified in the 4 ml column effluent with a scintillation counter.

Preparation of nuclear extracts

A modification of the method of Schreiber (Schreiber *et al*., 1989) was used. At the end of each experimental period, BAEC were washed twice with PBS, scraped and transferred to microcentrifuge tubes. Cell pellets were resuspended in 400 μl of Buffer B (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 1 $\mu g\ ml^{-1}$ of leupeptin, antipain, trypsin inhibitor and pepstatin A). After 15 min at 4°C, 25 μl of 10% Nonidet P-40 was added. Tubes were vigorously vortexed for 10 s and nuclei were sedimented for 30 s at 15,000 rpm. Nuclear pellets were resuspended in 50 μl of Buffer C (20 mM HEPES pH 7.9,

400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and protease inhibitors at 1 μg ml^{-1}) and vigorously rocked at 4°C for 15 min. They were then centrifuged for 5 min, 15,000 rpm at 4°C and the supernatants aliquoted at −80°C. Before use their protein content was determined by the BCA protein assay (Pierce, Rockford, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were synthesized in a Beckman Oligo 100 M DNA synthesizer. The oligonucleotide sequence corresponding to the AP-1 site in the bovine eNOS promoter was: 5′ GGC CCC CAA CTT **GAG TCA** CAG GGG GTG 3′ 3′ GGG GGT TGA **ACT CAG T**GT CCC CCA CGG 5′.

Primers were annealed by incubation for 5 min at 85°C in Buffer D (50 mM Tris-HCl pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$ and 5 mM DTT) and slowly cooled down to room temperature. Two hundred nanograms of these primers were end-labelled with Klenow DNA polymerase (Pharmacia, U.S.A.) in the presence of 20 μCi of [^{32}P]-dCTP. For binding reactions, approximately 6,000 c.p.m. of the end-labelled oligonucleotide probes were incubated with 7 μg of nuclear extract and 1 μg of poly (dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 4% glycerol, 1 mM DTT) at 4°C for 30 min. Protein-DNA complexes were separated by electrophoresis, in a 6% non-denaturing polyacrylamide gel in 0.25 × TBE buffer at 20 mA, and visualized by autoradiography. For competition experiments 125 fold molar excess of competitor DNA was coincubated with the labelled oligonucleotide probe.

Materials

CsA was a generous gift from Sandoz Pharma (Basel, Switzerland). Salmon sperm DNA, phenylmethylsulphonyl fluoride (PMSF), formaldehyde, glucose oxidase, xanthine, xanthine oxidase, hydrogen peroxide, pirrolidin dithiocarbamate, superoxide dismutase, catalase, leupeptin, antipain, trypsin inhibitor, pepstatin, dithiothreitol (DTT) were purchased from Sigma Chemical (St. Louis, MO). Guanidinium thiocyanate, formamide, Nonidet P-40 were from Fluka (St. Louis, MO). The fluorescent probes dihydrorhodamine 123 (DHR123) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, U.S.A.). RPMI 1640 medium, bovine calf serum, trypsin-EDTA (0.02%) and penicillin-streptomycin were purchased from Bio-Whittaker (Walkersville, MD). Culture plates were from Nunc (Kamstrup, Denmark). Dowex AG 50W-X8 cation-exchange resin (100–200 mesh) was from Bio-Rad (Richmond, CA). The nylon filters, deoxy-[^{32}P]-cytidine triphosphate and DNA labelling kit (Rediprime) were purchased from Amersham (Buckinghamshire, U.K.). L-[^{3}H]-arginine (40–70 Ci mmol^{-1}) was obtained from New England Nuclear (Wilmington, DE). X-OMAT films were from Kodak (Rochester, NY).

Statistical analysis

In every case, the data shown are the mean ± s.e.mean of a variable number of experiments (see legends of the figures). In the L-citrulline quantification assay each individual result was the mean of triplicate wells, and in the trypan blue experiments of 4 wells. Data were compared with the Friedman test. $P<0.05$ was considered statistically significant.

