Nephrol Dial Transplant (2013) 28: 2066–2072
doi: 10.1093/ndt/gft020
Advance Access publication 15 May 2013

ORIGINAL ARTICLE

A new recombinant MnSOD prevents the Cyclosporine A-induced renal impairment

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Keywords: cyclosporine, nephrotoxicity, reactive oxygen species, superoxide dismutase

ABSTRACT

Background. Cyclosporine A (CsA) is one of the most frequently used anticalcineurinic drugs for preventing graft rejection and autoimmune disease. Its use is hampered by nephrotoxic effects, namely an impairment of the glomerular filtration rate (GFR) and hypertension. Evidence suggests that reactive oxygen species (ROS) play a causal role in the nephrotoxicity. The present study aims to investigate *in vivo* the effects of a new recombinant mitochondrial manganese-containing superoxide dismutase (rMnSOD), a strong antioxidant, on the CsA-induced nephotoxicity.

Methods. Rats were treated with CsA (25 mg/kg/day) alone or in combination with rMnSOD (10 μg/kg/day) for 7 days. At the end of the treatment, GFR was estimated by inulin clearance (mL/min/100 g b.w.) and the mean arterial pressure (MAP) was recorded through a catheter inserted in the carotid artery. Superoxide concentration within the cells of the

abdominal aorta was quantified from the oxidation of dihydroethidium (DHE). In kidney tissues, ROS levels were measured by the 2′7′ dichloroflurescin diacetate assay. Renal morphology was examined at the histochemistry level.

Results. CsA-treated rats showed a severe decrease in GFR (0.34 ± 0.17 versus 0.94 ± 0.10 in control, $P < 0.001$) which was prevented by rMnSOD co-administration (0.77 ± 0.10). CsA-injected animals presented with higher blood pressure which was unaffected by rMnSOD. ROS levels both in the aorta and in renal tissue were significantly increased by CsA treatment, and normalized by the co-administration with rMnSOD. This effect was, partly, paralleled by the recovery from CsA-induced morphological lesions.

Conclusions. Administration of rMnSOD prevents CsA-mediated impairment of the GFR along with morphological alteration. This effect could be related to the inhibition of ROS.

INTRODUCTION

Cyclosporine A (CsA) is one of the most frequently used anticalcineurinic drugs for preventing graft rejection and autoimmune disease. CsA administration significantly improved long-term survival in the case of solid organ transplantation [1]. Unfortunately, its use is hampered by the nephrotoxic effects, including impairment of the glomerular filtration rate (GFR) and hypertension [1]. The pathogenesis of hypertension is not completely clear. There is evidence to suggest that CsA-induced hypertension is associated with sodium and water retention [1], and involvement of reactive oxygen species (ROS) has been postulated [1, 2].

Several lines of proof suggest that CsA treatment stimulates ROS production. For example, the administration of CsA markedly increased renal cortical lipid peroxidation and urinary excretion of ROS that were trapped by the spin-trapping agent α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone [3]. Clinical studies showed that plasma hydroperoxide levels were markedly raised in kidney and heart transplant patients treated with CsA [4]. In addition, treatment with CsA significantly increases superoxide release from isolated rat aortic rings incubation [5], whereas pre-treatment with a superoxide dismutase (SOD) restored the normal tone in arteries of rats treated with CsA [6]. These observations suggest that an increased ROS production contributes to CsA-induced endothelial dysfunction which might be responsible for the development of hypertension. Finally, it has been recently demonstrated that CsA-induced hypertension and increased ROS production are associated with elevated Ang II levels [7].

MnSOD is a member of a family of structurally unrelated SODs encoded by different genes; it is found exclusively in the mitochondrial matrix [8]. Overexpression studies show the capacity of MnSOD to alter many properties typical of cancer cells (growth rate, invasiveness, anchorage-independent growth, etc.) both *in vitro* and *in vivo* [9].

