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Dietary Glycine and Renal Denervation Prevents Cyclosporin A-Induced Hydroxyl Radical Production in Rat Kidney

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ABSTRACT

Cyclosporin A (CsA) nephrotoxicity is associated with renal hypoxia and increases in free radicals in the urine. This study was designed to elucidate the mechanism of radical production caused by CsA. Pretreatment of rats with CsA (25 mg/kg, i.g.) for 5 days decreased glomerular filtration rates by 65%, an effect largely prevented by both dietary glycine (5%) or renal denervation. CsA dissolved in olive oil produced a 6-line α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN)/free radical signal in the urine, which partitioned predominantly into the aqueous phase after chloroform extraction (i.e., it is water soluble). Dimethyl sulfoxide (DMSO) is attacked by the hydroxyl radical to produce a methyl radical; administration of CsA with [^{12}C]DMSO produced two radical species in urine, one with hyperfine coupling constants similar to the 4-POBN/methyl radical adduct found in aqueous solution. CsA given with [^{13}C]DMSO produced a 12-line spectrum, confirming the formation of hydroxyl radicals. The methyl radical produced by the hydroxyl radical represented 62% of radicals detected in urine but only 15% in bile. Therefore, hydroxyl radicals are produced largely in the kidney. Free radicals in urine were increased about 5-fold by CsA, an effect completely blocked by the inhibitory neurotransmitter, glycine, or by renal denervation. CsA infusion for 30 min increased efferent renal nerve activity 2-fold, and dietary glycine (5%) totally blocked this phenomenon. Taken together, these data are consistent with the hypothesis that CsA increases hydroxyl radical formation by increasing renal nerve activity resulting in vasoconstriction and hypoxia-reoxygenation. Glycine blunts the effect of CsA on the renal nerve, which explains, in part, prevention of nephrotoxicity.

Cyclosporin A (CsA) is an immunosuppressive agent used after organ transplantation and in the treatment of several autoimmune diseases (Wood et al., 1983; Berg et al., 1986). It has a number of serious side effects, with kidney damage being the most common with grave consequences. Moderate to severe renal dysfunction occurs in about 30% of patients receiving CsA (Farthing and Clark, 1981; Young et al., 1995).

A previous study showed that binding of a 2-nitroimidazole hypoxia maker, pimonidazole, in the kidney was increased nearly 3-fold by CsA, indicating marked tissue hypoxia (Zhong et al., 1998). Moreover, free radicals in urine were also increased dramatically after CsA treatment (Zhong et al., 1998), and vitamin E attenuated CsA-induced lipid peroxidation and nephrotoxicity (Wang and Salahudeen, 1995); however, the mechanism by which CsA increases free radical formation is not clear. It is also known that CsA increases renal nerve activity (Moss et al., 1985) resulting in vasoconstriction in the kidney (Murray et al., 1985; Mehring et al., 1992). It also causes vasoconstriction directly in isolated renal arterioles (Lanese and Conger, 1993; Lanese et al., 1994). These alterations could theoretically lead to a classical hypoxia-reoxygenation injury involving free radicals. In addition, free radicals could be derived directly from CsA or its metabolites. Consistent with this possibility, CsA increased lipid peroxidation in isolated hepatic microsomes, the major metabolic site for CsA (Inselmann et al., 1990). Therefore, it is possible that metabolism of CsA by cytochrome P-450 in the kidney could directly enhance the production of free radicals. It has been shown that CsA inhibits mitochondrial respiration in renal tubular cells (Jung and Pergande, 1985); however, clear mechanisms remain unknown. The purpose of present study was to investigate the mechanism by which CsA causes free radical formation.

Recent studies in this laboratory demonstrated that dietary glycine could prevent CsA-induced alterations in renal function and pathological changes including proximal tubular dilatation, cellular necrosis, and infiltration of macrophages (Thurman et al., 1997). Glycine is a neurotransmitter

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ABBREVIATIONS: CsA, cyclosporin A; GFR, glomerular filtration rate; ESR, electron spin resonance; 4-POBN, (α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone; DMSO, dimethyl sulfoxide.

with inhibitory effects (Ito and Cherubini, 1991); therefore, it could decrease renal sympathetic nerve firing thus inhibiting hypoxia/reperfuion injury caused by CsA (Heyman et al., 1992). Indeed, severing the renal nerves diminished CsA nephrotoxicity (Murray et al., 1985). Accordingly, this study also evaluates the hypothesis that dietary glycine can minimize CsA-induced nephrotoxicity by blocking increases in renal sympathetic nerve activity associated with CsA treatment.

