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Cyclosporine A and NAC on the inducible nitric oxide synthase expression and nitric oxide synthesis in rat renal artery cultured cells

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Background. The immunosuppressor cyclosporine A (CsA) presents the nephrotoxicity as its major side effect that is mostly attributed to a renal vasoconstriction. This may be due to an excessive generation of vasoconstrictors like reactive oxygen species (ROS), or due to a reduction of vasodilators such as the nitric oxide, which in turn, can be caused by increased amounts of ROS. We evaluated the effect of CsA and the antioxidant N-acetylcysteine (NAC) on inducible nitric oxide synthase (iNOS) mRNA expression and nitric oxide synthesis, in rat renal artery vascular smooth muscle cells (rVSMCs) primary culture.

Methods. In cells treated during 72 hours with CsA (10 μg/mL), its vehicle (control) (10 μL/mL), *Escherichia coli* lipopolysaccharide (LPS) (100 μg/mL), CsA + LPS, NAC (6.13 mmol/L), or CsA + NAC, we determined the nitric oxide synthesis (Griess and chemiluminescence methods), iNOS expression [reverse transcription-polymerase chain reaction (RT-PCR)] and cell viability (acridine orange method).

Results. In rVSMCs, LPS increased nitric oxide and iNOS expression; CsA decreased basal and LPS-induced nitric oxide and iNOS expression; NAC increased nitric oxide and blunted the nitric oxide reduction caused by CsA, with no effect on iNOS. CsA reduced cell viability.

Conclusion. In this study, CsA reduced nitric oxide synthesis in rVSMCs, both through iNOS down-regulation and reduction of cell viability, which could be responsible for the vasoconstrictive effect of the CsA. In the effect of CsA on nitric oxide, probably a role is also played by free radical production, as this effect was blunted by NAC.

Key words: cyclosporine A, N-acetylcysteine, nitric oxide, nitric oxide synthase, renal artery cell culture, nephrotoxicity.

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Cyclosporine A (CsA) is a powerful immunosuppressive agent, widely used in the prevention and treatment of organ transplant rejection episodes [1]. However, the clinical application of CsA is limited due to its several side effects, and among them nephrotoxicity is one of the most important. The nephrotoxicity is attributed to an intense vasoconstriction caused by CsA [2] which could be due to an increased production of vasoconstrictors like reactive oxygen species (ROS) or due to a reduction in vasodilators such as the nitric oxide.

Nitric oxide is produced by most cells of the human body and has as its substrate the L-arginine, which under the effect of nitric oxide synthase (NOS) generates L-citrulline and nitric oxide, that is metabolized to nitrite (NO_2^-) and nitrate (NO_3^-) [3, 4]. NOS presents two isoforms: the constitutive (cNOS) and the inducible (iNOS); iNOS can be induced by simple agents as, for instance, lipopolysaccharide (LPS), that is associated with the induction of sepsis [5]. There is evidence that LPS stimulates cytokine formation in macrophages, leading to an increased production of nitric oxide [6]. However, a mechanism independent of cytokines or perhaps a direct effect of LPS is suggested in studies using isolated rat aortic rings [7], LLC-PK_1 cells [8], or vascular smooth muscle cells (VSMCs) in culture [9], where no leukocytes were present, yet the cells produced nitric oxide in response to this drug.

It has been demonstrated that nitric oxide participates in the regulation of renal blood flow through its vasodilatory effects on the renal artery, and in the regulation of glomerular filtration, acting on the glomerular ultrafiltration coefficient [10].

Many studies indicate the participation of nitric oxide as a regulator of renal hemodynamics, its altered synthesis being related to ischemic [11] or nephrotoxic [12] acute renal failure (ARF).

In rat renal mesangial cells exposed to CsA it was observed a suppression of interleukin-1β (IL-1β) induced expression of NOS [13].

Currently, a great participation in cell injury has also been attributed to an excessive production of ROS and several drugs known as antioxidants were already demonstrated to prevent such injuries. Among these drugs, N-acetylcysteine (NAC), a thiol of low molecular weight, stands out regarding removal of free radicals [14].

DiMari et al [15] observed that NAC improves ischemic renal failure, and although these authors did not know which was the mechanism of action of NAC in this process, they believe that it could be due to both its antioxidant effect and a possible interaction between NAC and nitric oxide.

Therefore, in spite of the known nephrotoxic effect of CsA, a local action of this drug on the renal artery, along with a possible relationship between this and the synthesis of nitric oxide, are not enough elucidated yet.

The aim of the present study was to evaluate the effect of CsA and NAC on iNOS mRNA expression and nitric oxide synthesis in rat renal artery vascular smooth muscle cells (rVSMCs) primary culture.

METHODS

rVSMCs

Wistar male rats weighing 250 to 300 g were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), by intraperitoneal injection [16]. The kidneys were isolated through an abdominal incision and the renal arteries were removed and washed in cold phosphate-buffered saline (PBS). We used the "explant" technique for smooth muscle cell primary culture.