Results

Figure 1 shows the effect of CsA on ROS synthesis in cultured BAEC. In these experiments, cells were incubated with different CsA concentrations or for different incubation times with the drug, and ROS synthesis was measured with DHR123. As it can be observed, CsA induced a significant oxidative burst at concentrations over 10 nM (Figure 1a), and this effect appeared after 60 min of incubation (Figure 1b). When ROS production was evaluated with H_2DCFDA, an increased oxidative burst was also detected, but at higher CsA concentrations. In fact, 100 nM CsA did not modify the fluorescent signal of H_2DCFDA, whereas this parameter increased by 146 ± 6% and 178 ± 6% with respect to control values, after 60 min and 4 h of incubation with 1 μM CsA, respectively (in both cases, values are the mean ± s.e.mean of 5 different experiments and are expressed as % of the control values).

Xanthine plus xanthine oxidase increased the mRNA expression of eNOS, in a time-dependent fashion. The effect

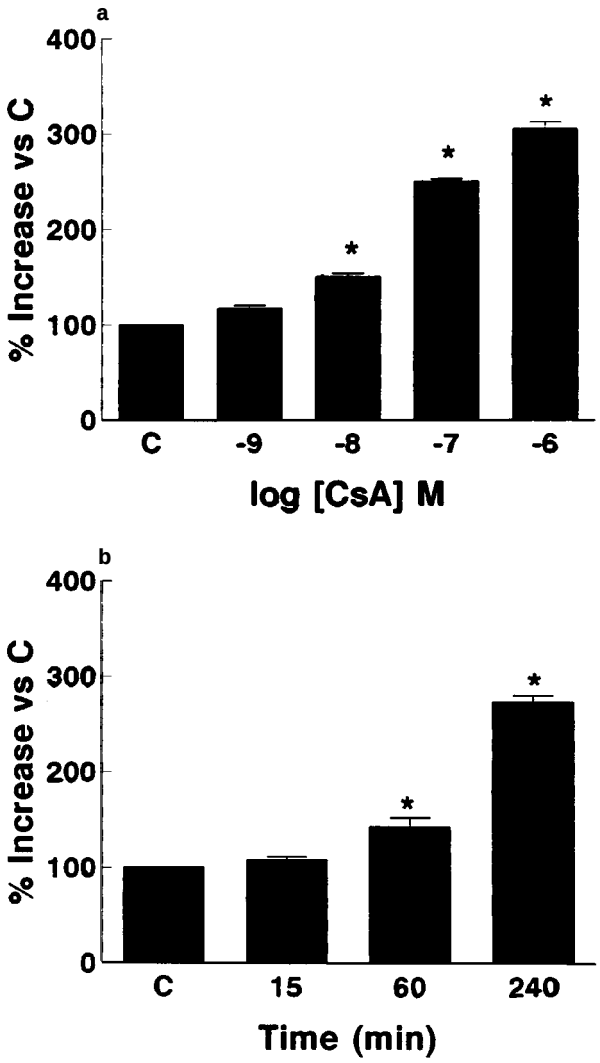

Figure 1 Synthesis of reactive oxygen intermediates (ROS) in bovine cultured aortic endothelial cells exposed to cyclosporine A (CsA). Cells were incubated with various CsA concentrations (a) for 4 h or for different times with 1 μM CsA (b), at 37°C, and the ROS production was evaluated with an ROS-sensible fluorescent probe, dihydrorhodamine (DHR123). Results shown are the mean ± s.e.-mean of 5 different experiments, and are expressed as % of the control values (C). *$P<0.05$ vs C.

appeared as soon as 4 h after the start of the incubations, reached a maximum after 8 h, and then returned to basal levels after 24 h (Figure 2a). This XXO-dependent increased expression of eNOS mRNA, after 8 h of incubation, was completely blunted by the presence of catalase in the incubation media, without any significant effect of SOD

