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LABORATORY INVESTIGATION

A possible role for nitric oxide in modulating the functional cyclosporine toxicity by arginine

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A possible role for nitric oxide in modulating the functional cyclosporine toxicity by arginine. The renal damage consequent to cyclosporine A (CsA) administration ranges from hemodynamic alterations to irreversible chronic lesions. The initial vasoconstriction depends upon the imbalance between the various modulators of the renal vascular tone, among which the most powerful are endothelins and nitric oxide (NO). CsA could play a crucial role by inhibiting the Ca^{++}/calmodulin-mediated activation of the constitutive NO synthase (NOS) isoform, which converts L-arginine (L-Arg) into NO and citrulline, with a 1:1 stoichiometry. To investigate the possibility of modulating CsA nephrotoxicity with L-Arg we studied six groups (G) of Lewis rats treated with daily gavage up to eight weeks: G1, CsA 40 mg/kg; G2, G1 plus L-Arg 300 mg/kg; G3, G2 plus the competitive inhibitor of NOS, N^G-nitro-L-Arg (L-NNA); G4, L-Arg alone; G5, L-NNA alone; and G6, controls receiving vehicle alone. After eight weeks L-Arg treated rats were protected against the toxic effects of CsA [creatinine (Cr) values, G2, 0.62 ± 0.05 mg/dl vs. G1, 0.99 ± 0.16 mg/dl, $P < 0.001$; proteinuria (P), G2, 7.2 ± 1.02 mg/day vs. G1, 15.1 ± 1.9 mg/day, $P < 0.01$]. The administration of L-NNA abolished the protective effect of L-Arg (G3, Cr 1.23 ± 0.16 mg/dl; P 16.9 = 2.3; $P < 0.02$ and $P < 0.005$, respectively vs. G2). The levels of Cr in G2 rats were superimposable to control groups. The NOS activity, evaluated by measuring [^{3}H]citrulline formation from [^{3}H]L-Arg in kidney homogenates, was blocked by L-NNA in G5 (0.019 ± 0.009 pmol/min/mg proteins vs. G6 0.047 ± 0.002, $P < 0.01$). NOS activity was significantly increased versus controls in G1 (0.110 ± 0.032, $P < 0.01$) and G2 (0.088 ± 0.009, $P < 0.02$), while L-NNA reversed this phenomenon (G3, 0.052 ± 0.03). The expression of mRNA encoding for cNOS and iNOS was only slightly increased in CsA-treated rats. We suggest that CsA treatment increases NOS activity in the kidney by a mechanism which does not require a *de novo* synthesis of the enzyme. Such an increase, that may be devoted to counterbalance the vasoconstrictive effects of the drug, is unable to reduce the toxic effect of CsA in the absence of exogenous L-Arg. Administration of L-Arg is likely to reduce CsA nephrotoxicity by accomplishing the higher request of activated NOS for its substrate, thus potentiating NO synthesis in the kidney.[1]

The full potential employment of cyclosporine A (CsA) in the prevention of rejection in organ transplantation [1, 2] and in many other therapeutical applications [3] is limited by the occurrence of several important side effects, particularly nephrotoxicity [3, 4].

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Renal disfunction can occur at any time and ranges from an early reversible functional damage to a late progression to irreversible chronic renal failure. Acute nephrotoxicity may appear soon after transplantation or after weeks or months, with oliguria, acute decrement of glomerular filtration rate and renal plasma flow [3–7]. Due to the lack of histological lesions, the acute toxicity is usually believed to be consequent to renal vasoconstriction [3–7]. Conversely, after prolonged CsA administration chronic nephrotoxicity is characterized by a progressive and mostly irreversible impairment of renal function, and it is supported by histological lesions ranging from striped fibrosis to ischemic collapse of the tuft, glomerular sclerosis and tubular atrophy [8]; however, the increase in interstitial matrix preceding the interstitial fibrosis might be due to a direct toxic effect of CsA [4, 6].

Several factors contribute to CsA-induced vasoconstriction, including adrenergic mediators and renin-angiotensin axis hyperstimulation [9–11]. Moreover, the capacity of CsA to be inserted into the lipid bilayer of the cell membrane and activate phospholipase A_2 has suggested a possible interference of this drug in the arachidonic acid metabolism [12–14]. A pivotal role in CsA toxicity seems to be played by endothelins (ET) [15, 16], as the synthesis of both ET and vasoconstrictive eicosanoids implicates an increase in cytosolic Ca^{++}. Several attempts to modulate CsA nephrotoxicity through the employment of Ca^{++} antagonists have been proposed [5, 17, 18].

