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Viral delivery of superoxide dismutase gene reduces cyclosporine A-induced nephrotoxicity

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Viral delivery of superoxide dismutase gene reduces cyclosporine A-induced nephrotoxicity.

Background. Cyclosporine A (CsA) increases free radical formation in the kidney. Accordingly, this study investigated whether gene delivery of superoxide dismutase (SOD) reduced radical production and nephrotoxicity caused by CsA.

Methods. Rats were given adenovirus (Ad) carrying *lacZ* or *Cu/Zn-SOD* genes three days prior to CsA treatment. Histology, glomerular filtration rates (GFRs) and free radical adducts in urine were assessed.

Results. SOD activity was increased 2.5-fold three days after viral infection and remained at 2- and 1.6-fold higher 10 and 17 days later. Treatment with CsA for seven days decreased GFR by 70% in rats infected with Ad-lacZ as expected; however, the decrease was diminished significantly in rats receiving Ad-SOD. CsA treatment for two weeks caused a loss of brush border and dilation of proximal tubules, necrosis, and increased leukocyte infiltration into the kidney; these effects were minimized by SOD. Dimethyl sulfoxide (DMSO) was attacked by the hydroxyl radical to produce a methyl radical. Indeed, administration of CsA with ^{12}C-DMSO in rats infected with Ad-lacZ produced a radical adduct with hyperfine coupling constants similar to 4-POBN/methyl radical adduct and another unknown radical adduct. CsA given with ^{13}C-DMSO produced a 12-line spectrum, confirming the involvement of hydroxyl radicals. Free radical adducts detected in urine were increased approximately fivefold by CsA, an effect blocked completely by SOD.

Conclusions. CsA increases free radical formation. Gene delivery of SOD blocks formation of free radicals, thereby minimizing nephrotoxicity caused by CsA.

Cyclosporine A (CsA), a widely used immunosuppressive agent, causes moderate to severe renal dysfunction in approximately 30% of patients [1, 2]. Mechanisms of nephrotoxicity of CsA remain unclear; however, vitamin E attenuates CsA-induced lipid peroxidation and nephrotoxicity [3], consistent with the hypothesis that oxidative stress is responsible, at least in part, for its nephrotoxicity. Indeed, free radicals detected in urine were increased dramatically after CsA treatment [4]. Oxidative stress leads to formation of superoxide radicals that, in turn, are converted to hydroxyl radicals via H_2O_2 or $ONOO^-$. Methyl radicals derived from hydroxyl radical attack on dimethyl sulfoxide (DMSO) represent approximately 65% of radicals detected in urine but only 15% of radicals detected in bile [5], consistent with the clinical observation that the kidney is the major target organ of CsA toxicity. Dietary glycine, which blocks hydroxyl radical production most likely by minimizing renal nerve-dependent hypoxia-reoxygenation, prevents nephrotoxicity caused by CsA [5].

Superoxide dismutase (SOD), a potent antioxidant enzyme, has been shown to minimize a variety of oxidative injuries in vitro and in vivo [6–11]. If oxidative stress plays a role in CsA-induced nephrotoxicity, increased SOD in the kidney via gene delivery should be protective. Three major isoforms of SOD are found in mammalian species [12], namely cytosolic (Cu/Zn-SOD), mitochondrial (Mn-SOD), and extracellular (Cu/Zn-SOD) SOD. The Cu/Zn-SOD form accounts for approximately 95% of SOD activity in the kidney [12]. Accordingly, this study was designed to test the hypothesis that gene delivery of cytosolic Cu/Zn-SOD can minimize CsA-induced nephrotoxicity by blocking free radical formation.

Key words: immunosuppression, free radicals, adenovirus, oxidative stress, antioxidant enzyme.

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METHODS

Reagents

Sandimmune oral solution and powdered CsA were Novartis products (Basel, Switzerland), and glycine diets were provided by Novartis Nutrition (Minneapolis, MN, USA). Creatinine assay kits and α-(4-pyridyl 1-oxide)-

N-tert-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and ascorbate oxidase paddles were obtained from Boehringer Mannheim Inc. (Indianapolis, IN, USA). ^{12}C-dimethyl sulfoxide (DMSO; containing 1.1% natural abundance ^{13}C) and $^{13}C_2$-DMSO (containing minimum 99 atom percentage ^{13}C) were obtained from Isotech, Inc. (Miamisburg, OH, USA).