We have recently isolated a new recombinant MnSOD (rMnSOD) from a human pleiomorphic liposarcoma cell line [10]. This rMnSOD is not localized in the mitochondrial matrix, but it is secreted in the media. Given its antioxidant activity, we set out to determine whether rMnSOD treatment could prevent the nephrotoxic side effects caused by CsA. Data will be shown demonstrating that rMnSOD is able to completely prevent CsA-induced impairment of the GFR. This effect is associated with improvement of the CsA-induced renal morphological alterations and vascular ROS damage.

MATERIALS AND METHODS

Experiments were performed on rats treated with humane care, in compliance with the indications of the *Guide to the Care and Use of Experimental Animals*. A total of 48 Sprague–Dawley rats were used, with weights ranging from 200 to 230 g. They were housed under controlled environmental conditions (temperature 22°C and a 12-h light–dark cycle). Animals were fed a standard diet; food and water were given ad libitum. The rats, randomly divided into four groups, were treated as follows: Group 1: 12 rats injected i.p. with CsA vehicle for 9 days; Group 2: 12 rats treated i.p. with 25 mg/kg of b.w./day CsA, Sandimmune Neoral®, i.v. preparation, containing Cremophore EL and alcohol as vehicle 2:1, from Novartis (Basel, Switzerland) for 7 days; Group 3: 12 rats pretreated i.p. for 2 days with rMnSOD (10 μg/kg) and then receiving for 7 days both rMnSOD (10 μg/kg) and CsA (25 mg/kg) administered simultaneously; Group 4: 12 rats treated i.p. with rMnSOD (10 mg/kg) for 9 days. The dose and length of CsA and rMnSOD administration was chosen according to previous experiments [11]. An additional set of rats (five per each group) was used for blood CsA and Hb determination and to evaluate the interstitial fibrosis.

Whole kidney clearance

Glomerular filtration rate was measured at the end of the experimental treatment. The rats were anaesthetized with an i.p. injection of Inactin (Sigma-Aldrich, St Louis, MO, USA), 120 mg kg^{-1} b.w., tracheostomized, placed on a thermo-regulated table (37°C) and prepared for renal clearance evaluation as previously described [12]. In brief, the right carotid artery was catheterized to monitor blood pressure through a blood pressure recorder (BP1 by WPI, USA) and to take blood samples for inulin concentration measurements. The left jugular vein was cannulated with polyethylene PE-50 tubing and used for i.v. infusion via a syringe pump (Braun, Melsungen) of 0.74 mg · 100 g b.w./min inulin in 10% saline solution. The surgical procedure also included bladder catheterization with PE-50 tubing. After a 60-min equilibration period, the first of four 30-min urine collections began. Arterial blood samples (100 μL) were taken at the start and end of each collection period. Inulin concentrations in plasma and urine were measured by the colorimetric method. Glomerular filtration rate was calculated using the standard clearance formula [13]. Determination of the Hb was performed by an instant measurement of arterial blood from the aorta by the HemoCue® system. Measurement of the level of CsA in the

serum was performed by a chemiluminescent microparticle immunoassay, Architect i 2000 (Abbot diagnostic).

ROS measurement assay

To measure the level of ROS in the kidney, we performed a 2′7′ dichloroflurescin diacetate assay.

At the end of the *in vivo* treatment, rats were anaesthetized and then sacrificed by exsanguinations and the right kidney was quickly stored at −80°C. Later, the kidneys were thawed on ice and weighed. Kidneys were homogenized in 5 mL of Tris–Hcl 40 mM (pH 7.4) with Ultraturrax. Protein concentration was assessed by the Bio-Rad protein assay.

2′7′ dichloroflurescin diacetate 5 μM (Molecular Probes) was utilized for the assay from a 500 μM stock. The tissue samples were diluted in a range from 10 000 to 2500 times. The spectrum was analysed from 480 to 525 nm at different times (T0, T10, T30) using a spectrophotometer Jasco FP-777. The levels of ROS were expressed as intensity fluorescence (IF) normalized for grams of tissue and micrograms of proteins.