The cells were grown in 25 cm^2 culture flasks, in Dulbecco's modified Eagle's medium (DMEM) with $NaHCO_3$ (2 g/L), Hepes (2.6 g/L), and penicillin (10.000 IU/L) supplemented with 20% fetal bovine serum (FBS) and incubated in a humidified atmosphere with 5% CO_2, at 37°C. After the first passage, DMEM with 10% FBS was used.

The cells were used in the experiments between the third and tenth passages. After the semiconfluency was reached, the medium was replaced by DMEM with 10% FBS and without phenol red to avoid interference in the Griess colorimetric method, and the cells were treated according to the following groups:

Experimental groups

The experimental groups included the following: (*1*) control (CTL_{CsA}), 10 μL/mL of olive oil in dimethyl sulfoxide (DMSO) (1:100), as CsA vehicle; (*2*) *Escherichia coli* lipopolysaccharide (LPS), 100 μg/mL, which was used as a positive control of nitric oxide production; (*3*) CsA, 10 μg/mL in DMSO (1:100), used in a 1 mg/mL solution; (*4*) CsA + LPS; (*5*) CTL_{NAC}, 10 μL/mL of water, as NAC vehicle; (*6*) NAC, 6.13 mmol/L; and (*7*) CsA + NAC.

After 72 hours, the cell culture media were collected and stored at −20°C for nitric oxide determination. Then, the cells were lysed with sodium dodecyl sulfate (SDS) 2% and stored at −20°C for protein determination.

Dose-response curve

A distinct group of cells was treated during 72 hours with CsA (1, 5, 10, or 50 μg/mL) or NAC (0.61, 6.13, or 61.3 mmol/L) and a group of cells was treated simultaneously with 10μg/mL of CsA and NAC at 0.61, 6.13, or 61.3 mmol/L. The dose-response curve of nitric oxide synthesis was obtained through nitric oxide measurement in the cell culture media.

Time-response curve

The supernatant from the cells treated with CsA (10 μg/mL) or NAC (6.13 mmol/L), during different periods of time (24, 48, or 72 hours), was collected and the nitric oxide synthesis time-response curve was obtained.

Nitric oxide determination

Nitric oxide was determined in the cell culture media by the Griess colorimetric method [17] and by the chemiluminescence method [18].

Griess colorimetric method. Briefly, a vol:vol mixture of 1% sulfanilamide (in 5% H_3PO_4) and 0.1% naphthylethylenediamine solution was added to the samples and the absorbance at 546 nm was measured using a GENESYS 2 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY, USA). NO_2^-, one of the stable metabolites of nitric oxide, was then estimated from a standard curve constructed using $NaNO_2$.

Chemiluminescence method. We used the Model 280 Nitric Oxide Analyzer (NOA^{TM}) from Sievers Instruments, Inc. (Boulder, CO, USA), a high-sensitive detector for measuring nitric oxide, based on a gas-phase chemiluminescent reaction between nitric oxide and ozone:

$$NO + O_3 \rightarrow NO_2^- + O_2$$
$$NO_2^- \rightarrow NO_2 + hv$$

The emission from electrically excited nitrogen dioxide is in the red and near-infrared region of the spectrum, and it is detected by a thermoelectrically cooled red-sensitive photomultiplier tube. The sensitivity for measurement of nitric oxide and its reaction products in liquid samples is ∼1 picomole.

Intracellular protein

It was measured by the method of Lowry et al [19] and used as a cell number correction factor in the corresponding culture flasks.

Cell viability

Cellular viability in all experiments was assessed by the exclusion of the fluorescent dyes acridine orange and ethidium bromide [20]. After the cells were trypsinized, a 10 μL sample of cell suspension was incubated with 0.3 μL acridine orange/ethidium bromide solution (100 μL/mL of each dye). Acridine orange is cell permeable and binds to either the double-stranded DNA emitting a green fluorescence (excitation 502 nm and emission 525 nm) or single-stranded RNA emitting a reddish-orange fluorescence (excitation 460 nm and emission 650 nm). In contrast, ethidium bromide binds only to the double-stranded DNA and also emits a red fluorescence (excitation 510 nm and emission 595 nm); however, the cell is not permeable to the dye when the plasma membrane is intact, and therefore, only necrotic cells will take up this dye (with the red fluorescence quashing the green/orange of acridine orange). Cell suspensions were observed under a fluorescent microscope at 40× magnification, and 200 cells from a number of microscopic fields were counted. Cells emitting a green fluorescence were considered viable, and those emitting a red fluorescence, nonviable. Viable cells were expressed as percentage of the total counted cells.