Animals

Male Sprague-Dawley rats (200 to 250 g) were fed a semisynthetic powdered diet (AIN 76A) beginning three days prior to infection with virus. Recombinant adenovirus containing the transgene for either Cu/Zn-SOD (Ad-SOD) or β-galactosidase (Ad-*lacZ*) was prepared by the UNC vector core as described elsewhere [13, 14]. Briefly, the plasmid shuttle vector pAd5.CMV.*lacZ* was constructed by standard cloning protocols as described by Sambrook et al and Graham and Prevec [15, 16]. The adenoviral shuttle plasmid was transfected into the permissive HEK 293 host cell line to generate recombinant Ad-*lacZ* adenovirus. The virus isolates were plaque purified and propagated in HEK 293 cells, isolated, concentrated, and titered by plaque assay. Recombinant adenovirus containing the transgene for human Cu/Zn-SOD (Ad-SOD) was received as a seed stock from Dr. John Engelhardt of the University of Iowa (Iowa City, IA, USA) [17] and propagated in a similar manner as Ad-lacZ. Purified virus (1×10^9 pfu) was diluted in 1 mL normal saline and injected intravenously. Three days after viral infection, rats were treated with CsA (25 mg/kg, orally) in olive oil or an equivalent volume of vehicle daily for 14 days. Previous studies showed that CsA at doses ranging from 25 to 50 mg/kg causes nephrotoxicity in the rat characterized by reduced glomerular filtration rates (GFRs), increased serum creatinine and pathological changes involving proximal tubular cell swelling and necrosis, infiltration of macrophages, and interstitial fibrosis [2, 18–20]. All animals received humane care in compliance with institutional guidelines, and viral experiments were approved by the Institutional Biosafety Committee.

Glomerular filtration rates and histology

To estimate GFRs, animals were placed in metabolic cages, and urine was collected daily. Creatinine levels in urine and serum were determined using commercially available kits (Sigma). GFRs were calculated from the ratio of creatinine in the urine/blood and the volume of urine produced in 24 hours and were corrected for body weight [21]. In some experiments, inulin was infused intravenously, and inulin in urine and blood was measured as described elsewhere [22]. Glomerular filtration rates calculated from inulin (data not shown) and creatinine clearance were nearly identical under these conditions.

On the day of sacrifice, the kidneys were rinsed with 10 mL normal saline and were perfusion fixed with 10% buffered formaldehyde. Sections were stained with hematoxylin eosin and were analyzed microscopically.

Detection of β-galactosidase

Approximately 100 mg of kidney tissue were homogenized in 500 μL of buffer containing 40 mmol/L Tris, 140 mmol/L NaCl, and a protease inhibitor cocktail, including aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and dithiothreitol (pH 7.6) [23]. The homogenate was centrifuged at $10,000 \times g$ for 10 minutes at 4°C, and the supernatant was collected. The activity of β-galactosidase in the supernatant was quantitated by the cleavage of O-nitrophenyl-β-D-galactopyranoside (ONPG) to nitrophenol, which was determined spectrophotometrically at 420 nm as described elsewhere [24]. Protein was measured by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

Western blotting for superoxide dismutase

Kidneys were perfused with 10 mL of normal saline and frozen in liquid nitrogen. Samples were homogenized in a buffer containing 40 mmol/L Tris, 140 mmol/L NaCl, and the protease inhibitors (pH 7.6), and were centrifuged at $900 \times g$ for 10 minutes at 4°C. The supernatant was analyzed for SOD using Western blotting as described elsewhere [24]. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad).

Activity of superoxide dismutase

Kidneys were homogenized in a buffer containing 40 mmol/L Tris, 140 mmol/L NaCl, protease inhibitors (aprotinin, leupeptin, and PMSF, pH 7.6) and dithiothreitol, and were centrifuged at $10,000 \times g$ for 10 minutes at 4°C. Superoxide dismutase SOD activity in the supernatant was measured by inhibition of the reduction of ferricytochrome c [25]. Supernatant (10 μL) was added to a solution containing 50 mmol/L K_2HPO_4, 0.1 mmol/L Na_2EDTA, 0.5 mg/mL cytochrome c, and 0.25 mg/mL xanthine. Generation of superoxide was initiated by the addition of 0.004 units of xanthine oxidase. After a 10-minute incubation at room temperature, the absorbance at 550 nm was measured. Superoxide dismutase activity was estimated based on a standard curve generated using purified bovine erythrocyte SOD (Boehringer Mannheim, Mannheim, Germany). Uric acid in kidney homogenates was measured using a commercially available kit from Sigma, and uric acid production in the presence of renal tissue, exogenous xanthine and xanthine oxidase was monitored by absorbance changes at 290 nm as described elsewhere [26]. Tissue contents of uric acid were minimal and were similar in all groups studied (data not shown). In addition, uric acid production rates were not different among the Ad-lacZ and

Ad-SOD groups as expected [26]. Therefore, the effect of uric acid on the assay for SOD activity, if any, is the same for both the Ad-lacZ and Ad-SOD groups.