Materials and Methods

Reagents. Sandimmune oral solution was the Novartis product (Novartis Pharmaceutics, Basel, Switzerland); [^{12}C]CsA (containing 1.1% natural abundance of ^{13}C) and [$^{13}C_3$]CsA (containing an additional ^{13}C-labeled alanine residue: purity > 98%; see Fig. 1) were synthesized by Dr. Rolf Voges (Novartis Pharmaceutics) with standard techniques, and glycine diets were provided by Novartis Nutrition (Minneapolis, MN). The creatinine assay kits, deferoxamine mesylate and α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St. Louis, MO). Ascorbate oxidase paddles were obtained from Boehringer Mannheim Inc. (Indianapolis, IN). [^{12}C]dimethyl sulfoxide (DMSO) (containing 1.1% natural abundance of ^{13}C) and [$^{13}C_2$]DMSO (containing minimum 99 atom % ^{13}C) were obtained from Isotech, Inc. (Miamisburg, OH).

Animals. Male Sprague-Dawley rats (200–250 g) were fed a semisynthetic powdered diet containing 5% glycine and 15% casein (glycine diet) or 20% casein (control diet) (Rose et al., 1997). Diets were begun 3 days before bilateral renal denervation or the sham operation. For renal denervation, rats were anesthetized with metofane, the upper abdomen was opened with a vertical midline incision, and kidneys were carefully freed from the surrounding tissues. Renal nerves were identified along the dorsal side of renal vessels using an operating microscope and severed 5 mm from the renal hili. In sham-operated (control) animals, kidneys were also freed from surrounding tissues, and gentle manipulation was performed on kidneys and renal vessels to mimic the conditions of denervation surgery. Four days after surgery, rats were treated with CsA (25 mg/kg, p.o.) or an equivalent volume of olive oil daily for 5 days. Previous studies showed that CsA at doses ranging from 25 to 50 mg/kg caused nephrotoxicity 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 in rats fed standard chow diets (Farthing and Clark, 1981; Thomson et al., 1984; English et al., 1987; Thurman et al., 1997). Higher doses of CsA are required in rats than in humans to cause renal damage. This effect was probably due to the lower sensitivity of rats to CsA (English et al., 1987; Farthing and Clark, 1981; Thomson et al., 1984). In this study, CsA (25 mg/kg dissolved in olive oil at a concentration of 10 mg/ml) or an equivalent volume of olive oil vehicle was given by oral gavage daily for 5 days. All animals received humane care in compliance with institutional guidelines.

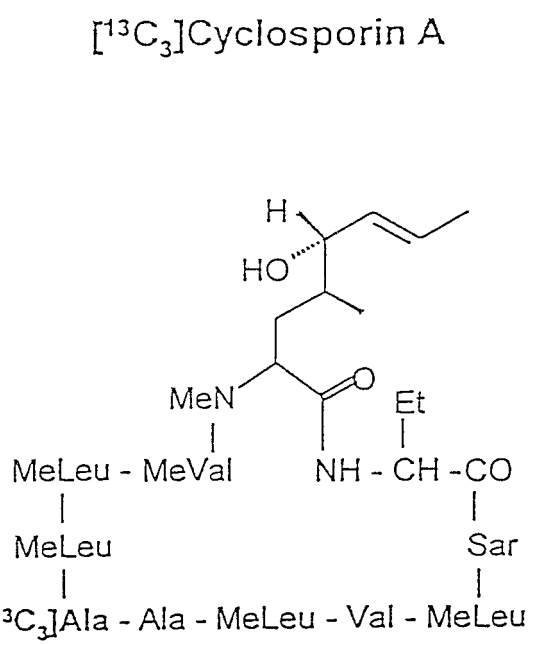

Fig. 1. Structure of [$^{13}C_3$]CsA. [$^{13}C_3$]CsA was synthesized with standard technique using ^{13}C-labeled β-alanine.