Reverse transcription-polymerase chain reaction (RT-PCR) of iNOS

Total RNA was extracted from cultured cells by the Trizol extraction method. The resulting RNA was used to generate the cDNA fragment corresponding to the iNOS by RT-PCR amplification using the primers 5′-CCG GATCCTCTTTGCTACTGAGACAGG-3′ and 5′-CCG AATTCGGGATCTGAATGCAATGTT-3′. The cycling parameters were hot start at 95°C for 3 minutes, followed by 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 7 minutes, and 4°C. The RT-PCR products were resolved on a 3% agarose gel and visualized by ethidium bromide staining. The housekeeping gene β-actin was used for internal control. The band density was analyzed by scanning densitometry using image Quant 5.2 (Storm) (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). To compare nitric oxide between the groups we used the one-way analysis of variance (ANOVA). The RT-PCR was quantified by densitometry, and the difference in the iNOS/β-actin ratio between the groups was compared by ANOVA with Tukey's test. Statistical significance was defined as $P < 0.05$.

Chemicals

Acridine orange, copper sulfate, DMEM, folin, Hepes, LPS (from *E. coli* serotype O111:B4), NaK-tartrate, naphthylethylenediamine, ortho-phosphoric acid, ethidium bromide, PBS, penicillin, SDS, sodium bicarbonate, sodium hydroxide, sodium nitrate, sodium nitrite, sulfanilamide, trypsin-ethylenediaminetetraacetic acid (EDTA) solution and vanadium were purchased from Sigma Chemical Company (St. Louis, MO, USA). FBS was obtained from Gibco BRL (São Paulo, Brazil) or Cultilab (Campinas, Brazil). Ketamine and xylazine were obtained from Virbac (São Paulo, Brazil). Molecular biology chemicals, including agarose, bovine serum albumin (BSA), dithiothreitol (DTT), magnesium chloride solution, Moloney-murine leukemia virus (M-MLV) reverse transcriptase, M-MLV RT 5 × reaction buffer, primers, Taq DNA polymerase, and Trizol® reagent, were purchased from Invitrogen Life Technologies (Gaithersburg, MD, USA). Dietilpirocarbonate (DEPC), ethidium bromide solution, and loading buffer were purchased from Sigma Chemical Company. Deoxynucleoside triphosphate (dNTP) and RNasin® were purchased from Promega Corporation (Madison, WI, USA). Finally, oligo(dT) primer was purchased from Pharmacia Biotech (Uppsala, Sweden).

RESULTS

In order to determine the synthesis of the nitric oxide we used two different methods, the Griess and the chemiluminescence method.

We present the results of nitric oxide synthesis analyzed by both methods in a cultured rVSMC after treatment with CsA, LPS, or CsA + LPS, during 72 hours (Table 1). By the Griess method, we verified that in LPS group there was an increase of nitric oxide when compared to control (410 ± 62.1 vs. 18.1 ± 0.5 nmol/mg protein, respectively ($N = 6$ for both groups) ($P < 0.05$). The CsA group (7.3 ± 1.2 nmol/mg protein) ($N = 6$) ($P < 0.05$), presented a significant reduction in the synthesis of nitric oxide when compared to control and LPS groups. The treatment with CsA + LPS also caused a significant reduction in nitric oxide production (78.5 ± 11.7 nmol/mg protein) ($N = 6$) ($P < 0.05$) when compared to LPS group.

By the chemiluminescence method we verified the same behavior for each group. The LPS group presented a significant increase in the nitric oxide synthesis when compared to control (2250.1 ± 157.3 vs. 207.9 ± 23 nmol/mg protein, respectively) ($N = 6$ for both groups) ($P < 0.05$). The CsA group showed a significant reduction when compared to control or LPS groups (78.5 ± 9.2 nmol/mg protein) ($N = 6$) ($P < 0.05$). When we used

Table 1. Nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) treated with cyclosporine A (CsA) (10 μg/mL) and/or lipopolysaccharide (LPS) (100 μg/mL) during 72 hours

	CTL_{CsA}	LPS	CsA	CsA + LPS
Griess	18.1 ± 0.5	410 ± 62.1[a]	7.3 ± 1.2[a,b]	78.5 ± 11.7[a,b]
Nitric oxide analyzer	207.9 ± 23	2250.1 ± 157.3[a]	78.5 ± 9.2[a,b]	660 ± 78.9[a,b]

$N = 6$ for all groups; mean ± SEM; one-way analysis of variance (ANOVA)
[a] $P < 0.05$ vs. control; [b] $P < 0.05$ vs. LPS.

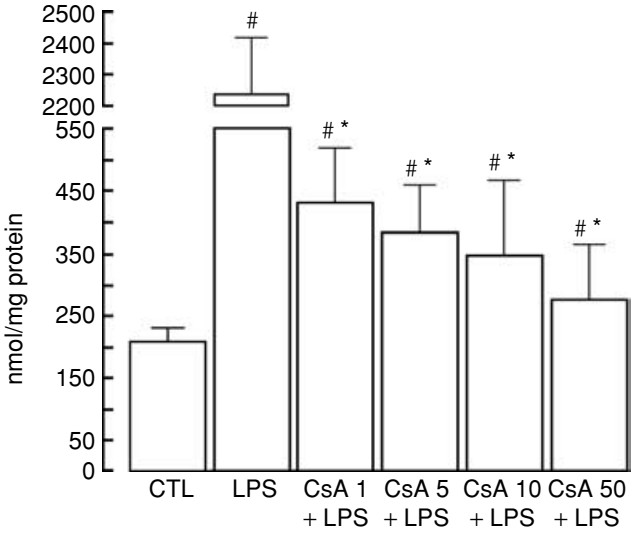

Fig. 1. Dose-response curve of nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) exposed to cyclosporine A (CsA) (1, 5, 10, or 50 μg/mL) and/or lipopolysaccharide (LPS) (100 μg/mL) during 72 hours, analyzed by the chemiluminescence method. Mean ± SEM ($N = 6$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. LPS.