Experimental evidence suggests an important role of the decrease of vasorelaxant mediators in the pathogenesis of the renal vasoconstrition initiating CsA nephrotoxicity [19–21]. Among these substances, the endothelium-derived relaxing factor (EDRF), now biochemically characterized as nitric oxide (NO), is one of the most effective [22, 23]. Endothelium-derived NO has been demonstrated to play an important role in the local regulation of blood flow in kidney and in other organs [22]. It is produced in many tissues by a family of NO synthases (NOS), which transform L-arginine (L-Arg) into NO and L-citrulline, with a 1:1 stoichiometry [22, 24, 25]. A constitutive Ca^{++}/calmodulin-dependent form (cNOS) is activated by agonists that increase intracellular Ca^{++} and is blocked by the binding of CsA to calmodulin via the formation of the complex CsA/cyclophilin [5, 23, 24]. Another NOS isoform is Ca^{++}-independent and inducible by endotoxin

and many cytokines (iNOS) [22]. Recently a Ca^{++}-dependent, calmodulin-independent NOS has been described [22]. NO, by stimulating the cytosolic guanylate cyclase, is able to induce an increase of cyclic GMP (cGMP) production [26] which can account for the vasodilating effect of NO. An NO-mediated increase of cGMP has been observed not only in smooth muscle cells but also in other contractile cells, such as endothelium, pericytes and mesangial cells [27, 28]. It has been demonstrated that CsA chronic administration impairs NO production by rat kidney arteries [19, 20].

At the time of the first description of renal vasoconstriction induced by CsA, the role of nitric oxide had not been characterized. Therefore we aimed this study to: (1) investigate the effects of *in vivo* administration of CsA on the NOS activity in the rat kidney; (2) evaluate the possibility of modulating CsA nephrotoxicity by administrating L-Arg to increase the substrate availibility for NO synthesis and then to counteract the renal vasoconstriction.

Methods

Experimental design

Experiments were conducted in 90 male Lewis rats (150 to 170 g body weight) (Charles River, Calco, Italy). Animals were allowed free access to tap water and each had 16 g/day of standard rat laboratory diet.

Six groups of rats were identified.

The first group (G1) received CsA (Sandoz Pharmaceutical, Morris Plains, USA) by daily intragastric gavage at the dose of 40 mg/kg, using as vehicle a 10% fat emulsion (Intralipid, Kabivitrum, Stockholm, Sweden). G2 received CsA plus L-Arg 300 mg/kg by the same intragastric route. G3, together with CsA and L-Arg also received N^G-nitro-L-arginine (L-NNA), a competitive antagonist of L-Arg, 5 mg/kg.

G4 to G6 were the pair-fed control groups: G4 rats were treated with L-Arg alone, G5 with L-NNA alone and G6 with the lipophilic vehicle alone.

In every group eight animals were sacrificed at the fourth week and the remaining seven at the eighth week. At the end of the experiment, before sacrifice, 24-hour urine collections from rats in individual metabolic cages and blood samplings were performed. One kidney was used for morphologic studies and the other was snap frozen and stored at −70°C until the check of NOS activity and RNA extraction. Data from three separate experiments, each including all six groups of rats, were pooled because the results were superimposable.

Five rats in group G1, four rats in G2 and four controls (G6) underwent a measurement of radioisotopic glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) before sacrifice at four weeks.

Blood and urine analysis

CsA blood levels. CsA in whole blood was assayed 24 hours after the last administration by means of a RIA kit employing a monoclonal antibody (Cyclo-Trac^R, Incstar Corporation, Stillwater, MN, USA).

Plasma creatinine levels. After centrifugation of blood samples collected in EDTA 0.2 M, serum creatinine was determined using the Jaffé's colorimetric method on a Cobas-Miras Instrument (Roche Diagnostic System, Milan, Italy).

Proteinuria. After the 24 hour collection, urines were centrifuged to eliminate cells and the dosage was performed on the supernatant by evaluating the turbidity induced by 3% sulfosalicilic acid and 7% sodium sulfate in distilled water. The protein concentration was expressed in mg/24 hr with human albumin as standard.

Urinary excretion of cGMP. Urinary cGMP was evaluated using a commercial RIA kit (UVWR, Prague, Czechoslovakia).

Measurement of NOS activity on renal tissue homogenates

Renal tissues from each experimental animal were frozen in 1 ml of reaction buffer (mM: Hepes 20, EDTA 0.5, dithiothreitol 1, pH 7.2), homogenated and then sonicated on ice with three 10-second bursts. In each test tube, the following reagents were added to 100 μl homogenate at the final concentrations: 2 mM NAPDH, 1.5 mM $CaCl_2$, 1 to 100 μM L-arginine, 2.5 μCi (=0.4 μM) L-[2, 3, 4, 5-^{3}H]arginine monohydrochloride (62 Ci/mmol; Amersham International, Bucks, UK) [29, 30]. After a 30 minute incubation at 37°C, the reaction was stopped by adding 2 ml of 20 mM Hepes-Na pH 6 containing 2 mM EDTA. The whole reaction mixture was applied to 2 ml columns of Dowex AG50WX-8 (Na^+ form; Aldrich, Milano, Italy) and eluted with 4 ml of water. At pH 6 arginine is negatively charged, while citrulline is neutral: the Dowex resin is a cationic exchanger which binds arginine but not citrulline in these conditions. The radioactivity corresponding to [^{3}H]citrulline content in 6 ml eluate was measured by liquid scintillation counting. NOS activity was expressed as pmol citrulline/min/mg protein. The protein content of homogenates was assessed with the modified micro-Lowry method (Kit from Sigma, St. Louis, MO, USA).