Detection of free radical adducts

To assess free radical formation, powdered CsA (25 mg/kg) was dissolved in 0.2 mL ^{12}C- or ^{13}C-DMSO and administered by oral gavage. Three hours after CsA treatment, the spin trapping reagent α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (4-POBN; 1 g/kg body weight) was dissolved in 2.0 mL normal saline and injected slowly into the tail vein. The urinary bladder was always emptied because of handling. Urine was collected into 30 mmol/L dipyridyl (50 μL) to prevent ex vivo radical formation. At the end of three hours of urine collection, all rats were sacrificed, and the lower abdomen was opened. Urine in the bladder was aspirated using a syringe and pooled with other urine samples. Samples were kept at −80°C until analysis. Urine was placed in a quartz electron spin resonance (ESR) flat cell and bubbled with oxygen for 10 minutes followed by nitrogen for 5 minutes. After the ESR spectrum was obtained, an ascorbate oxidase paddle was inserted into the sample, and the gas treatment was repeated. These treatments oxidize the ascorbate to dehydroascorbate, an ESR-silent substance, and the ESR-silent, reduced hydroxylamine form of the free radical adducts to the ESR-active nitroxide. Free radical adducts were detected with a Bruker 200 ESR spectrometer. Instrument conditions were as follows: 20 mW microwave power, 0.63 G modulation amplitude, and 80 G scan range [27]. Spectral data were stored on an IBM compatible computer and were analyzed for ESR hyperfine coupling constants by computer simulation [28]. The magnitude of the ESR signal was measured at the low-field line (the first line from left) at identical gains and expressed in arbitrary units (1 unit = 1 cm chart paper).

Statistical analysis

For all statistics, analysis of variance (ANOVA) and the Student-Newman-Keuls post hoc tests were used. $P < 0.05$ was selected prior to the study to indicate significance.

RESULTS

Expression of β-galactosidase and superoxide dismutase in the kidney

Three days after infection with recombinant adenoviral (Ad) vectors containing lacZ, β-galactosidase, which is encoded by lacZ, was increased approximately twofold in the kidneys compared with the groups receiving normal saline or Ad-SOD. (Fig. 1). These results indicate that adenovirus can effectively infect kidney cells, thereby delivering genes of interest.

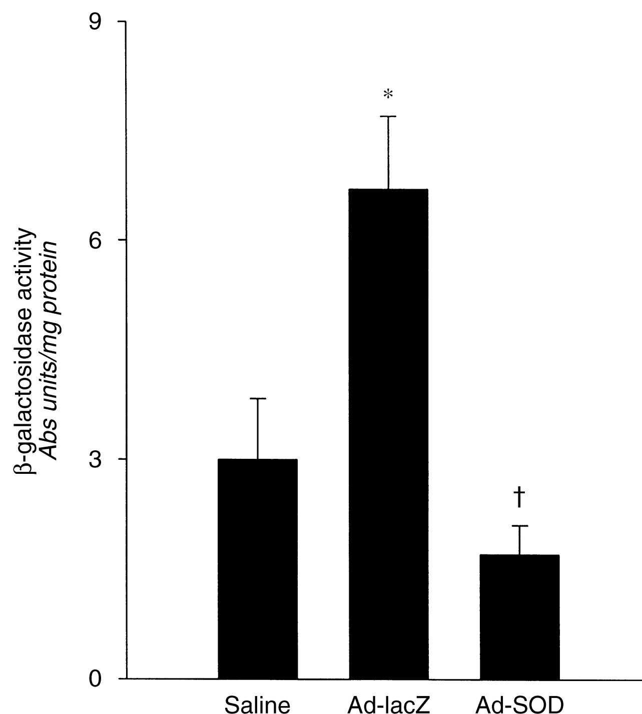

Fig. 1. Activity of β-galactosidase in the kidney. Recombinant adenoviral vectors (Ad) containing lacZ (1×10^9 pfu) diluted in 1 mL normal saline or an equal volume of vehicle were injected intravenously. Three days after viral infection, rats were sacrificed, and β-galactosidase in kidney homogenates was detected by cleavage of O-nitrophenyl-β-D-galactopyranoside as described in the **Methods** section. Values are means ± SEM (ANOVA, $N = 4$ in each group). $^*P < 0.05$ compared with saline; $^†P < 0.05$ compared with Ad-lacZ (Student-Newman-Keuls post hoc test).

Three days after infection, human SOD (19 kD) protein was detected in kidney from rats infected with Ad containing the SOD gene but not in kidney from rats receiving Ad-lacZ or saline (Fig. 2A). Moreover, three days after viral infection, SOD activity in kidney homogenates was approximately threefold higher in rats receiving Ad-SOD than rats receiving normal saline and about 2.5-fold greater compared with rats receiving Ad-lacZ (Fig. 2B). SOD activity was twofold higher 10 days and 1.6-fold 17 days later. Therefore, it is clear that viral gene delivery significantly increases SOD protein expression and enzyme activity in the kidney.