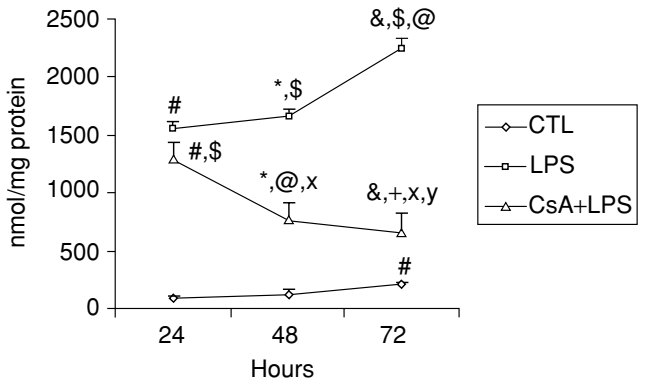

Fig. 2. Time-response curve of nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) exposed to cyclosporine A (CsA) (10 μg/mL) and lipopolysaccharide (LPS) or LPS alone (100 μg/mL) during 24, 48, or 72 hours, analyzed by the chemiluminescence method. Mean ± SEM ($N = 6$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. control (CTL_{24}); $^{*}P < 0.05$ vs. CTL_{48}; $^{\&}P < 0.05$ vs. CTL_{72}; $^{\$}P < 0.05$ vs. LPS_{24}; $^{@}P < 0.05$ vs. LPS_{48}; $^{+}P < 0.05$ vs. LPS_{72}; $^{\times}P < 0.05$.vs. CsA + LPS_{24}; $^{y}P < 0.05$ vs. CsA + LPS_{48}.

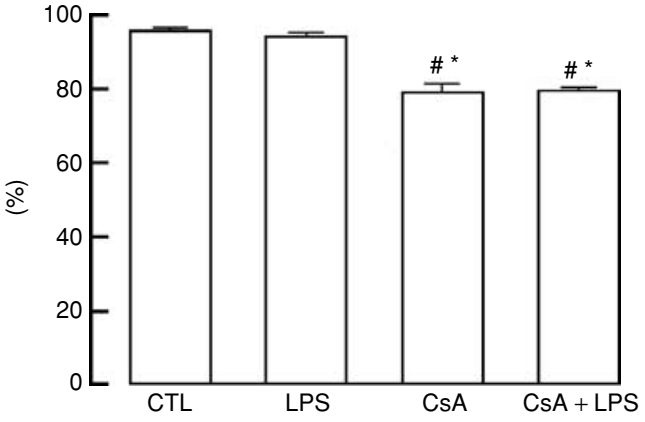

Fig. 3. Cellular viability (%) in rat renal artery vascular smooth muscle cells (rVSMCs) exposed to cyclosporine A (CsA) (10 μg/mL) and/or lipopolysaccharide (LPS) (100 μg/mL) during 72 hours. Mean ± SEM ($N = 4$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. LPS.

CsA and LPS simultaneously we observed that there was a reduction in the nitric oxide synthesis when compared to LPS group (660 ± 78.9 nmol/mg protein)($N = 6$) ($P < 0.05$).

In the dose-response curve of nitric oxide synthesis (Fig. 1) analyzed by the chemiluminescence method, we observed a significant nitric oxide increase in LPS group when compared to control (2250.1 ± 157.3 vs. 207.9 ± 23 nmol/mg protein) ($N = 6$ for both groups) ($P < 0.05$). We observed that CsA at all concentrations (1, 5, 10, or 50 μg/mL) was capable to inhibit the nitric oxide induced by LPS (429.3 ± 91.2, 382.2 ± 74.9, 346.3 ± 124, and 274 ± 90.5 nmol/mg protein, respectively) ($N = 6$ for all groups) ($P < 0.05$) and this effect increased for each increase of CsA concentration.

For the time-response curve (Fig. 2), rVSMCs were incubated with CsA (10 μg/mL) and/or LPS (100 μg/mL) during 24, 48, or 72 hours. We observed that in control (91.5 ± 8.7, 116.1 ± 42.6, and 207.9 ± 23 nmol/mg protein, respectively) ($N = 6$) and LPS (1556.7 ± 146.1, 1663.3 ± 148.7, and 2250.1 ± 157.3 nmol/mg protein, respectively) ($N = 6$) the synthesis of nitric oxide increased significantly and gradually according to the increase of incubation time. This increase in LPS group was significant when compared to control at all incubation periods. In CsA + LPS group (1290.7 ± 66.7, 759.2 ± 60.8, and 660 ± 78.9 nmol/mg protein, respectively) ($N = 6$), there was a significant reduction in nitric oxide synthesis, according to the incubation time, when compared to LPS at all periods.