mRNA analysis of inducible (iNOS) and constitutive (cNOS) forms of the enzyme in renal tissues

Renal tissue from three animals of each esperimental group was processed for molecular biology studies. Total RNA was obtained by the guanidine isothiocyanate/cesium chloride method [31]. Twenty-five micrograms of total RNA were electrophoresed on a 1% agarose gel containing 6.3% formaldehyde in 4-morpholinepropanesulfonic acid (MOPS, Sigma) buffer and blotted on a Nylon Duralon-UV membrane (Stratagene, La Jolla, CA, USA) by the traditional capillary system in 10 × SSC [32]. Prehybridization and hybridization steps were performed overnight in 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, with 100 mg/ml denaturated salmon sperm DNA at 42°C. The cDNA for endothelial, constitutive cNOS [33] (a gift from Dr. T Michel, Brigham and Women's Hospital, Boston, MA, USA) and iNOS [34] (a gift from Dr. C. Nathan, Cornell University of Medical College, New York, NY, USA) were labeled with [α^{32}-P]dCTP (3000 Ci/mmol, Amersham) at 2.2×10^8 cpm/mg specific activity, by the random primer labeling kit (Amersham) according to the manufacturer's instructions. Post-hybridization washes were performed at high stringency (twice in 2× SSC + 0.1% SDS for 30 min at room temperature, twice in 0.2× SSC + 0.1% SDS for 30 min at room temperature, and twice in 0.1× SSC + 0.1% SDS for 30 min at 55°C), and the membrane was exposed on autoradiography with Hyperfilm-MP (Amersham) and intensifying screen at −80°C for 20 days. RNA loading and transfer to membranes was checked by examination under UV light. The optical density of the autoradiography bands integrated for the band area was

evaluated by a digitized image analyzer (RAS 3000, Coat System, Amersham).

Radioisotopic measurement of GFR and ERPF

The measurements were performed on conscious rats to avoid the hemodynamic modifications induced by anesthetics. The experimental method described by Tauxe [35] was employed with modifications, using [^{51}Cr] EDTA (Amersham) and o-^{125}I-hippuran (Sorin, Italy) as tracers. A total of 0.2 μCi/100 g body wt of [^{51}Cr] EDTA and 0.4 μCi/100 g body wt of o-^{125}I-hippuran were injected in the caudal vein and blood sampling was performed 30, 60 and 90 minutes after the injection. The blood samples were centrifuged at 3,000 rpm for 10 minutes and the sera counted for 10 minutes in a Packard Autogamma 5130 gamma counter.

GFR was calculated according to the following formula to evaluate the distribution volume:

$$\text{standard counts(cpm)} \times \text{(weight dose)(g)/(weight standard)(g)} \times 1000 \text{ divided by intercepts on } T_0$$

and from this value:

$$\text{GFR (ml/min)} = \text{distribution volume} \times 0.65 \times 1000 \text{ divided by T1/2}$$

Effective renal plasma flow was calculated using the following formula [35]:

$$\text{ERPF (ml/min)} = 1126.2 \times [1 - C^{-0.008} \times (v30 - 7.8)$$

where v30 represents the theoretical distribution volume 30 minutes after the injection.

Renal tissue morphology studies

Longitudinal sections of kidneys were fixed in Serra fluid for 24 hours at room temperature, dehydrated with ethanol and xylene and embedded in paraffin. Section at 4 μm thickness were stained with hematoxylin eosin, periodic acid Schiff reagent and Masson trichrome.

Statistical analysis

The statistical analyses were performed using the Statview^{512+} software (Brainpower, Calabasas, CA, USA) on a Macintosh Personal Computer (Cupertino, CA, USA). Data are given as mean values ± SD. The different groups were compared using analysis of variance (ANOVA test) and statistical significance was defined as $P < 0.05$.

Results

CsA blood levels

Mean blood levels of CsA were very high in the treated groups (G1, G2, G3), confirming a good enteric absorption of the drug (G1 6840 ± 842 ng/ml, G2 8053 ± 745, G3 8128 ± 793 ng/ml). No significant difference was observed among these groups.

Serum creatinine and urinary protein losses

After four weeks and even more after eight weeks of daily treatment with CsA, G1 rats displayed serum creatinine levels significantly higher than controls (G6) ($P < 0.02$ at 4 weeks, $P < 0.001$ at 8 weeks; Fig. 1). In this group creatinine values were twofold higher after four weeks and threefold higher after eight weeks than in rats treated with the vehicle alone [G6].