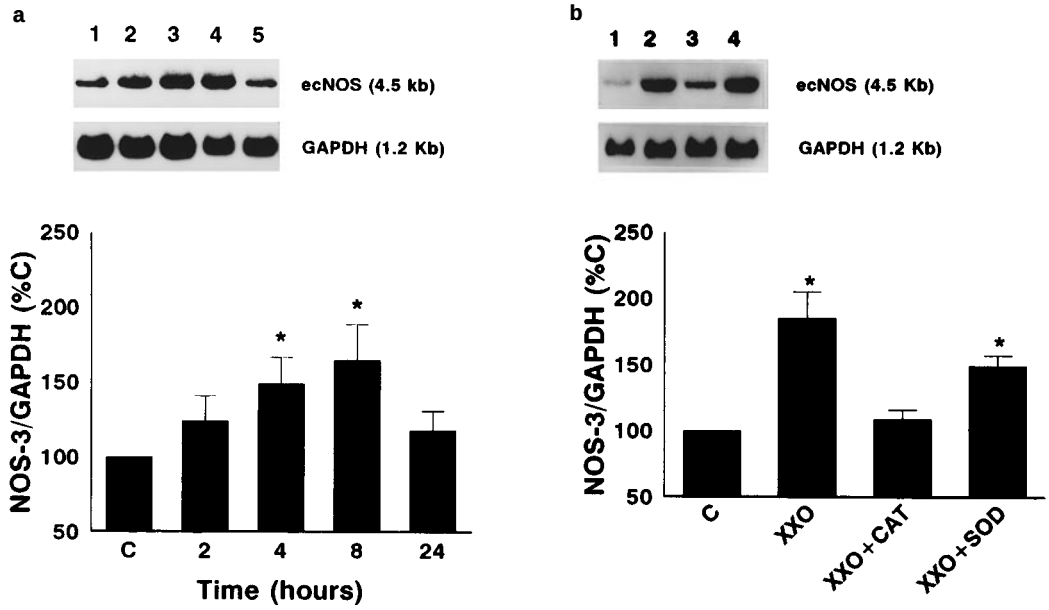

Figure 2 Effect of xanthine plus xanthine oxidase (XXO) on eNOS mRNA expression in bovine cultured aortic endothelial cells. (a) Cells were incubated with xanthine (X, 100 μM) plus xanthine oxidase (XO, 1 mu ml^{-1}) for different times. Lane 1: control; lane 2: XXO, 2 h; lane 3: XXO, 4 h; lane 4: XXO, 8 h and lane 5: XXO, 24 h. (b) Cells were incubated with xanthine (X, 100 μM) plus xanthine oxidase (XO, 1 mu ml^{-1}) for 8 h, in the presence of catalase (CAT, 80 u ml^{-1}) or superoxide dismutase (SOD, 100 u ml^{-1}). Antioxidant enzymes were added to the incubation media 10 min before XXO, without removing them from the incubation media for the whole experimental period. Lane 1: control; lane 2: XXO, 8 h; lane 3: XXO + CAT, 8 h and lane 4: XXO + SOD, 8 h (b). A representative Northern blot experiment of hybridization with eNOS and GAPDH probes is shown in the upper part of the figure. The lower part depicts the ratios between eNOS and GAPDH mRNA, after the denistometric analysis. These results are the mean ± s.e.mean of 4 different experiments, and are expressed as % of the control values (C). *$P<0.05$ vs C.