Regarding cellular viability (Fig. 3), the groups CsA and CsA + LPS presented a significant reduction (78.4 ± 2%, 78.7 ± 0.4%, respectively) ($N = 4$) ($P < 0.05$) when

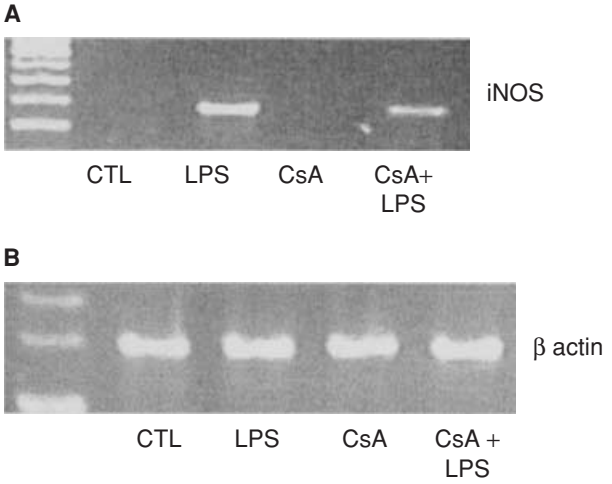

Fig. 4. Effects of cyclosporine A (CsA) and/or lipopolysaccharide (LPS) on inducible nitric oxide (iNOS) mRNA expression in rat renal artery smooth muscle cells (rVSMCs). (*A*) Representative reverse transcription-polymerase chain reaction (RT-PCR) of four independent experiments showing a 464 bp product corresponding to the iNOS isoform, only in the LPS and CsA + LPS groups. (*B*) RT-PCR products (191 bp) using the primers for β-actin. Densitometric analysis of iNOS/β-actin mRNA showed a significant decrease on CsA + LPS (0.24 ± 0.14 relative densitometric units) compared to LPS group (0.65 ± 0.12) ($P < 0.05$).

Table 2. Nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) treated with N-acetylcysteine (NAC) (6.13 mmol/L) + NAC or cyclosporine A (CsA) (10 μg/mL) during 72 hours, analyzed by the chemiluminescence method

CTL_{NAC}	204 ± 8.0
NAC	326.8 ± 22.5[a]
CsA + NAC	365.4 ± 28.6[a]

$N = 7$ for all groups. Mean ± SEM; one-way analysis of variance (ANOVA)
[a] $P < 0.05$ vs. control.

compared to control (95.5 ± 0.5%) ($N = 5$) ($P < 0.05$). The LPS group showed a cellular viability of 93.8 ± 0.7, similar to control.

By RT-PCR, there was no detection of iNOS in rVSMCs treated with CsA, as well as in the control. However, iNOS mRNA was up-regulated in cells treated with LPS or CsA + LPS. Densitometric analysis of iNOS mRNA corrected for β-actin expression (relative densitometric units) of four independent experiments showed a significant decrease on CsA + LPS compared to LPS group (0.24 ± 0.14 vs. 0.65 ± 0.12, respectively) ($P < 0.05$) (Fig. 4).

We present the results of nitric oxide synthesis obtained through the chemiluminescence method after treatment with NAC or CsA + NAC during 72 hours (Table 2). NAC (326.8 ± 22.5 nmol/mg protein) ($N = 7$) showed a significant increase of nitric oxide when compared to control (204 ± 8.0 nmol/mg protein) ($N = 7$). When CsA and NAC were used simultaneously, we also observed

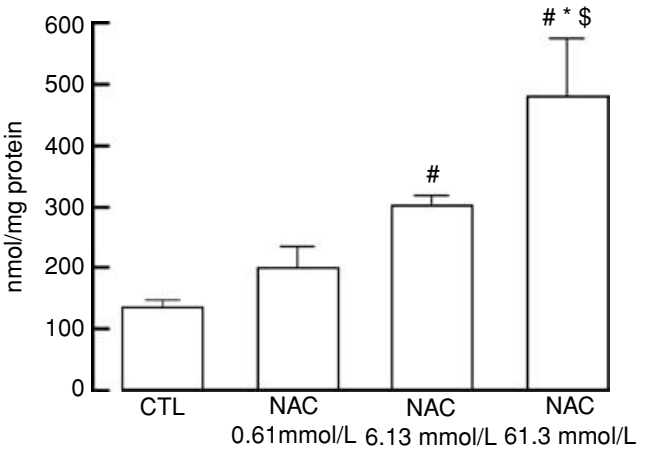

Fig. 5. Dose-response curve of nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) exposed to N-acetylcysteine (NAC) (0.61, 6.13, or 61.3 mmol/L) during 72 hours, analyzed by the chemiluminescence method. Mean ± SEM. ($N = 7$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. NAC 0.61 mmol/L; $^{\$}P < 0.05$ vs. NAC 6.13 mmol/L.

a significant increase in nitric oxide synthesis (365.4 ± 28.6 nmol/mg protein) ($N = 7$) comparatively to control.