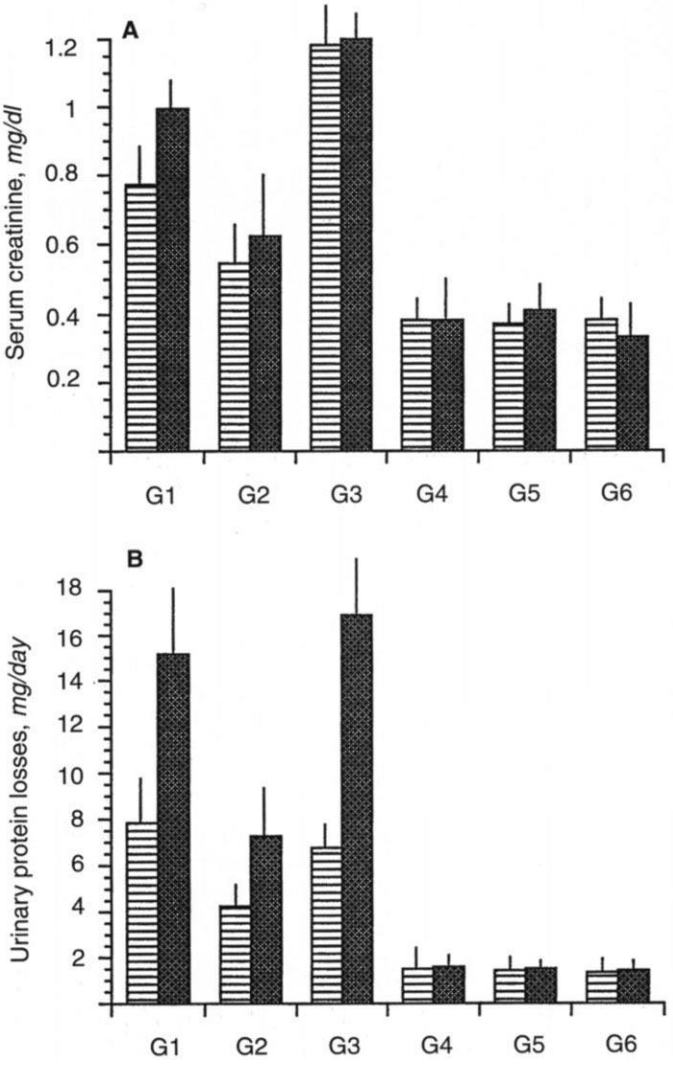

Fig. 1. *Serum creatinine levels (mg/ml) in G1 to G6 rats at 4 weeks* ▤ *and 8 weeks* ▩. For the statistical significance see the text.

In comparison to the rats receiving CsA alone (G1), the rats treated with both CsA and L-Arg (G2) showed significantly lower creatinine levels at four ($P < 0.05$) and eight weeks ($P < 0.01$). In comparison to G1, G2 rats also displayed lower urinary protein loss at four ($P < 0.05$) and eight weeks ($P < 0.01$). The protective effect induced by L-Arg appeared particularly evident after four weeks of treatment, when serum creatinine and urinary protein excretion values were only slightly and not significantly higher than in rats treated with L-Arg alone (G4). After eight weeks of treatment the protective effect induced by L-Arg on CsA functional nephrotoxicity was still present, even though less strong, and creatinine levels and proteinuria in G2 were significantly higher than in G4 (creatinine $P < 0.05$; proteinuria $P < 0.02$).

Moreover, in these G2 rats a significant increase in serum creatinine values ($P < 0.01$) and urinary protein excretion ($P < 0.02$) was evident from the fourth to the eighth week, indicating a progression of CsA-induced nephrotoxicity.