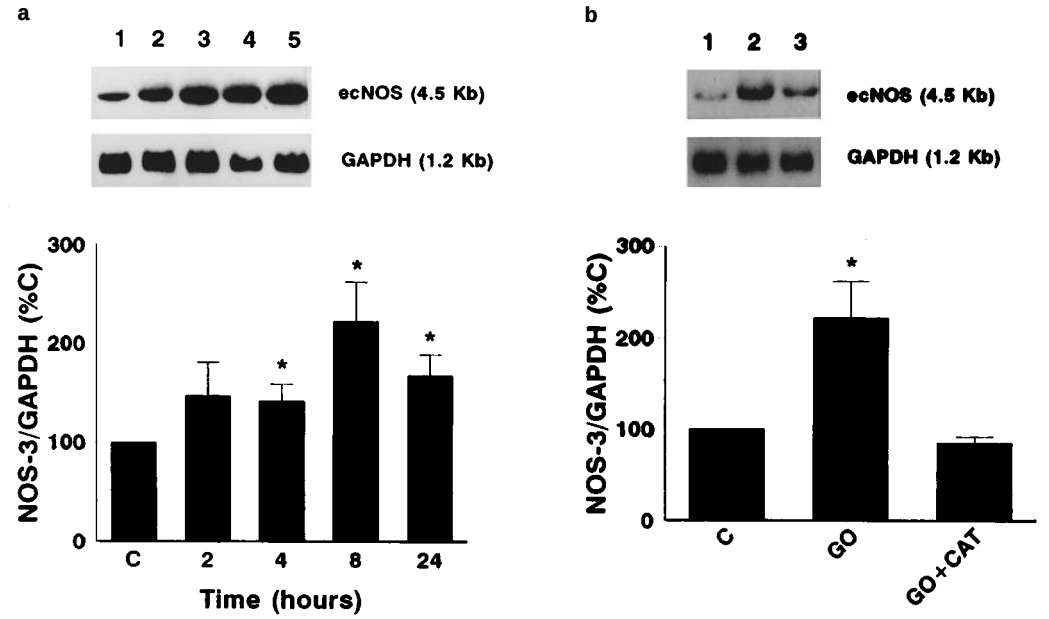

Figure 3 Effect of glucose oxidase (GO) on eNOS mRNA expression in bovine cultured aortic endothelial cells. (a) Cells were incubated with glucose oxidase (1 mu/ml^{-1}) for different times. Lane 1: control; lane 2: GO, 2 h; lane 3: GO, 4 h; lane 4: GO, 8 h and lane 5: GO, 24 h. (b) Cells were incubated with glucose oxidase (GO, 1 mu ml^{-1}) for 8 h, in the presence of catalase (CAT, 80 u ml^{-1}). Catalase was added to the incubation media 10 min before GO, and was present in the incubation media for the whole experimental period. Lane 1: control; lane 2: GO, 8 h and lane 3: GO + CAT, 8 h (b). A representative Northern blot experiment of hybridization with eNOS and GAPDH probes is shown in the upper part of the figure. The lower part depicts the ratios between eNOS and GAPDH mRNA, after the densitometric analysis. These results are the mean ± s.e.mean of 6 different experiments, and are expressed as % of the control values (C). *$P<0.05$ vs C.

(Figure 2b). Similar results were obtained when glucose oxidase was added to the glucose-containing incubation media: a time-dependent increase of the eNOS mRNA was detected (Figure 3a), an effect which was abolished when catalase was present in the incubation media (Figure 3b). As shown in Figure 4, the activity of the enzyme reflected the changes in the eNOS mRNA. Both, XXO and GO, increased the rate of conversion of L-arginine to L-citrulline by endothelial cells. This effect was detected only after 8 h of incubation with the ROS generating systems, and remained over control values after 24 h of incubation. Short-term incubations with XXO or GO did not modify the activity of eNOS (data not shown).

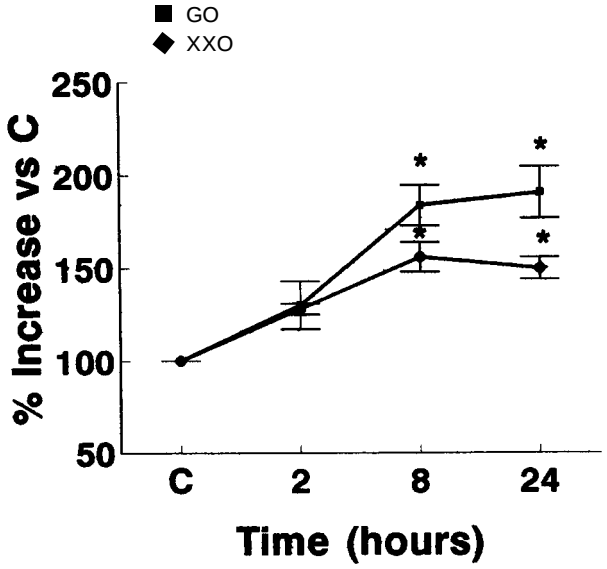

Figure 4 Effect of ROS on eNOS activity in bovine aortic endothelial cells. Cells were incubated for different times with 100 μM xanthine plus 1 mu ml^{-1} xanthine oxidase (XXO) or with 1 mu ml^{-1} glucose oxidase (GO). Results are the mean $\pm$ s.e.mean of 6 different experiments, and are expressed as % of the control (C) values (631 $\pm$ 25 c.p.m./well). *$P<0.05$ vs C.