We show the dose-response curve of nitric oxide synthesis obtained by chemiluminescence method, in cultured rVSMCs treated with different concentrations of NAC (0.61, 6.13, and 61.3 mmol/L) (Fig. 5). We observed that NAC is capable to increase the nitric oxide production at all concentrations (198 ± 9.5, 300.3 ± 18.0, and 479.4 ± 33.3 nmol/mg protein) ($N = 7$) when compared to control (135.1 ± 8.7 nmol/mg protein) ($N = 7$). Only at NAC 6.13 and NAC 61.3 mmol/L the nitric oxide increase was significant when compared to control; at the dose of 61.3 mmol/L, the increase was significant in relation to 6.13 mmol/L.

The time-response curve of nitric oxide synthesis obtained by the chemiluminescence method (Fig. 6), when rVSMCs were incubated with NAC (6.13 mmol/L) during 24, 48, or 72 hours, presented both in controls (102.3 ± 4.7, 132.3 ± 4.7, and 195.6 ± 10.7 nmol/mg protein, respectively) ($N = 7$ for all groups) and NAC groups (128 ± 7.3, 191.7 ± 13, and 326.8 ± 22.5 nmol/mg protein) ($N = 7$ for all groups) a gradual and significant increase of nitric oxide at each increase of incubation time.

In the Figure 7 we show the dose-response curve of nitric oxide synthesis, analyzed by chemiluminescence method in cultured rVSMCs exposed to NAC (0.61, 6.13, or 61.3 mmol/L) during 72 hours, in the presence of CsA (10 μg/mL). We observed that CsA reduced the nitric oxide synthesis (100.9 ± 14.8 nmol/mg protein) ($N = 5$) when compared to control (245.9 ± 21.1 nmol/mg protein) ($N = 5$) ($P < 0.05$). NAC, in all concentrations, increased nitric oxide even in the presence of CsA (301.2 ± 47.5, 388.7 ± 27.4, and 538.1 ± 8.57 nmol/mg protein)

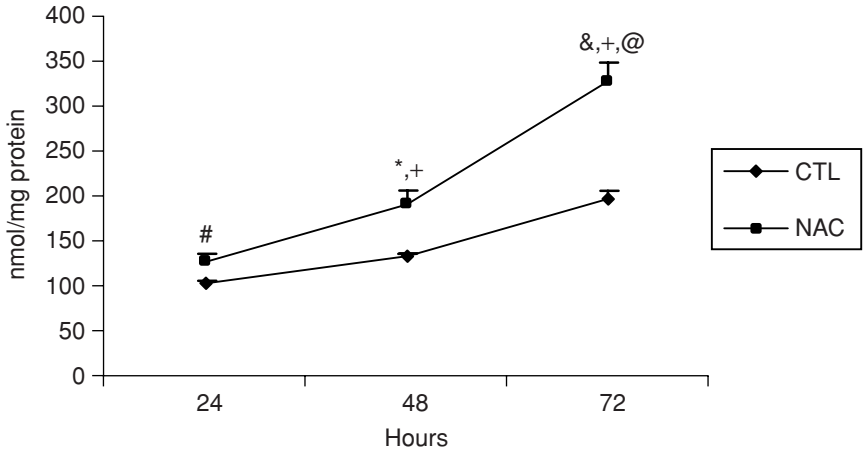

Fig. 6. Time-response curve of nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) exposed to N-acetylcysteine (NAC) (6.13 mmol/L) during 24, 48, or 72 hours, analyzed by the chemiluminescence method. Mean ± SEM ($N = 7$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. CTL_{24}; $^{*}P < 0.05$ vs. CTL_{48}; $^{\&}P < 0.05$ vs. CTL_{72}; $^{+}P < 0.05$ vs. NAC_{24}; $^{@}P < 0.05$ vs. NAC_{48}.