After four weeks of treatment serum creatinine levels in G3

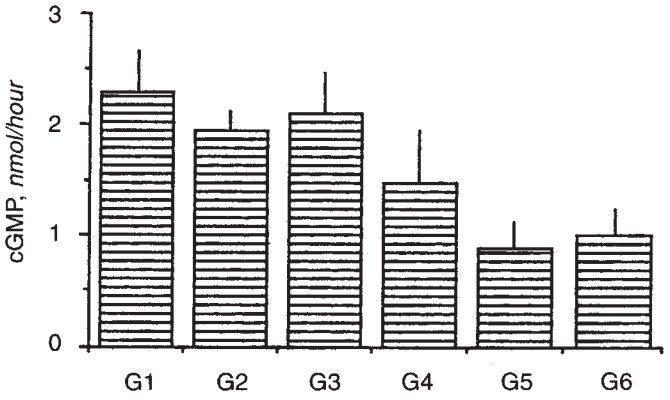

Fig. 2. *cGMP measurement in urine (nmol/hr).*

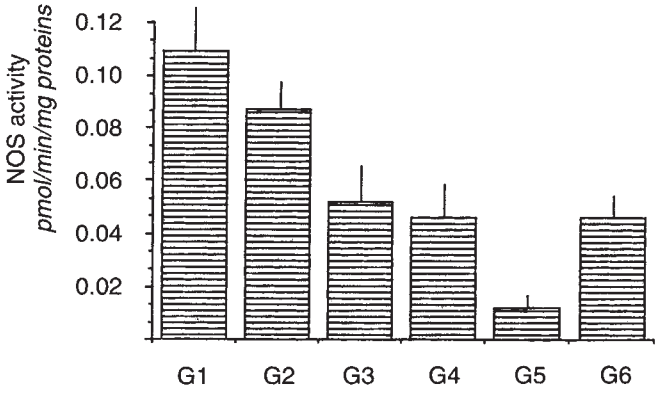

Fig. 3. *NOS activity (pmol/min/mg proteins) in renal tissue homogenates.* For statistical significance see the text.

were significantly higher than in G2 ($P < 0.005$) and G1 ($P < 0.02$). At the eighth week of treatment serum creatinine levels in G3 were still higher than in G1 ($P < 0.05$) and G2 ($P < 0.02$). After four weeks, urinary protein excretion values in G3 were not significantly different than in the groups receiving CsA (G1 and G2), but at the eighth week the values were significantly higher than in the rats given CsA and L-Arg (G2) ($P < 0.005$). These data suggest that the protective effect exerted by L-Arg was mediated via its conversion to NO.

Moreover in G3 rats a progressive impairment of renal function from the fourth to the eighth week was observed ($P < 0.05$) and urinary protein excretion values increased significantly during this time ($P < 0.02$).

Urinary cGMP excretions

A slight increase of urinary cGMP excretion was observed in CsA-treated groups (G1, G2, G3) (Fig. 2) in comparison to the control groups (G4, G5, G6) without any significant difference between the groups.

NOS activity in renal tissue homogenates

NOS activity was high in the group treated with CsA alone (G1 vs. G6, $P < 0.01$). The increase of enzyme activity was completely abolished in animals receiving both the inhibitor and CsA (G3). *In vitro* renal NOS activity was increased in rats receiving L-Arg only if they were simultaneously given CsA (G2 vs. G4, $P < 0.02$). Administration of L-NNA, a competitive inhibitor of both cNOS and iNOS, induced a stable blockage of NOS activity in renal tissue from G5 rats (Fig. 3).

mRNA encoding for iNOS and cNOS in renal tissue

To investigate the mechanism leading to increased NOS activity in rats treated with CsA, the expression of iNOS and cNOS transcripts was studied. Figure 4 shows representative Northern blots whereas Figure 5 summarizes the results as arbitrary densitometry units.

G2 and G3, independently from the administration of L-Arg or L-NNA contemporary to CsA, had some increase in cNOS and to a lesser extent iNOS mRNA expression (Fig. 4). The amounts of both iNOS and cNOS transcripts were not significantly different among the various groups (Fig. 5).

Renal hemodynamics

After four weeks of CsA treatment in comparison to the control rats (G6) G1 rats displayed a clear-cut decrease of GFR values ($P < 0.02$) and of ERPF ($P < 0.01$) (Fig. 6). The filtration fraction (FF) resulted significantly increased ($P < 0.05$).

L-Arg treatment in association to CsA in G2 rats was able to limit CsA hemodynamic effects: GFR and ERPF in G2 were significantly greater than in G1 ($P < 0.05$) while FF was significantly lower ($P < 0.05$). Interestingly, GFR, ERPF and FF values in G2 were similar to control G6 (P = NS).

Renal morphology

Light microscopy did not show evidence of lesions either at the fourth or eighth week. In particular vascular and tubular changes as well as striped interstitial fibrosis were not observed.

Discussion

The daily CsA dose administered in our model (40 mg/kg/day), although similar to other protocols reported in the literature [6], is much higher than that employed in human kidney transplantation. High CsA blood levels are necessary to overcome the natural resistance of the rat to the renal toxic effects of this drug [36] and to exert a maximal effect. A simple transposition of a dose-effect relationship from rats to humans is therefore impossible. The L-Arg doses given per os were high as well (300 mg/kg) and analogous doses administered i.v. were able to worsen hypothension until death in endotoxin shock models [37]. L-NNA at the employed dose of 5 mg/kg is able to block the enzymatic activity of both iNOS and cNOS, the last able to be inhibited by even lower amounts [37].