Effects of cyclosporine A and superoxide dismutase on glomerular filtration rates

Glomerular filtration rate, a classic indicator of renal function, was approximately 0.65 mL/min/100 g body wt in controls and was not altered by adenoviral infection. GFR in rats receiving Ad-lacZ declined by about 70% after seven days of treatment with CsA as expected and

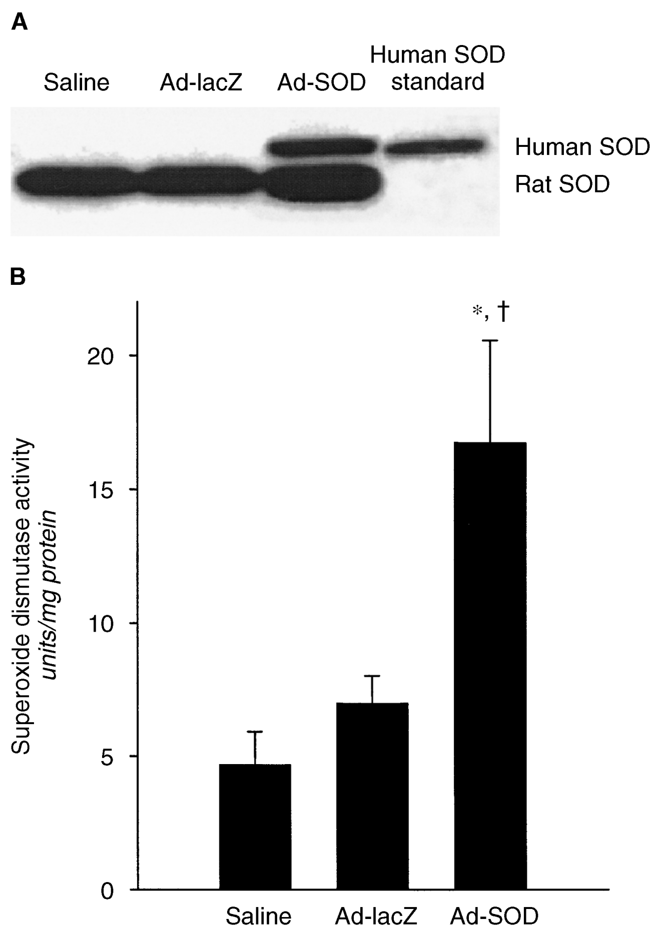

Fig. 2. Superoxide dismutase (SOD) expression (*A*) and activity (*B*) in the kidney. Recombinant adenoviral vectors (Ad) containing lacZ or SOD (1×10^9 pfu) diluted in 1 mL normal saline were injected intravenously. Three days after viral infection, rats were sacrificed, and the kidney was homogenized in a buffer containing 40 mmol/L Tris, 140 mmol/L NaCl, and protease inhibitors. SOD protein in the supernatant was detected by Western blotting, and activity was measured by the inhibition of reduction of ferricytochrome c. (A) Representative image of Western blotting for SOD. (B) Activity of SOD. Values are means ± SEM (ANOVA, $N = 4$ in each group). $^*P < 0.05$ compared with saline; $^\dagger P < 0.05$ compared with Ad-lacZ (Student-Newman-Keuls post hoc test).

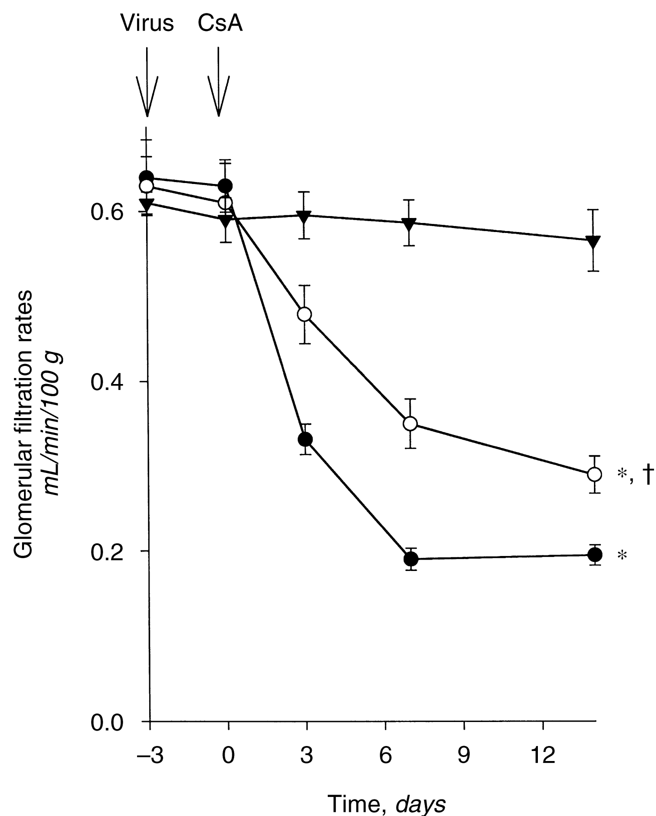

Fig. 3. Glomerular filtration rates (GFRs). Rats were given adenoviral vectors (Ad) containing lacZ (●) or SOD (○; 1×10^9 pfu) diluted in 1 mL normal saline intravenously three days prior to CsA treatment. Rats were treated with CsA (25 mg/kg, orally) or an equivalent volume of olive oil (▼, Ad-lacZ) daily for 14 days. Urine samples were collected using metabolic cages, and GFRs were calculated from the ratio of creatinine in the urine/blood, the volume of urine produced in 24 hours, and the body weight. Values are means ± SEM (analysis of variance, $N = 4$ in each group). $^*P < 0.05$ compared with Ad-lacZ; $^\dagger P < 0.05$ compared with Ad-lacZ + CsA group (Student-Newman-Keuls post hoc test).

reached a new steady-state level in approximately two weeks (Fig. 3). Delivery of the *SOD* gene significantly blunted decreases in GFRs caused by CsA (Fig. 3) [20].