Superoxide assay in the dissected aorta

Superoxide concentrations within the cells of the abdominal aorta were evaluated by the oxidation of dihydroethidium (DHE; Molecular Probes). DHE can enter the cell and be oxidized by superoxide to yield ethidium (Eth), which binds DNA producing bright red fluorescence. The increase in Eth-DNA fluorescence indicates peroxide production within cells [14].

The paraformaldehyde (PFA)-perfused aorta was dissected and frozen at −80°C. Sections (10-μm thick) were cut at the cryostat (Leica CM 1850) and then incubated with a 10 μM DHE solution for 30 min at room temperature. Cover slips were mounted with Dako Fluorescence Mounting Medium. Pictures were acquired at the Leica DMI6000 B inverted microscope. Fluorescence intensity was quantified by Image-j software. Briefly, average pixel intensity was quantified from three equal random areas from each sample. The average pixel intensity was compared among groups.

Renal histology

Rats were anaesthetized by inactin (120 mg kg^{-1} b.w.). The left kidney was washed by retrograde perfusion via the abdominal aorta by 0.01 M PBS (pH 7.4) for 30 s and then fixed with 4% PFA for an additional 3 min. All procedures have been described in detail previously [12]. Briefly, the kidney was embedded in paraffin, and 4-μm thick sections were then cut at Leica Reichert-Jung 2030 BIOCUT Microtome. After overnight immersion in xylene, sections were dehydrated in ethanol, then washed in milli Q-water and stained with haematoxylin and eosin. Cover slip was mounted with Bio-optica O. Kindler GmbH EUKITT. Masson's trichrome staining and the semi-quantitative fibrosis evaluation were performed as previously described [12]. Briefly, two blinded investigators independently scored randomized pictures of the renal cortex taken by an external investigator. The pictures were taken at a ×200 magnification ($n = 5$/section was examined). Interstitial fibrosis was scored as zero if there were no signs of fibrosis, 1, if <25%, or 2, if 26–50%, or 3, if 51–75%, or 4, if > 76% of the observed field was affected, respectively. Images were acquired at Leica DM16000 inverted microscope.

Statistical analysis

All data are mean ± SD. Statistical analysis was performed by an unpaired *t*-test or one-way ANOVA followed by the unpaired *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Physiological parameters

CsA-treated rats showed a loss of weight that was prevented by rMnSOD co-treatment (Table 1). Equal treatment among the CsA and the CsA + rMnSOD groups has been validated by similar serum concentration of CsA (Table 1). The administration of CsA induced a significant increase in the mean arterial pressure (MAP), measured at the time of the clearance study by carotid catheterization (Table 1). rMnSOD did not affect this haemodynamic parameter neither alone nor when administered in combination with CsA. The urinary flow rate was significantly decreased in rats treated with CsA when compared with control rats ($P < 0.01$) and rMnSOD co-treatment did not reverse it. No differences in the level of Hb were seen among the studied groups (Table 1).

Table 1. Physiological parameters

Parameter	Control	rMnSOD	CsA	CsA + rMnSOD
Body weight gain (g)	48 ± 7	45 ± 8	−37 ± 15**	47 ± 100
MAP (mmHg)	96 ± 3	101 ± 2	141 ± 5**	139 ± 4**
Urinary flow (μL/min)	14 ± 5	9 ± 5	5 ± 2**	7 ± 3**
Hb (g/dL)	15 ± 0.6	14.5 ± 0.6	16 ± 0.3	15 ± 0.5
Serum CsA (ng/mL)	ND	ND	4442 ± 29	4106 ± 375

Values are mean ± SD (** is for $P < 0.01$ compared with the control group), $n = 5$ in all the groups. For Hb analysis in CsA and CsA + rMnSOD group $n = 4$.