GFRs. To estimate GFRs over time, 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 h, and corrected for the body weight (Laiken and Fanestil, 1985). In some experiments, inulin was infused i.v., and inulin in urine and blood was measured as described elsewhere (Davidson and Sackner, 1963). GFRs calculated from inulin clearance and creatinine clearance were nearly identical under these conditions.

Detection of Free Radical Adducts. To assess free radical formation, powdered [^{12}C]- or [$^{13}C_3$]CsA (25 mg/kg) was dissolved with 0.2 ml DMSO and administered by gavage. In some experiments, [^{12}C]- or [$^{13}C_3$]CsA were dissolved in 0.1 ml acetone, added to olive oil (0.25 ml/100 g), and bubbled with nitrogen for 30 min to remove the acetone. Three hours after CsA treatment, the spin-trapping reagent 4-POBN (1 g/kg b.wt.) was dissolved in 2.0 ml normal saline and injected slowly into the tail vein. After injection of 4-POBN, the urinary bladder was always voided due to handling. Urine was collected using metabolic cages for 3 h after injection of 4-POBN. At the end of each experiment, all rats were sacrificed, the lower abdomen was opened, and urine in the urinary bladder was aspirated using a syringe and pooled with other urine samples. In other experiments, rats were anesthetized with metofane, the upper abdomen was opened by a vertical midline incision, and a polyethylene tubing (PE-10) was inserted into the common bile duct and secured with a 6–0 suture. Bile was collected for 1 h via a polyethylene tube into 50 μl of 30 mM deferoxamine mesylate to prevent free radical formation ex vivo. Blood samples were collected into 50 μl of 30 mM deferoxamine mesylate at the end of bile collection. Samples were kept on dry ice until analysis, and some bile and urine samples were extracted with an equal volume of chloroform. Bile and urine samples were placed in a quartz electron spin resonance (ESR) flat cell and bubbled with oxygen for 10 min followed by nitrogen for 5 min. After the ESR spectrum was obtained, an ascorbate oxidase paddle was inserted into the sample and the gas treatment repeated. This second treatment completely removed the ascorbate free radical from the sample. All data shown received both treatments. The aqueous phases of the chloroform extraction were treated similarly. The organic phases of the extractions were bubbled only with nitrogen. In vitro preparation of the 4-POBN/$\cdot CH_3$ radical adduct involved reacting DMSO (250 mM) with hydroxyl radicals produced by a Fenton reaction occurring between H_2O_2 (2 mM) and $FeSO_4$ (2 mM) in pH 7 phosphate buffer in the presence of 4-POBN (50 mM). To compare coupling constants in identical environments, 50 μl of the 4-POBN/$\cdot CH_3$ solution were added to 500 μl of urine or bile from untreated rats followed by gas treatments and ESR analysis identical with the samples from CsA-treated rats. Free radical adducts were detected with either a Bruker 200 ESR spectrometer or a Varian E-109 ESR spectrometer. Instrument conditions were as follows: 20-mW microwave power, 0.63-G modulation amplitude, and 80-G scan range (Knecht et al., 1990). Spectral data were stored on an IBM-compatible computer and were analyzed for ESR hyperfine coupling constants by computer simulation (Duling, 1994). Quantitation of free radical adducts was achieved by double integration of

ESR spectra using the calculation function of the ESR program (Duling, 1994).

Recording of Nerve Activity. Because prolonged recording of nerve activity in conscious animals is very difficult, and data interpretation is confounded by numerous factors, our neurophysiological recording protocol was performed in terminal experiments on anesthetized rats as described elsewhere (Moss et al., 1985). In brief, animals were anesthetized by i.p. injection with pentobarbital (50 mg/kg b.wt.). The right femoral artery was cannulated with a polyethylene tube (PE-50) for measurement of blood pressure, and the right jugular vein (PE-50) for continuous infusion of CsA. Normal saline was infused into the jugular vein at a rate of 3% of body weight/h to achieve a modest extracellular volume expansion. This procedure counteracts the dehydration and neural excitation that accompanies anesthesia and surgery, thus stabilizing basal nerve activity and providing a level that is able to react in both directions to show either excitatory or inhibitory response (Petersen and Di-Bona, 1994; Badoer et al., 1998). Anesthesia was maintained with intrajugular injections of pentobarbital whenever the corneal reflex reappeared.