Histology

The histology of the kidney cortex after two weeks of CsA treatment from this study is shown in Figure 4. Figure 4A depicts a kidney cortex with normal renal architecture from a rat infected with Ad-lacZ that received olive oil. CsA caused loss of brush border and dilation of the proximal tubules, swelling, necrosis, and white blood cell infiltration, as has been reported previously (Fig. 4B) [1, 20]. These pathological changes were largely blocked by delivery of the *SOD* gene (Fig. 4C).

Free radical formation

A previous study from this laboratory showed that CsA causes hypoxia and hydroxyl radical formation in the kidney [4]. To detect hydroxyl radical formation, DMSO was given to rats along with CsA. Because hydroxyl radicals attack DMSO, producing a methyl fragment readily trapped by 4-POBN leading to a stable adduct, DMSO significantly increases the sensitivity of detection of hydroxyl radicals with spin-trapping reagents [29].

The ESR signal of free radicals was minimal in urine from rats that were infected with virus but did not receive CsA (Fig. 5A, B). When CsA was administered with ^{12}C-DMSO, a six-line ESR spectrum due to 4-POBN radical adducts was detected in urine (Fig. 5C) and was reduced significantly by Ad-SOD (Fig. 5D). Computer simulation of the spectrum demonstrated two free radical species. Hyperfine coupling constants of species I (20%