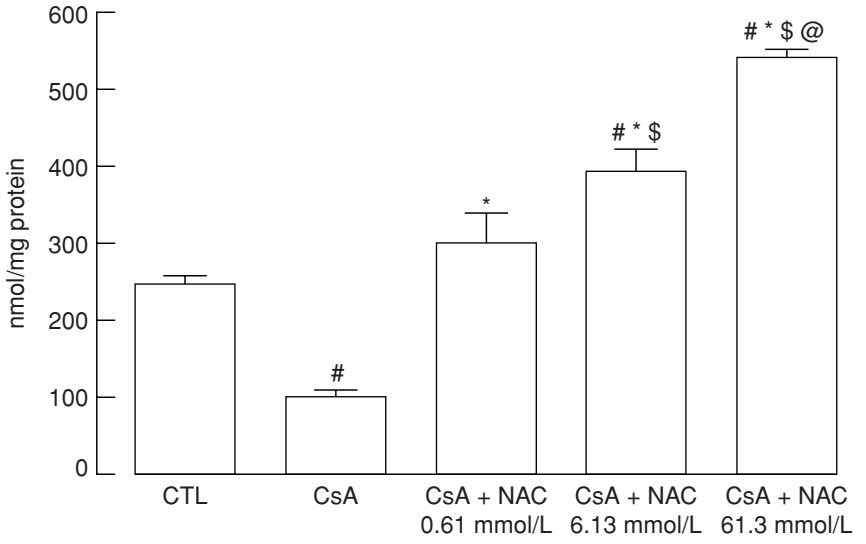

Fig. 7. Dose-response curve of nitric oxide synthesis (nmol/mg protein) in cultured rat renal artery vascular smooth muscle cells (rVSMCs) exposed to N-acetylcysteine (NAC_ (0.61, 6.13, or 61.3 mmol/L) in the presence of cyclosporine A (CsA) (10 µg/mL) during 72 hours, analyzed by the chemiluminescence method. Mean ± SEM ($N = 5$ for all groups). One-way analysis of variance (ANOVA). $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. CsA; $^{\$}P < 0.05$ vs. CsA + NAC 0.61 mmol/L; $^{@}P < 0.05$ vs. CsA + NAC 6.13 mmol/L.

($N = 5$ for all groups) ($P < 0.05$ vs. control for CsA + NAC 6.13 and 61.3 mmol/L).

In the cells treated with NAC, there was no expression of iNOS mRNA, as well as in the control, CsA, and CsA + NAC groups. On the contrary, in the LPS group, as previously seen, RT-PCR demonstrated the presence of iNOS (Fig. 8).

DISCUSSION

The nephrotoxicity caused by CsA, a powerful immunosuppressive agent, is a limitant factor for its clinical use and its pathophysiology is still controversial. In experimental models it was observed that CsA increases the renal vascular resistance and decreases the renal blood flow [21]. In addition, this immunosuppressor induced an intense constriction of the renal microcirculation, which was dependent on continuous production of nitric oxide [2].

Previous reports suggest the participation of nitric oxide in ischemic ARF since L-arginine, the substrate for nitric oxide synthesis, had a beneficial effect on the recovery phase of renal failure in rats [22]. However, in other studies nitric oxide seems to increase the renal injury [23] probably through its reaction with the superoxide radical (O_2^-), generating the very cytotoxic peroxynitrite [24]. Besides, there are no conclusive data yet, regarding the nitric oxide production in ARF, as some studies show increased amounts [11, 25, 26] whether others suggest decreased synthesis of this molecule [12].

In this study we evaluated the expression of iNOS and the nitric oxide synthesis in cultured rVSMCs treated with CsA, as well as the influence of the antioxidant NAC on the nitric oxide production in these cells.

It is known that cells in culture present a very low basal production of nitric oxide; therefore, we used two methods for the detection of this molecule: Griess and chemiluminescence. Through the chemiluminescence method we obtained higher values of nitric oxide in relation to

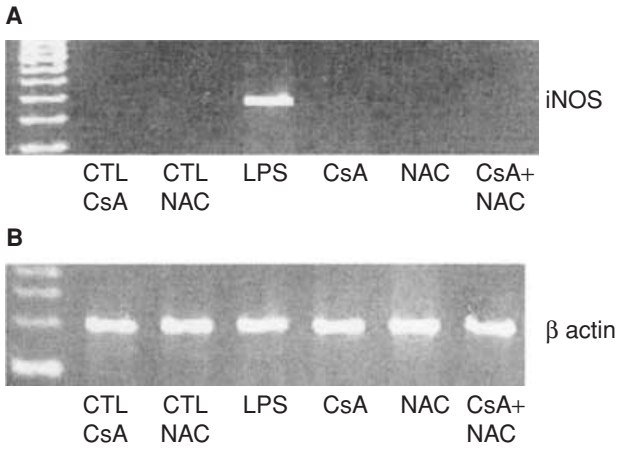

Fig. 8. Effects of cyclosporine A (CsA), lipopolysaccharide (LPS), or N-acetylcysteine (NAC) on inducible nitric oxide synthase (iNOS) mRNA expression in rat renal artery smooth muscle cells (rVSMCs). (*A*) Representative reverse transcription-polymerase chain reaction (RT-PCR) of four independent experiments showing a 464 bp product corresponding to the iNOS isoform, only in the LPS group. (*B*) RT-PCR products (191 bp) using the primers for β-actin. Densitometric analysis of iNOS/β-actin mRNA showed (iNOS) mRNA expression only in the LPS group (0.65 ± 0.12 relative densiometric units).

the Griess method, since the former is considered more sensitive, as showed for example, by Kanamaru et al [27]. In addition, unlike the Griess method, which only detects the NO_2^-, through the chemiluminescence we can detect both NO_2^- and NO_3^-.

In this study we used cultured cells collected from renal arteries. It should be very interesting if VSMCs were grown from renal arterioles, where CsA probably plays an important constrictive effect.