The effect of CsA on the mRNA expression of eNOS was tested in the absence and presence of different antioxidants after 24 h of incubation, and the results are included in Figure 5. SOD and catalase abrogated the CsA-dependent increased expression of eNOS mRNA. In control cells, these two antioxidants alone also inhibited the basal expression of the mRNA of the enzyme. In contrast, PDTC not only did not block eNOS mRNA expression but also actually increased it, both in basal conditions and in the presence of CsA.

In an effort to investigate potential mechanisms responsible for the effects of ROS, and therefore of CsA on the expression of eNOS we looked at the potential activation of AP-1, a transcription factor with an identified cis-regulatory sequence in the eNOS promoter. As shown in Figure 6 both CsA and glucose oxidase augmented AP-1 binding to the AP-1 oligonucleotide containing the cis-regulatory sequence of the eNOS promoter between 1.5 and 2 fold, as determined by EMSA. In the case of glucose oxidase the maximum binding occurred at 30 min, in contrast to the effect of CsA which had a peak around 4 h.

Table 1 shows the cell toxicity, assessed by the trypan blue dye exclusion method. The presence of XXO in the incubation media, and the combined treatment with CsA plus SOD slightly but significantly decreased the ability of cells to exclude the trypan blue dye. Under the other experimental conditions tested, only minor but statistically non-significant changes in cell viability were detected.