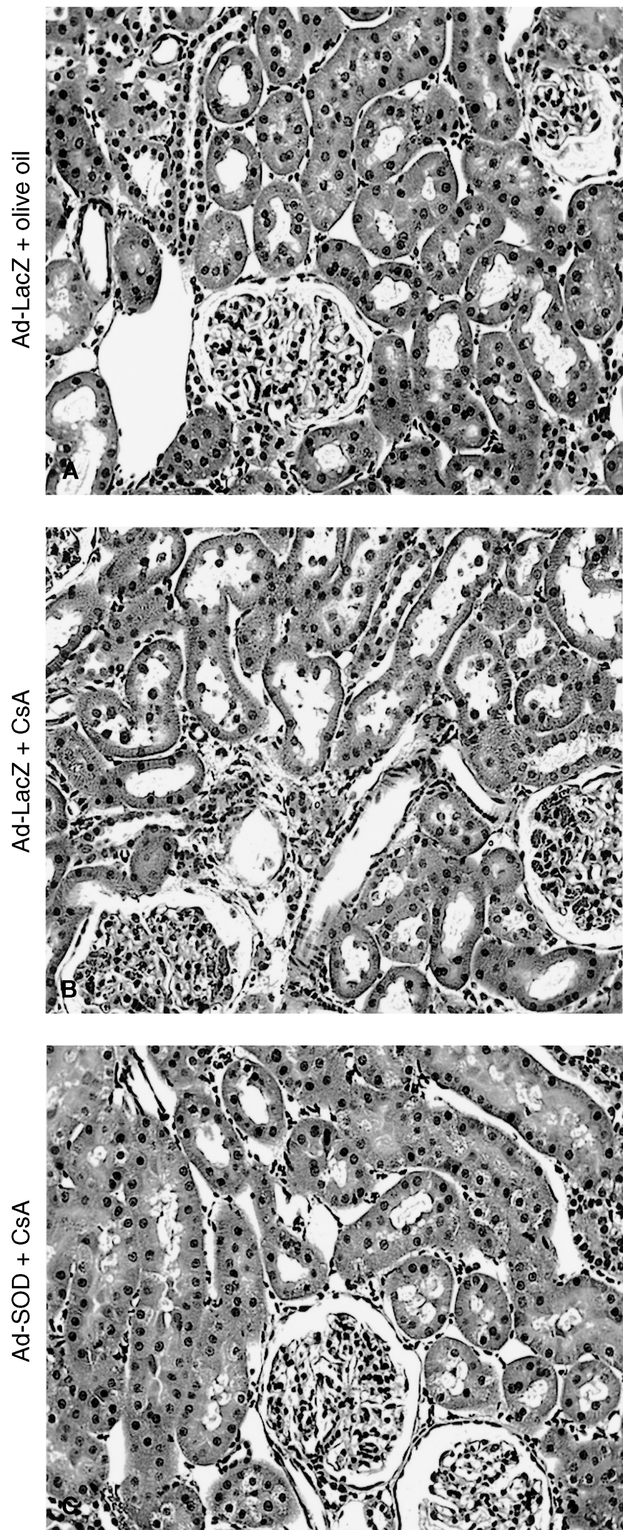

Fig. 4. Effects of cyclosporine A (CsA) and superoxide dismutase (SOD) on renal histology. The conditions are the same as in Figure 3. Rats were treated with CsA (25 mg/kg, orally) or an equivalent volume of olive oil daily for 14 days. Data represent typical images of hematoxylin eosin-stained sections of perfusion-fixed kidneys. Original magnification ×200. (*A*) Section from animals infected with Ad-lacZ and olive oil. (*B*) Section from animals infected with Ad-lacZ and treated with CsA. (*C*) Section from animals infected with Ad-SOD and treated with CsA.

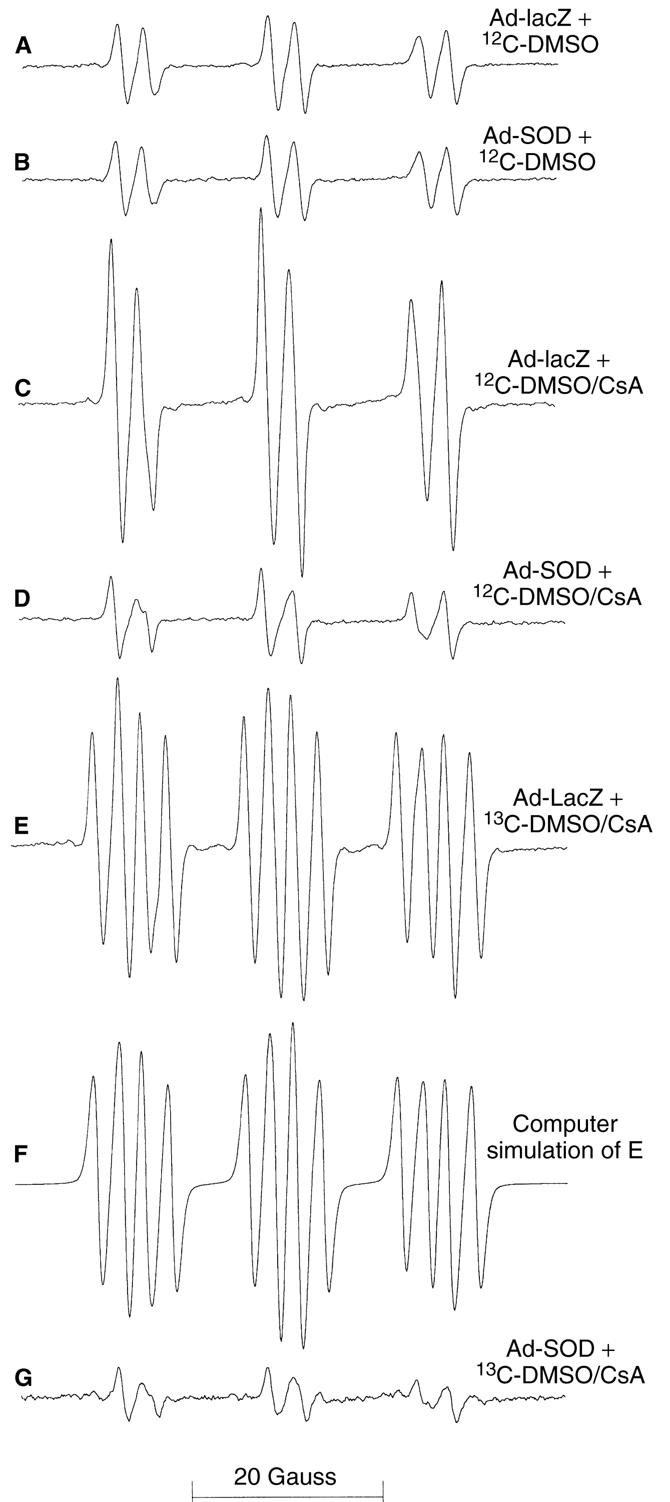

Fig. 5. Effects of cyclosporine A (CsA) and superoxide dismutase (SOD) on electron paramagnetic resonance (ESR) spectra of free radical adducts in urine. After pretreatment with CsA for seven days, powdered CsA was dissolved in 0.2 mL ^{12}C- or ^{13}C-DMSO and given to the rat by oral gavage. Three hours after the last dose of CsA, the spin-trapping reagent α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (4-POBN; 1 g/kg body weight) was dissolved in 2.0 mL normal saline and injected slowly into the tail vein. Urine was collected using metabolic cages for three hours. Free radical adducts in urine were detected with a Bruker ESP 200 ESR spectrometer. Typical spectra: (*A*) rat received Ad-lacZ and ^{12}C-DMSO; (*B*) Ad-SOD and ^{12}C-DMSO; (*C*) Ad-lacZ and CsA in ^{12}C-DMSO; (*D*) Ad-SOD and CsA in ^{12}C-DMSO; (*E*) Ad-lacZ and CsA in ^{13}C-DMSO; (*F*) computer simulation of the radical adduct spectrum of "E"; (*G*) Ad-SOD and CsA in ^{13}C-DMSO.