The cells treated by CsA presented a significant decrease in the nitric oxide production, both by the Griess and chemiluminescence methods, as compared to control. The CsA concentrations used in this study, including the lowest value in the dose-response curve, are above the therapeutic peak. Previous studies by Meyer-Lehnert and Schrier [28] showed that 10 μg/mL of CsA was able to induce VSMC contraction, and in our laboratory, we observed that CsA in the same concentration caused reduction of nitric oxide synthesis by LLC-PK_1 cells in culture [8]. This decrease of nitric oxide was also observed when we incubated the cells with CsA + LPS, demonstrating once again the effect of CsA, which was able to reduce the LPS-stimulated production of this molecule. When the cells were exposed to LPS with different concentrations of CsA (dose-response curve), there was a progressive reduction in nitric oxide production when compared to LPS alone, and from 1 μg/mL of CsA on, this reduction was already significant. The effect of CsA reducing the nitric oxide synthesis induced by LPS was also time-dependent.

A similar effect was reported in the studies of Conde et al [29] who observed that the CsA inhibited the basal or the interferon γ + LPS stimulated production of NO_2^- in cultured macrophages. Nitric oxide inhibition was also demonstrated in mesangial cells exposed to CsA, by the evaluation of the IL-1β–induced NOS expression [13]. A similar effect to that of CsA inhibiting nitric oxide synthesis by a direct action was observed in VSMCs [30] and also in macrophage culture [31].

Ribeiro et al [9], in our laboratory, showed that rVSMCs, exposed to different types of radiocontrast, which are nephrotoxic agents as CsA, presented a reduction in the synthesis of nitric oxide. Although this effect on nitric oxide was more pronounced in the cells treated with the high osmolality ioxitalamate, by using mannitol they showed that the osmolality was not related to the nitric oxide reduction.

There are several hypotheses on the mechanism of action of CsA in the reduction of nitric oxide synthesis. The blockade of tumor necrosis factor-α (TNF-α), which is one of the several stimuli for nitric oxide production [32], can be one of these mechanisms. This effect is compatible with the decreased nitric oxide production obtained by Lima et al [8] when they treated the LLC-PK_1 cells with TNF-α and CsA. The reduction in the viability of LLC-PK_1 cells treated with CsA observed by these authors, which was also observed in the rVSMC in our study, could explain the nitric oxide reduction caused by CsA. However, the intense reduction in nitric oxide synthesis (60%) caused by CsA may not be totally explained by the cellular death caused by this substance, which was of the order of 18%.

In the present study, we analyzed the iNOS mRNA. In rVSMCs treated with LPS the expression of iNOS mRNA was increased when compared to control, but lower in the CsA + LPS when compared to LPS, which suggests a blockade of nitric oxide synthesis by CsA, at the transcriptional level. Other factors might be involved, perhaps in association with the described ones [e.g., the depletion of a cofactor such as tetrahydrobiopterin, essential in the mechanism of nitric oxide production, or the nuclear factor kappaB (NF-κB), which can reduce nitric oxide by inhibiting the iNOS mRNA transcription].

There is also the possibility that CsA can induce an excessive formation of the O_2^-, which reacts with nitric oxide, reducing its levels. Cellular markers suggesting ROS generation and lipid peroxidation were, for example, detected during ischemia/reperfusion injury [33]. Shah and Walker [34] demonstrated that in nephrotoxic ARF caused by glycerol, treatment with N′, N′-dimethylthiourea (DMTU) and sodium benzoate, an antioxidant that preferentially scavenges hydroxyl radical (OH^-), preserved the renal function. In addition, they showed that the use of deferoxamine, an iron chelator, which can block the generation of OH^-, protected the renal function.

Other studies showed that the nephrotoxicity caused by radiocontrasts can be attenuated by NAC [35, 36], and

although the mechanism of action of NAC in this process is still unknown, according to DiMari et al [15] it might be due to both its antioxidant properties and to a possible interaction between NAC and nitric oxide.

NAC is a thiol with low molecular weight, with mucolytic action, which has an antioxidant effect by removing free radicals, and it is also responsible for the synthesis of intracellular glutathione, a natural antioxidant [14].

In our study, when rVSMCs were exposed to NAC, there was a significant increase in the nitric oxide production, which was also observed after incubating these cells with CsA + NAC, indicating that somehow, NAC completely prevented the nitric oxide reduction caused by CsA. We also analyzed the synthesis of iNOS mRNA after NAC treatment. The iNOS expression was not modified in rVSMCs treated with NAC, neither with CsA + NAC. These results suggest that NAC increases the potent vasodilator nitric oxide, through its antioxidant action, removing ROS and inhibiting the formation of peroxynitrite.

CONCLUSION

In our study CsA reduced the nitric oxide production in rVSMCs, in part by the decrease in cellular viability and in part due to down-regulation of iNOS mRNA. In the effect of CsA on nitric oxide, probably a role is also played by free radical production, as this effect can be blunted by NAC. The reduced synthesis of nitric oxide could be related to the vasoconstriction and, therefore, to the nephrotoxicity caused by this immunosuppressor. Clinical studies should be undertaken to verify if maneuvers to control CsA induced reduction of nitric oxide, including the use of NAC, can prevent this nephrotoxicity.

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