The administration of CsA over four weeks was planned to investigate an initial chronic effect of the drug, since acute toxicity is usually evaluated after seven days [6, 38]. The lack of histological lesions at four weeks in the subset of animals sacrificed in each group prompted us to continue the experiments until eight weeks, but not longer, since a pivotal study previously showed that at 10 weeks the rats treated with CsA plus L-Arg and L-NNA showed an initial sickness with weight loss until death. At that time, however, no histological signs of nephrotoxicity were detectable as well, according to the reports that interstitial fibrosis develops only after four months [8]. We did not carry out low sodium diet [39] or mononephrectomy [40], which would have

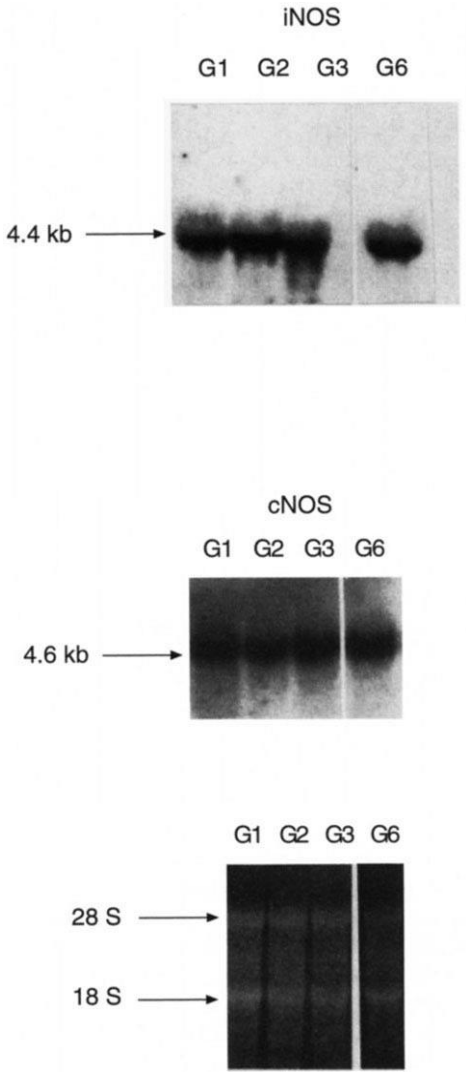

Fig. 4. *Expression of iNOS and cNOS mRNA in rat kidneys.* Total RNA (25 μg for each lane) extracted from kidneys of animals belonging to different experimental groups was examined by Northern blot analysis. Comparable amounts of total RNA were transferred to filters as shown by ethidium bromide staining (bottom of the figure). Integrity of RNA was evidenced by the clear bands.

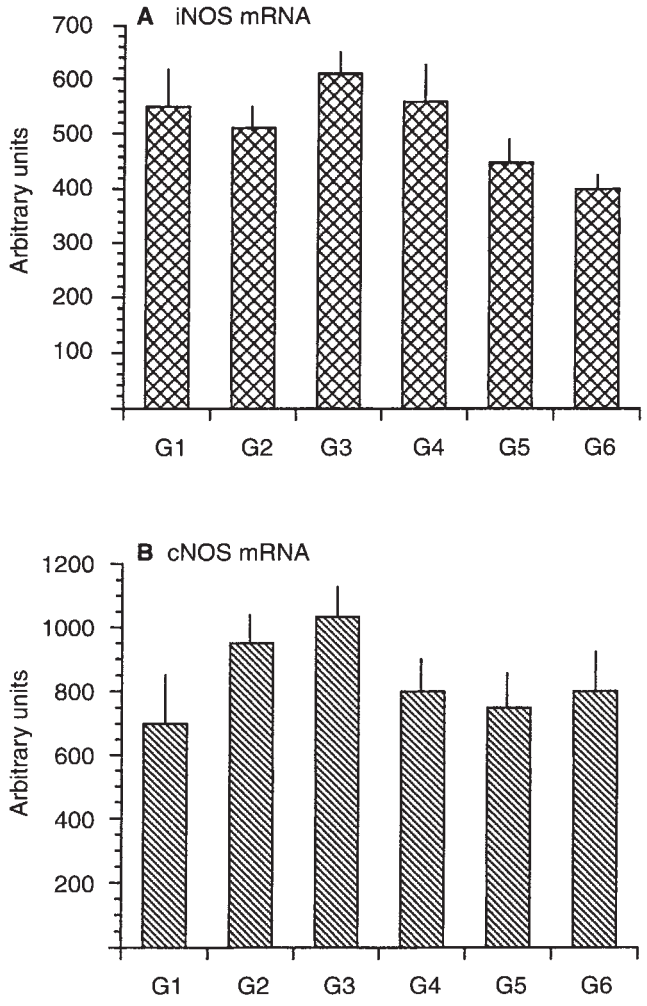

Fig. 5. *mRNA encoding for cNOS and iNOS.* Results are expressed in arbitrary units after densitometry measure.

enhanced the development of histological signs of nephrotoxicity, in order to avoid an additional increase of angiotensin II activity.

After four weeks of daily CsA administration we observed a significant increase of serum creatinine levels, which worsened after eight weeks and was inhibited by L-Arg and reversed by L-NNA, a well known NOS inhibitor.