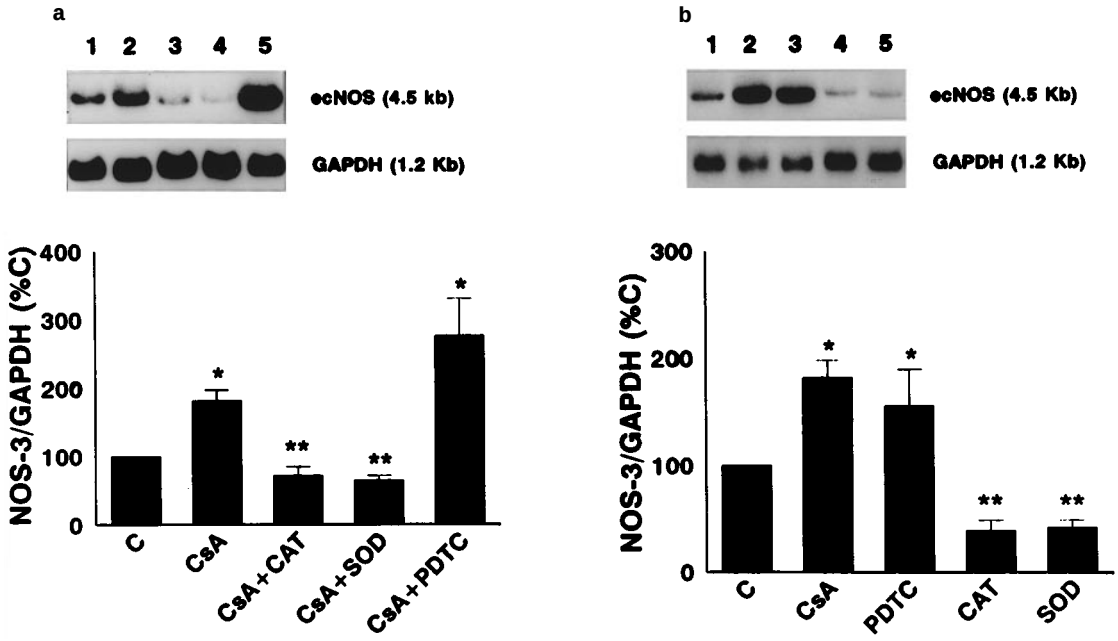

Figure 5 Effect of different antioxidants on the cyclosporine A (CsA)-induced up-regulation of eNOS mRNA expression in bovine cultured aortic endothelial cells. Cells were preincubated for 10 min with superoxide dismutase (SOD, 100 u ml^{-1}), catalase (CAT, 80 u ml^{-1}) or pirrolidin dithiocarbamate (PDTC, 100 μM), and then, without removing the antioxidants from the incubation media, 1 μM CsA was added to the cells for 24 h. In (a), the different combination of treatments are shown, lane 1: control; lane 2: CsA; lane 3: CsA + CAT; lane 4: CsA + SOD and lane 5: CsA + PDTC. In (b) only each treatment is shown, lane 1: control; lane 2: CsA; lane 3: PDTC; lane 4: CAT and lane 5: SOD. A representative Northern blot experiment of hybridization with eNOS and GAPDH probes is shown in the upper part of the figure. The lower part depicts the ratios between eNOS and GAPDH mRNA, after the densitometric analysis. These results are the mean $\pm$ s.e.mean of 4 different experiments, and are expressed as % of the control values (C). *$P<0.05$ vs C. **$P<0.05$ vs CsA.

Discussion

The present results suggest that the effect of CsA on the eNOS mRNA expression could be mediated by ROS. Three different results support this contention. Firstly, CsA was able to increase ROS synthesis in BAEC. Secondly, the ROS-generating systems selected for the present experiments, XXO and GO, mimicked the effect of CsA on eNOS mRNA expression (López-Ongil *et al*., 1996). Thirdly, SOD and catalase inhibited the effect of CsA on the expression of the mRNA of this enzyme. On the other hand, we provide data suggesting that the activation of the transcription factor AP-1 is associated with treatment of CsA and glucose oxidase, thus opening an avenue to explore its participation in the up-regulation of eNOS and other genes at the transcriptional level.

The CsA-induced overproduction of ROS was studied with two different fluorescent probes. DHR123 is a good sensor for different ROS, and it has been widely used for testing global ROS (Vowells *et al*., 1995; Yvonne *et al*., 1995). Although DHR123 does not specifically sense superoxide anion, it may react with peroxynitrite, which is formed after the reaction of superoxide with nitric oxide, as well as with hydrogen peroxide in the presence of significant amounts of Fe^{2+} (Koppenol *et al*., 1992; Henderson & Chappel, 1993; Royall & Ischiropoulos, 1993; Kooy *et al*., 1994; Crow *et al*., 1995). H_2DCFDA has been shown to be more specific for the detection of hydrogen peroxide, whereas it does not sense superoxide anion (Sanchez-Ferrer *et al*., 1990). These different specificities of the two fluorescent probes may account for the quantitative differences observed in the analysis of the intracellular oxidative burst when endothelial cells were treated with CsA: as DHR123