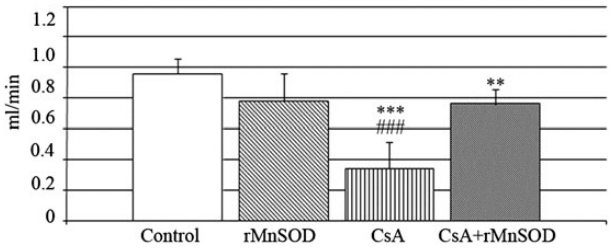

FIGURE 1: Effects of CsA on GFR measured by the clearance of inulin. CsA treatment significantly decreased GFR, while co-administration with rMnSOD partially reverses this effect. (values are mean ± SD; * is versus the control group; # is versus the CsA-rMnSOD; **P < 0.01, ***P < 0.001, ###P < 0.001)

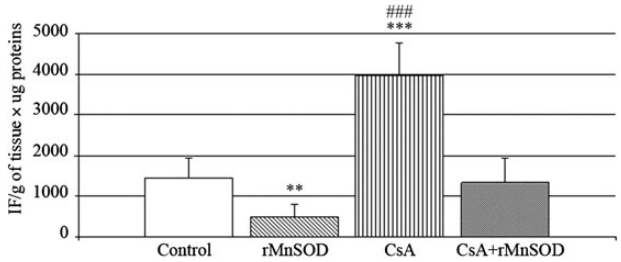

FIGURE 2: Effects of rMnSOD on CsA-induced ROS production (trichloroflurescin diacetate assay). CsA-treated rats showed a significant increase in ROS production compared with the control. rMnSOD + CsA co-treatment prevents CsA-induced ROS production. rMnSOD significantly reduces ROS production compared with the control group. (values are mean ± SD; *is versus the control group; # is versus the CsA-rMnSOD; **P < 0.01, ***P < 0.001, ###P < 0.001)

rMnSOD prevents the CsA-induced GFR impairment

To evaluate the effect of CsA on renal haemodynamics, GFR was measured by the clearance of inulin. CsA treatment significantly decreased GFR compared with control animals (0.34 ± 0.17 versus 0.96 ± 0.10 mL/min, P < 0.001 n = 6:6, respectively) (Figure 1). When CsA was co-administered with rMnSOD, the GFR value improved significantly when compared with the CsA-treated rats (0.77 ± 0.10 versus 0.34 ± 0.17 mL/min, n = 6:6, P-value < 0.001) and only partially reduced when compared with the control group (0.77 ± 0.10 versus 0.96 ± 0.10 mL/min, P < 0.01 n = 6:6, respectively). Single treatment with rMnSOD caused a slight decrease in the GFR compared with the control group (0.78 ± 0.18 mL/min versus 0.96 ± 0.10 mL/min, P = 0.0781, n = 5:6, respectively) (Figure 1).

rMnSOD prevents the CsA-induced ROS production

The production of ROS in the kidneys has been evaluated with the trichloroflurescin diacetate assay. The novel rMnSOD significantly reduces the ROS production compared with the control group (482 ± 300 versus 1459 ± 500 IF/g of tissue × µg of proteins; P < 0.01, n = 5:6, respectively) (Figure 2). As expected, CsA-treated rats presented with a larger ROS production than controls (3970 ± 500; P < 0.001 n = 6), whereas co-administration of both CsA and rMnSOD (1322 ± 600; P < 0.001 n = 6) reversed the ROS production to the control level.

Effects of rMnSOD on CsA-induced oxidative stress

The red fluorescence generated by the binding of the Eth–DNA complex showed that the abdominal aorta of CsA-treated animals present a red fluorescent signal significantly brighter than control (44 250 ± 4614 versus 25 306 ± 15 715 P < 0.05 n = 5:4, respectively) (Figure 3A, B and E). When the rats were treated with CsA plus rMnSOD, the fluorescence intensity was significantly lower than the CsA-treated group (36 463 ± 5322 versus 44 250 ± 4614, P < 0.05 n = 5:5 respectively) (Figure 3D), indicating that rMnSOD was able to completely prevent the production of superoxide induced by CsA. Analysis of samples from rats receiving only rMnSOD treatment indicates that the antioxidant administration had no effect (Figure 3C).