These data, in absence of histological lesions, are consistent with a vasocostrictive effect of the drug. Several observations indicate that CsA induces an uncompensated preglomerular vasoconstriction [7, 41]. CsA interferes with the normal endothelium-dependent relaxation of the afferent arteriole [19] and with the compensatory generation of angiotensin II in the kidney [42]. In an acute CsA toxicity model an afferent arteriolar vasoconstriction was demonstrated, which was reversed by L-Arg [38].

The glomerular hemodynamic investigations performed in this study by radioisotopic GFR and ERPF measurement confirmed the hypothesis that the major protective effect of L-Arg on CsA toxicity was mediated by opposing its hemodynamic modifications. After CsA administration at fourth week there was a 50% decrease of GFR and a 30% decrease of ERPF; these data were in agreement with the results obtained by other authors employing inulin and paraaminohippuric acid clearance [43]. We found a clear filtration fraction increase in CsA-treated animals; this is considered to be an index of glomerular hyperfiltration, and actually we observed a parallel 10-fold increase in urinary protein excretion. It is noteworthy that the group treated with both CsA and L-Arg showed a clear reduction of filtration fraction until normalization, and a significant protection against the development of proteinuria.

Taken as a whole, these data support the hypothesis that the severe glomerular hemodynamic alteration induced by CsA leads to glomerular hyperfiltration and permselectivity alterations which are counterbalanced by the administration of L-Arg, the physiological substrate of NOS.

However, since the hemodynamic indices were not completely

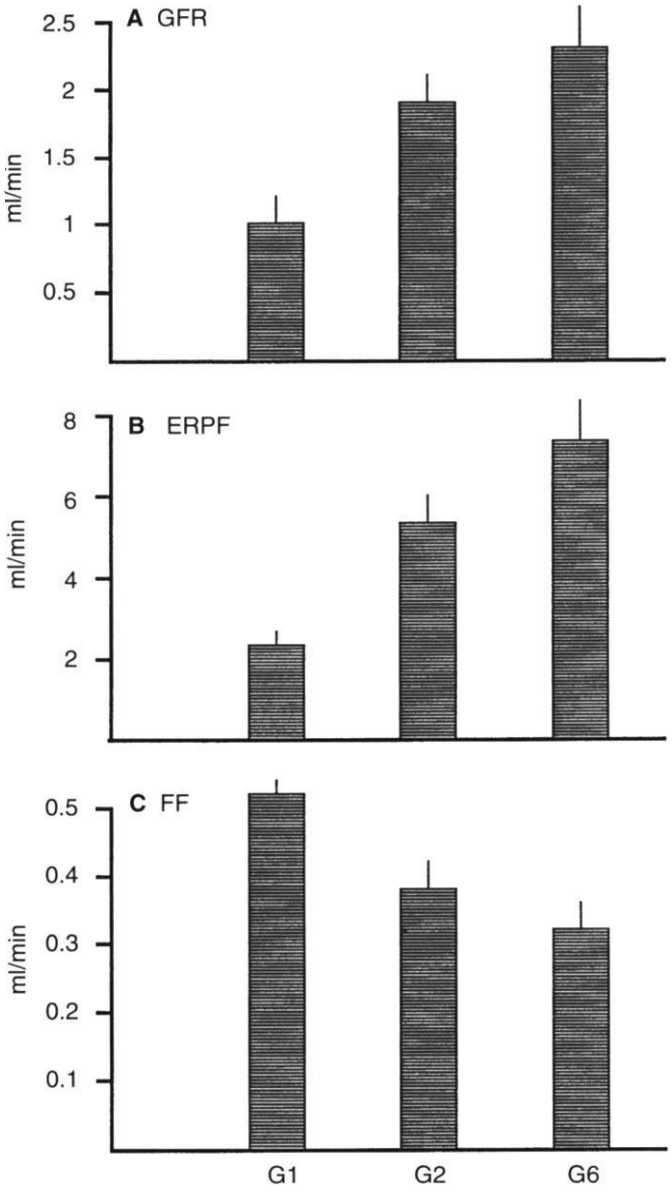

Fig. 6. *Renal hemodynamics in G1 rats treated with CsA alone, G2 (CsA + L-Arg) and G6 control group.* For the statistical significance see the text.

reversed by L-Arg, and a small increase in creatinine and proteinuria—already evident at the 4th week—became significant at eighth week, there admittedly are additional CsA effects on pressures and flow across the glomerular capillary wall unaffected by L-Arg administration.

To investigate whether the protective effects of L-Arg on CsA nephrotoxicity were mediated by NO-elicited guanylate cyclase activation we measured urinary cGMP. The rats treated with CsA showed a slight but not significant increase in urinary cGMP excretion in comparison to controls, and there were no differences between the three groups given CsA. Such a result could be accounted for by the very low levels of cGMP detectable in urine and by the fact that cGMP urinary excretion could derive from the action of other mediators, such as PGE_2 or atrial natriuretic factor. Moreover, cGMP is an unstable compound [22, 26] that can quickly lose its antigenic properties, becoming unrecognized by the antibody employed in the immunoenzyme assay adopted.