of total radicals) are a^{N} = 15.68 G and a^{H}_{β} = 2.61 G, which are identical to the unknown radical found in the urine from CsA-treated rats [4]. Hyperfine coupling constants of species II (80%) are a^{N} = 15.96 G and a^{H}_{β} = 2.74 G. Previously, a study showed that ex vivo formation of hydroxyl radicals initiated by the Fenton reaction with ^{12}C-DMSO and 4-POBN in urine produced a six-line radical signal with coupling constants of a^{N} = 15.96 G and a^{H}_{β} = 2.74 G [5], identical to species II detected in the urine of CsA/^{12}C-DMSO-treated rats. Proof that the radical adduct was DMSO derived was confirmed using ^{13}C-DMSO. In vivo administration of CsA with ^{13}C-DMSO yielded urine samples that produced a 12-line ESR spectrum (Fig. 5E, F; species I, a^{N} = 15.68 G and a^{H}_{β} = 2.61 G; species II, a^{N} = 15.96 G, a^{H}_{β} = 2.74 G and $a^{C\text{-}13}_{\beta}$ = 4.95 G) as expected [5]. The doubling of the number of ESR lines indicates the presence of a magnetic C-13 in the radical adduct, which in this case could arise only from the ^{13}C-DMSO [30], confirming hydroxyl radical formation in the kidney. Importantly, in rats treated with Ad-SOD and CsA/^{13}C-DMSO, this methyl adduct indicative of hydroxyl radicals could not be detected above basal levels (Fig. 5G). In addition, the unknown species was also reduced significantly. Overall, free radical formation caused by CsA was almost totally blocked by delivery of the Cu/Zn *SOD* gene (Fig. 6).

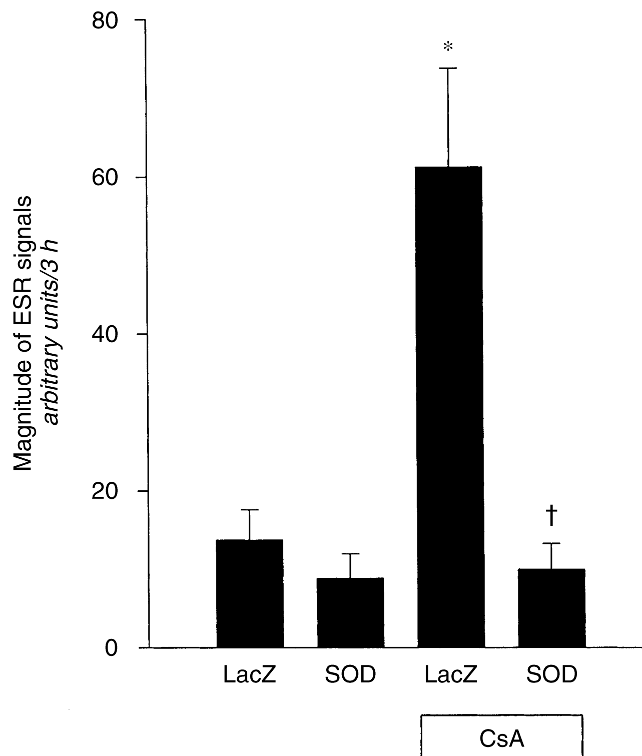

Fig. 6. Effects of superoxide dismutase (SOD) on average free radical production caused by CsA. The conditions are the same as in Figure 5. The magnitude of the ESR signal was measured at the low-field line (the first line from left) at identical gains and is expressed in arbitrary units (1 unit = 1 cm chart paper). Relative radical adduct production was calculated by multiplying the magnitude by the volume of urine collected during a three-hour sampling interval. Values are means ± SEM (ANOVA, N = 4 to 5 in each group). *$P < 0.05$ compared with Ad-lacZ controls. †$P < 0.05$ compared with the Ad-lacZ + CsA group (Student-Newman-Keuls post hoc test).

DISCUSSION

Cyclosporine A causes oxidative stress in the kidney

Cyclosporine A, a hydrophobic cyclic peptide produced by the fungus *Tolypocladium inflatum gams* [31], is an immunosuppressant that is critical in organ transplantation and is used in a variety of immune disorders, including rheumatoid arthritis and psoriasis [31–34]. Patients must take this drug over their lifetime, and unfortunately, CsA causes severe nephrotoxicity [1]. This injury is characterized by diminished renal blood flow and GFR as well as proximal tubular cell swelling, necrosis, and infiltration of neutrophils and macrophages [1]. If the dose of cyclosporine is not reduced, renal dysfunction and failure of the kidney will occur in approximately 30% of patients [1, 2].