Morphology

As demonstrated previously [12], we confirm that CsA treatment induces severe alteration in the renal morphology. Tubular vacuolization associated with a reduction in the height of the tubular epithelium and interstitial infiltrate is the predominant histological pattern in CsA-treated rats (Figure 4). These morphological alterations are mainly located in the cortex and outer stripe of outer medulla and they are common both to proximal and distal tubules. rMnSOD co-treatment reduces CsA-related morphological alteration; indeed tissue slices from rats receiving rMnSOD and CsA present few and less extensive areas of tubular vacuolization and interstitial infiltrates (Figure 4). rMnSOD treatment alone is not associated with significant morphological alteration, compared with the control group (Figure 4). Interstitial fibrosis has been evaluated by Masson's trichrome staining. CsA treatment induces a more severe interstitial fibrosis compared with the control group. Co-administration of rMnSOD and CsA is associated with a tendency towards a fibrosis score lower than the CsA group alone (Figure 5).

DISCUSSION

Previous studies have indicated that oxidative stress could be implicated in CsA-induced toxicity [15]. In the present investigation, we show that chronic treatment with CsA significantly boosts ROS formation in the kidney as revealed by the dichloroflurescin diacetate assay (Figure 2). In addition, the synthesis of ROS, following CsA administration, also occurs in other tissues, as demonstrated by directly measuring ROS formation in the aorta (DHE experiments) (Figure 3B) in agreement with the report of Galle *et al.* [5]. Since it has been shown that antioxidants may protect from CsA side effects, we have tested new recombinant mitochondrial manganese-containing superoxide dismutase (rMnSOD), a strong antioxidant, on an *in vivo* model of CsA-induced nephrotoxicity [10].

Our data show that rMnSOD completely counteracts CsA-induced oxidative stress in the kidney as indicated by the