A more direct approach is to measure NOS activity in renal tissue homogenates. This technique, which permits evaluation of the *in vitro* NOS activity in the presence of large excess of substrate, quantifies citrulline, which is produced equimolarly with NO from L-Arg. NOS activity in the kidney was blocked in the rats treated with L-NNA, an inhibitor of both inducible and constitutive forms of NOS. Our data suggest that renal NOS is inhibited by L-NNA. In fact, *in vivo* administration of the inhibitor caused a permanent decrease of the NOS activity measured *in vitro*. Such an irreversible blockage *ex vivo* has been already observed in the rat brain at concentrations similar to that used in our study [44]. CsA with or without L-Arg increased NOS activity. Conversely, the enzyme activity was reversed to the low values found in controls in animals receiving both L-NNA and CsA (G3). When L-Arg was given to the rats treated with CsA renal NOS activity was significantly increased, while the assumption of L-Arg alone did not modify the enzyme activity.

The *in vitro* increase of NOS activity elicited by CsA in the rat kidney may appear in contrast with the decrease of GFR and ERPF and the increase of protein excretion, which suggest that CsA evokes a renal vasoconstriction. L-Arg given in association with CsA reversed the hemodynamic conditions to baseline with normalization of GFR, ERPF, serum creatinine levels and urinary protein excretion. However, NOS activity in these rats was similar to that measured in the rats treated with CsA alone.

It is conceivable that, acting via a complex with cyclophilin, CsA causes an inhibition of the activity of the Ca^{++}/calmodulin-dependent cNOS in the kidney [5]; after a prolonged exposure, in the kidney such an effect could be counteracted by an increase of iNOS activity. Such an effect may render critical the availibility of L-Arg for NO synthesis: in the absence of an adequate substrate supply, the increase of NOS activity would not correspond to a parallelely increased production of NO. It has been already reported in endothelium that intracellular L-Arg concentration is sufficient for basal NO production, but is not enough to support the increased NOS activity following stimulation with cytokines, as during septic shock [45]. In the absence of L-Arg, NOS reduces O_2 to H_2O_2 [46] or O_2^- [47]. It is possible that CsA-evoked NOS hyperactivity is responsible of an oxidative stress, which could partly account for both acute and chronic nephrotoxic effects of the drug; L-Arg administration would prevent these side effects by shifting NOS from a oxygen reductase to its physiological activity.

The Northern blot analysis of cNOS and iNOS transcripts in renal tissues showed no significant difference in the six experimental groups. This finding is not suprising to us, considering a recent report in which some cytokines, such as IFN-γ, TNF-α and IL-1β were able to increase the NOS activity in human umbilical vein endothelial cells *in vitro* by an up-regulation of tethrahydrobiopterin synthesis without a parallel increase of NOS mRNA [48].

In conclusion, we demonstrate a protective effect of L-Arg on the nephrotoxicity induced by chronic administration of CsA at high doses in the rat.

A positive effect of L-Arg on intrarenal hemodynamics (SNGFR and SNRF) has been recently reported in an experimental model of short-term CsA administration in rats [49]. L-Arg was found to restore the glomerular and tubular response to glycine infusion after CsA treatment. Since glycine infusion causes renal

vasodilation mediated by NO, CsA was supposed to diminish the NO activity within the kidney which could be reversed by L-Arg feeding. In that study no methodological approach was done to measure NOS activity or mRNA encoding for NOS or cGMP as second mediator. We tried to give further insight on the mechanism governing not only the intrarenal hemodynamics but particularly the renal function consequences of CsA toxicity, including proteinuria and creatinine increase. Even though L-Arg has a clear beneficial effect on functional CsA nephrotoxicity, our data failed to give direct and unequivocal evidence that NO is the only mediator. Since the L-Arg-mediated decrease of proteinuria and creatinine was completely reversed by L-NNA inhibition, we suggest that NO may play a crucial role in L-Arg effect. We suggest that CsA treatment increases NOS activity in the kidney, by a mechanism which does not require a *de novo* synthesis of the enzyme. Such an increase, which may counterbalance the vasoconstrictive effects of the drug, is uneffective to reduce the toxic effect of CsA in the absence of exogenous L-Arg. Administration of L-Arg is likely to reduce CsA nephrotoxicity by accomplishing the higher request of activated NOS for its substrate, thus potentiating NO synthesis in the kidney. However, our data do not exclude that L-Arg might protect the kidney against CsA effects via other metabolic pathways.

Such effects occur not only by abolishing preglomerular vasoconstriction induced by CsA with drop in GFR and ERPF, but also by protecting from the consequent increase of filtration fraction and urinary protein excretion. Therefore L-Arg not only proved to be effective in abolishing the hemodynamic effects exerted by CsA but was able to also re-establish the glomerular permselectivity, the loss of which condition the evolution of the glomerular damage toward sclerosis in several experimental models.

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