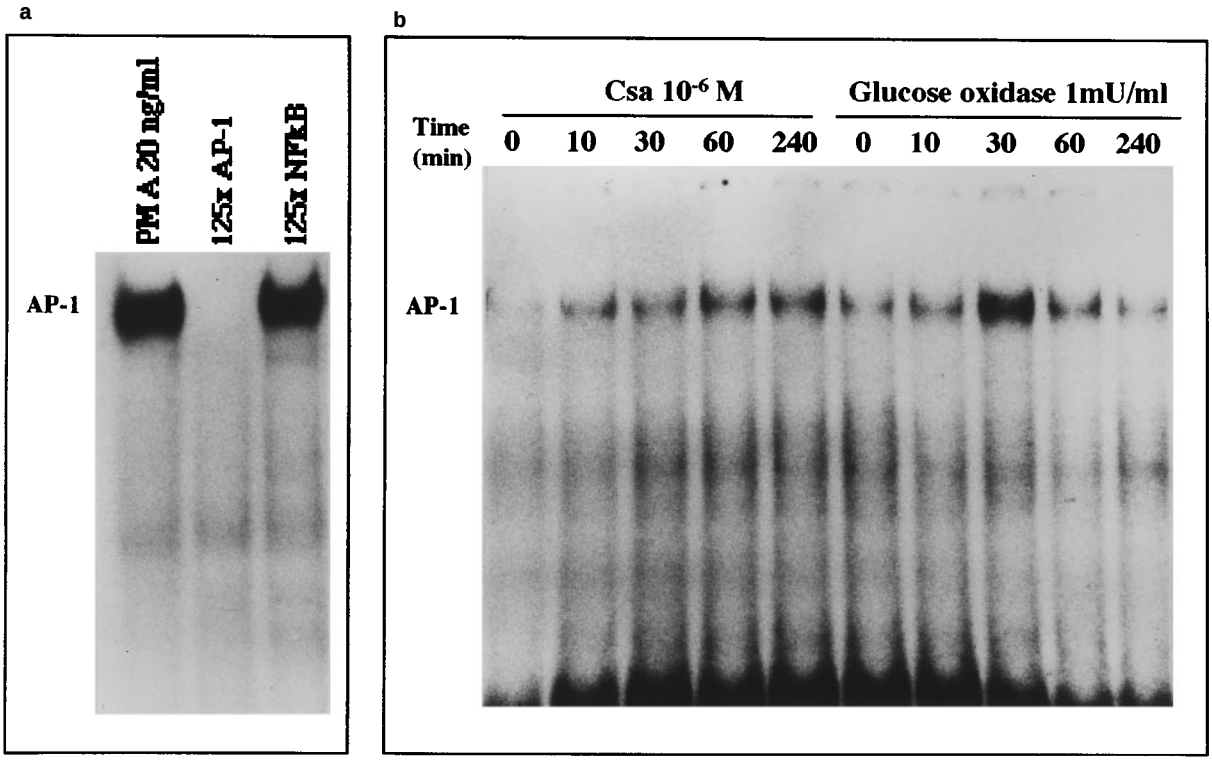

Figure 6 Effect of CsA and glucose oxidase on the binding of AP-1 to an oligonucleotide containing the AP-1 cis-regulatory sequence of the eNOS promoter. EMSA of nuclear extracts from BAEC treated as indicated were performed as described under Methods. (a) Controls for specificity of AP-1 binding. (b) BAEC treated with CsA 1 μM or glucose oxidase (GO, 1 mu ml^{-1}) for the indicated times. The blot shown is a representative experiment of 4 with nearly identical results.

Table 1 Cell toxicity assessed by the trypan blue dye exclusion

Treatments														
CsA	−	+	+	+	+	−	−	−	−	−	−	−	−	−
XXO	−	−	−	−	−	+	+	+	−	−	−	−	−	−
GO	−	−	−	−	−	−	−	−	+	+	+	−	−	−
SOD	−	−	+	−	−	−	+	−	−	+	−	+	−	−
CAT	−	−	−	+	−	−	−	+	−	−	+	−	+	−
PDTC	−	−	−	−	+	−	−	−	−	−	−	−	−	+
% trypan blue exclusion	100	100 ±2	81* ±6	89 ±5	95 ±3	85* ±7	83* ±5	80* ±4	100 ±3	96 ±4	93 ±5	88 ±7	89 ±8	97 ±3

Cells were incubated for 24 h at 37°C with different treatments tested on bovine aortic endothelial cells: cyclosporine A (CsA, 1 μM), xanthine-xanthine oxidase (XXO, 100 μM – 1 mu/ml^{-1}), glucose oxidase (GO, 1 mu/ml^{-1}), superoxide dismutase (SOD, 100 u/ml^{-1}), catalase (CAT, 80 u/ml^{-1}), pirrolidin dithiocarbamate (PDTC, 100 μM). These results are the mean ± s.e.mean of 3 different experiments, and are expressed as % of trypan blue dye exclusion of the control values (C). *$P<0.05$ vs C.)

senses not only hydrogen peroxide but also other ROS, the CsA-induced changes in the fluorescent signal was more intense with this probe.