Mechanisms of CsA nephrotoxicity remain unclear; however, increasing evidence suggests that oxidative stress is involved [3, 35, 36]. CsA treatment increases the oxidized glutathione/glutathione sulfhydryl (GSSG/GSH) ratio, malondialdehyde, and conjugated dienes in the kidney [3, 36] as well as urinary excretion of F2-isoprostanes [37]. Generation of superoxide and hydrogen peroxide by isolated glomeruli is also enhanced by CsA treatment [38], indicating oxidative stress. CsA increases malondialdehyde, a product of lipid peroxidation, in isolated hepatic microsomes [39], suggesting that metabolism of CsA by cytochrome P450 could directly lead to free radicals. In addition, cytochrome P450 could be a source of iron that could be involved in conversion of hydrogen peroxide to hydroxyl radicals. However, a recent study using the spin-trapping technique and ESR has shown that administration of ^{12}C- or $^{13}C_3$-CsA results in only a six-line ESR spectrum in urine and bile, indicating that free radials are not derived directly from the CsA molecule [5]. In contrast, a methyl radical produced from the attack of hydroxyl radical on DMSO is detected in urine after CsA (Fig. 5) [5], providing direct physical evidence for oxidative stress since the methyl fragment arises from the attack of hydroxyl radical on DMSO [29]. Previous studies showed that CsA increases the activity of the renal nerves [40] and causes vasoconstriction [18]. Hydroxyl radical production caused by CsA can be minimized by renal denervation [5], suggesting that CsA causes oxidative stress by inducing vasoconstriction, which leads to hypoxia reoxygenation. In support of this idea, vitamin E, N-acetylcysteine, and lazaroids attenuate CsA-induced nephrotoxicity in rats [3, 41, 42]. Glycine, which

decreases renal nerve activity and blocks free radical production, also minimizes kidney injury caused by CsA [5, 20]. These data support the hypothesis that CsA causes oxidative stress leading to nephrotoxicity. However, kidney injury is probably not totally due to oxidative stress since overexpression of SOD, which largely blocked free radical formation, only partially blunted CsA-induced pathological changes and inhibition of renal function.

Superoxide dismutase minimizes cyclosporine A-induced hydroxyl radical formation and renal injury

Oxidative stress causes formation of superoxide radicals, which, in turn, are converted to highly reactive hydroxyl radicals through H_2O_2 in the presence of transition metals [43]. However, in biological systems, catalase is highly effective at rapidly degrading H_2O_2; therefore, accumulation of H_2O_2 is unlikely. Superoxide radicals can also react with nitric oxide (NO) to form $ONOO^-$, which leads to hydroxyl-like species [44]. Thus, increased expression of SOD could decrease superoxide and prevent its reaction with NO. In addition to formation of hydroxyl radicals, superoxide can rapidly react with iron-sulfur centers, causing prolonged inhibition of mitochondrial function [44]. Therefore, overexpression of SOD will decrease superoxide as well as $ONOO^-$ and hydroxyl radical formation, thus protecting the kidney against oxidative stress.

The effect of SOD on oxidative stress has been controversial. In some studies, SOD minimizes ischemia/reperfusion injury in the heart, liver, kidney, and small intestine in vitro and in vivo [6–8, 11, 45–47]. However, SOD fails to protect against ischemia/reperfusion injury in some animal studies and clinical trials [10, 48, 49]. This discrepancy is probably due to the rapid degradation of SOD by circulating proteases leading to a short half-life, since constant infusion of SOD protects in the heart [46], while bolus doses do not [10]. Constant intravenous infusion is possible for acute studies; however, it is impractical in patient populations in which CsA is taken chronically. Therefore, a gene delivery technique was used in this study to achieve constant expression of SOD in the kidney.

Indeed, the *SOD* gene can be transduced and expressed effectively in kidney cells using adenoviral vectors leading to significant increases in SOD activity in the kidney (Fig. 2). Cyclosporine A dramatically increased POBN/methyl radical adducts in the urine, reflecting formation of hydroxyl radicals (Fig. 5). Importantly, infection with Ad-SOD almost totally blunted increases in hydroxyl radical formation caused by CsA (Figs. 5 and 6). Moreover, delivery of the *SOD* gene minimized CsA-induced pathological alterations (Fig. 4) and inhibition of renal function significantly (Fig. 3). Taken together, these results are consistent with the hypothesis that CsA causes renal injury, at least in part, by increasing reactive oxygen species. Importantly, this study demonstrated that this injury can be prevented by delivery of the *SOD* gene.

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APPENDIX

Abbreviations used in this article are: Ad, adenovirus; CsA, cyclosporine A; DMSO, dimethyl sulfoxide; GFR, glomerular filtration rate; ESR, electron spin resonance spectroscopy; 4-POBN, α-(r-pyridyl 1-oxide)-*N-tert*-butylnitrone; SOD, superoxide dismutase.

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