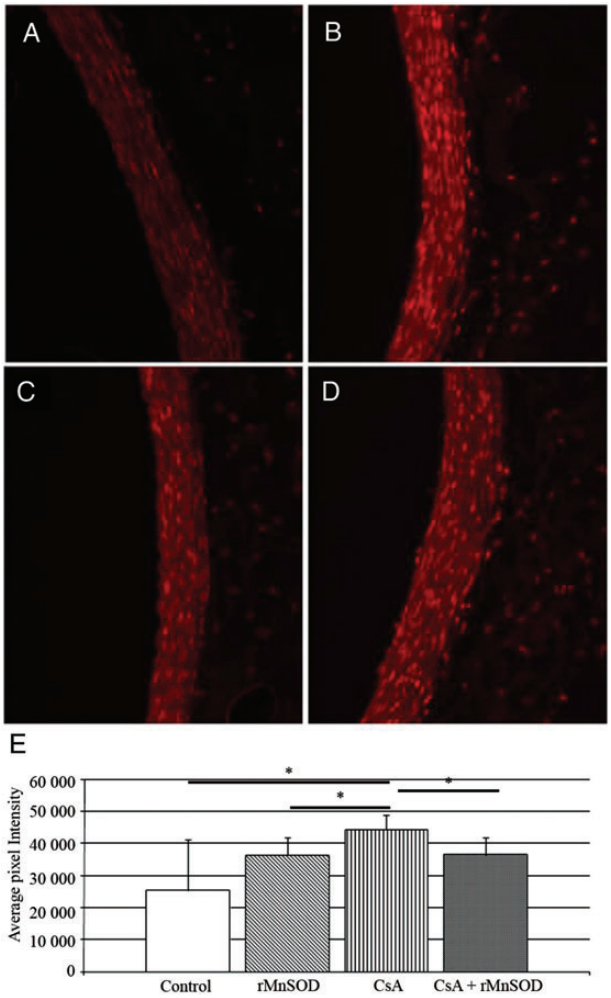

FIGURE 3: rMnSOD prevents CsA-induced injury on the aorta. Representative pictures of DHE staining on the rat aorta. CsA treatment induces a DHE fluorescence signal as the expression of superoxide-mediated intracellular injury (**B**). This effect is greater than in the control group (**A**). Co-treatment with rMnSOD and CsA prevents this effect (**D**). rMnSOD alone does not significantly affect DHE activation (**C**). (**E**) Fluorescence intensity quantification. Magnification ×200 (values are mean ± SD; * is for $P < 0.05$ compared with the CsA group).

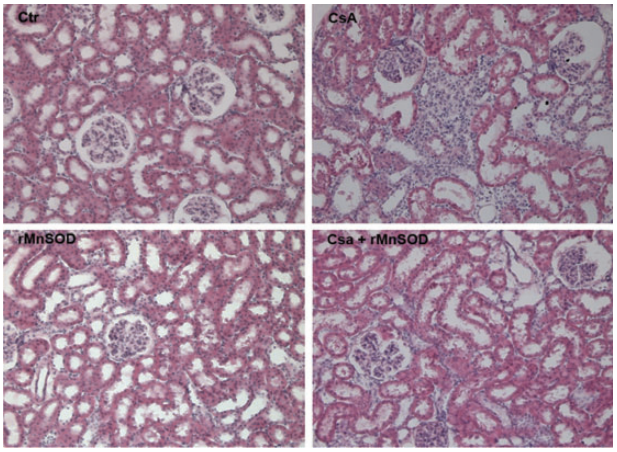

FIGURE 4: rMnSOD prevents CsA-induced morphological alteration. Representative pictures of haematoxylin and eosin staining from the renal cortex. CsA-treated rats show severe morphological alteration, including tubular vacuolization and atrophy associated with interstitial infiltrate. Co-treatment with rMnSOD is associated with a modest tubular injury and slight interstitial cellular infiltrate. rMnSOD-treated rats does not show morphological alteration compared with the control group. Magnification ×400.

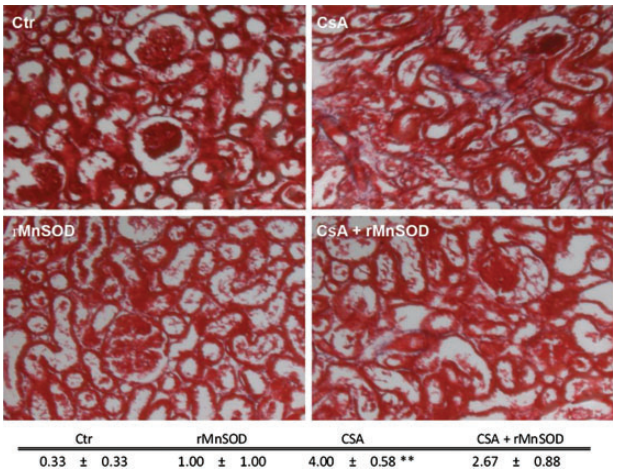

FIGURE 5: rMnSOD has a tendency to reduce the CsA-induced interstitial fibrosis. Representative pictures of Masson's trichrome staining from the renal cortex. CsA treatment is associated with an extensive degree of interstitial fibrosis (blue stripes). This pattern is almost absent in the control and rMnSOD. Co-treatment with rMnSOD and CsA leads to a tendency to a lower interstitial fibrosis. Magnification ×400. In the bottom, the fibrosis score reports a semi-quantitative evaluation ($n = 3$/group). ** is for $P < 0.01$ compared with the control group (one-way ANOVA plus unpaired *t*-test).

dichloroflurescin diacetate assay (Figure 2). Moreover, DHE experiments confirm that rMnSOD is able to quench CsA-induced ROS production in the aorta (Figure 3). The molecular mechanism responsible for these findings is most likely related to rMnSOD's remarkable ability to scavenge most of the radical species. It is very likely that this property may be related to the presence of a leader peptide that allows rMnSOD to enter the cells thus preventing its degradation by circulating proteases [10].