Both XXO and GO induced a significantly increased expression of the eNOS mRNA expression. XXO generates equimolar amounts of superoxide anion and hydrogen peroxide (Band *et al*., 1981; Royall & Ischiropoulos, 1993), whereas GO only generates hydrogen peroxide (Sanchez-Ferrer *et al*., 1990). In consequence, it is suggested that both superoxide anion and hydrogen peroxide may be involved in the CsA-dependent up-regulation of the eNOS mRNA expression. The fact that both SOD and catalase block the effects of CsA on the mRNA expression of eNOS strongly supports this contention.

All these results must be considered in the light of data regarding cell viability observed with the different pharmacological agents employed. Antioxidant enzymes, particularly superoxide dismutase, induced minor changes in cell viability, both in basal conditions and in the presence of CsA. However, inhibition by antioxidants of CsA-mediated eNOS mRNA up-regulation could not easily be justified by a possible toxic action, given the different magnitude of the two effects.

Results obtained with PDTC on eNOS mRNA expression raised different questions. In contrast with the results obtained with SOD and catalase, eNOS mRNA expression increased in presence of PDTC. However, PDTC has other well-recognized actions, in addition to its antioxidant properties. Particularly, this and other related compounds are able to modulate the activity of different transcription factors in several cellular systems, and it has been demonstrated that PDTC is able to promote the activation of AP-1 as well as to inhibit NF-κB (Meyer *et al*., 1993; Schenk *et al*., 1994; Del Arco *et al*., 1996; Muñoz *et al*., 1996). In contrast with previous studies demonstrating the presence of NF-κB cis-regulatory elements in the promoter region of the iNOS gene (Xie *et al*., 1993; Lowenstein *et al*., 1993), no sequence of this type has been described in the promoter of the eNOS gene, whereas an AP-1-responsive cis-regulatory sequence has been shown in the eNOS promoter (Marsden *et al*., 1993; Robinson *et al*., 1994). This fact, together with the similar effects of PDTC and CsA on the mRNA expression of the eNOS gene, raised the possibility that the CsA-induced up-regulation of the eNOS mRNA expression could share pathways dependent on AP-1 activation. Although a detailed analysis of this problem is beyond the objectives of the present work, the ability of CsA and ROS to stimulate the binding of this transcription factor to nuclear extracts from BAEC is consistent with the concept that both CsA and GO may operate in a similar way and supports the idea that ROS are mediating CsA effects on eNOS mRNA expression. Of interest, CsA has been shown to enhance calcium-dependent activation of AP-1 in a lymphoma cell line (Su *et al*., 1996). Moreover, ROS are capable of increasing the DNA binding activity of AP-1 (Goldstone *et al*., 1995), but no previous studies have dealt with their possible role in the regulation of eNOS mRNA expression.

In conclusion, the present experiments support a role for ROS, particularly superoxide anion and hydrogen peroxide, as mediators of the CsA-induced eNOS up-regulation. They may also contribute to reconcile apparently contradictory results obtained in studies looking at the effect of CsA on endothelial function. While there is consensus on the fact that endothelial-dependent vasodilatation is impaired by CsA, some authors have proposed that NO synthesis is decreased (Sudhir *et al*., 1994), while others actually find the opposite (López-Ongil *et al*., 1996; Stroes *et al*., 1997). In one study endothelial dysfunction was completely prevented by the addition of SOD (Diederich *et al*., 1994). Our data are compatible with both types of observations, as ROS synthesized in the presence of CsA could significantly impair NO availability, thus explaining the SOD-dependent improvement in endothelial dysfunction (Diederich *et al*., 1994), but also mediate CsA-dependent increase in eNOS activity and mRNA expression (López-Ongil *et al*., 1996; Stroes *et al*., 1997). Because of this ability to up-regulate eNOS mRNA expression, an important role for ROS in the haemodynamic changes associated with pathophysiological situations in which these metabolites are increased, is also possible.

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