It has also been shown that CsA administration provokes extensive alterations in renal morphology; the lesions are mainly interstitial and tubular. In the present experiments, we have found the typical alterations of the CsA-induced kidney injures. In particular, at the tubular level, we have observed cellular vacuolization associated with a reduction in the height of the tubular epithelium and interstitial infiltrate. The co-administration of rMnSOD with CsA prevented the documented lesions (Figure 4).

Clinical observations [16] and experimental evidence [17] indicate that CsA administration is associated with other major side effects, including hypertension. In our experiments, although we have measured BP in anaesthetized animals, there was an increase of ∼15 mmHg in BP values and the co-treatment with rMnSOD was not able to modify this parameter

(Table 1). The mechanisms involved in CsA-induced hypertension are still unclear, but it is generally thought that the primary causes may be related to dysfunction of several organs including the nervous system, the vasculature or the kidney [18]. With respect to the kidney, clinical and experimental studies showed that cyclosporine-induced hypertension was sodium dependent [1, 19] probably related to the up-regulation of the Na^+–K^+–$2Cl^-$ cotransporter (NKCC2) in the thick ascending limb of Henle [20, 21]. We may speculate that hypertension in CsA-treated animals is independent from the mechanism involved in GFR regulation. By inducing a salt-sensitive state, CsA may impair the pressure-natriuresis response [22] of the kidneys and thus sustain the hypertension. An additional side effect of chronic administration of CsA is severe impairment of renal haemodynamics. Indeed, we have found a profound decrease in the GFR measured by inulin clearance, a method that is considered the gold standard for the measurement of renal function. This model of CsA-induced renal failure was not associated yet with the development of anaemia, probably related to the short-time of the experimental study (7 days). Several mechanisms appear to be implicated in the CsA-inducing GFR reduction; however, there is general agreement that the major factor is mediated by its action on afferent arteriolar resistance through vasoconstriction of all contractile elements leading to vessel narrowing [23]. The almost full prevention of the reduction in GFR induced by rMnSOD is not related to a lower serum level of CsA.

rMnSOD administration does not increase the urinary flow rate together with the GFR. Increased water reabsorption along the connecting and collecting ducts (CDs) could play a role in this sense. The CsA-induced up-regulation of NKCC2 [20] could be a common molecular mechanism sustaining hypertension and water reabsorption along the CD. rMnSOD is the first substance able to prevent the CsA effects on kidney haemodynamics and to reduce the renal histological damage. Therefore, it looks that rMnSOD is superior to other agents previously used to prevent and/or ameliorate CsA nephrotoxicity [17]. For example, rMnSOD is more effective as compared with hydroxytyrosol, a natural antioxidant of olive oil that was unable to ameliorate the reduction in the GFR associated with CsA administration [12]. Moreover, even the overexpression of superoxide dismutase, by gene delivery, could only partially reduce CsA-induced pathological alterations and inhibition of the GFR [24]. Finally, the administration of the SOD-mimetic, tempol, that normalized BP, was less effective than rMnSOD on the GFR [25].

In conclusion, our data indicate that rMnSOD is able to prevent arterial and renal oxidative stress, the reduction in the GFR secondary to CsA administration and, in addition, to partly improve the renal morphology. Given these findings, rMnSOD may represent a novel therapeutic option in the treatment of CsA nephrotoxicity.

ACKNOWLEDGMENTS

This work was supported by a grant from the Italian Ministry Scientific Research to G.C.

CONFLICT OF INTEREST STATEMENT

Part of this work was presented as an oral presentation at the 44th Annual Meeting and Scientific Exposition of the American Society of Nephrology, Philadelphia (PA) 2011.

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Received for publication: 7.6.2012; Accepted in revised form: 10.1.2013