The retroperitoneal space was opened on the left side, the kidney was retracted to expose the renal pedicle, and the space was filled with mineral oil to prevent desiccation. Renal nerves were isolated carefully, severed near the kidney, and placed on a bipolar hook electrode to record the efferent activity of fibers. The electrical signal was magnified with a differential amplifier, passed through a band-pass filter with frequency cutoffs at 100 and 1000 Hz, and displayed on an oscilloscope. Impulses exceeding the background noise level were counted by nerve activity monitors.

Basal levels of renal efferent nerve activity were recorded in two to four 20-min periods until stable nerve activity was established. CsA (Sandimmune i.v. solution; Novartis) was added to the maintenance infusion of normal saline (0.67 mg/ml) and infused over 30 min to provide a total dose of 10 mg/kg b.wt. Nerve activity was monitored throughout the experiment and expressed as mean frequency (hertz). The percentage of change from the control period was assessed over three successive 10-min intervals. A previous study demonstrated that Cremophor EL (Novartis, Basel, Switzerland), the vehicle for the i.v. formulation of CsA, had no effects on renal nerve activity (Moss et al., 1985).

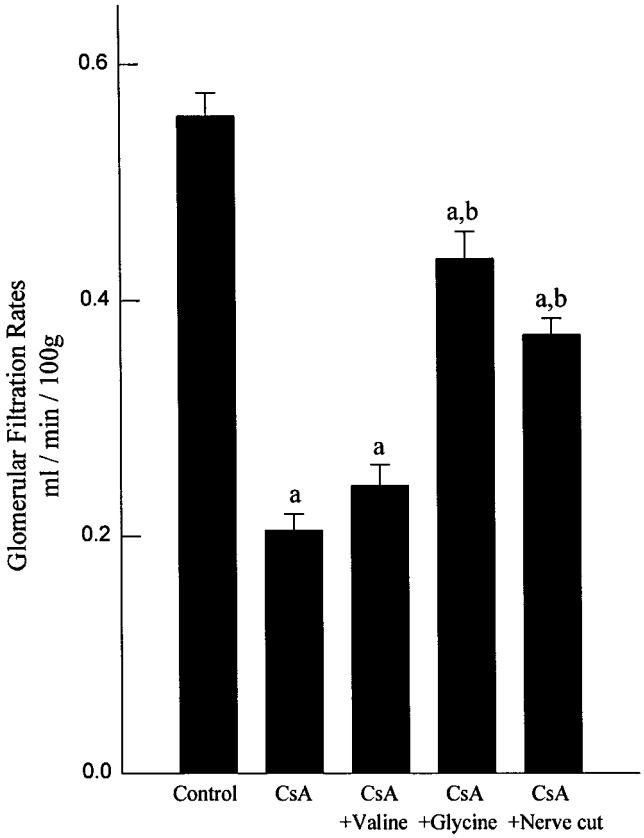

Fig. 2. GFR. Rats were fed a semisynthetic powdered diet containing 5% glycine and 15% casein (glycine diet), 5% valine and 15% casein (valine diet), or 20% casein (control diet; Rose et al., 1997) starting 3 days before severing the renal nerves or sham operation. In some rats fed the control diet, bilateral renal nerves were removed surgically; 4 days later, rats were treated with CsA (25 mg/kg p.o.) or an equivalent volume of olive oil daily for 5 days. Urine samples were collected with metabolic cages, and GFRs were calculated from the ratio of creatinine in the urine/blood, the volume of urine produced in 24 h, and the body weight. Values are means ± S.E.M. (ANOVA; $n = 4–15$ in each group). a, $p < .05$ compared with control; b, $p < .05$ compared with the CsA group.

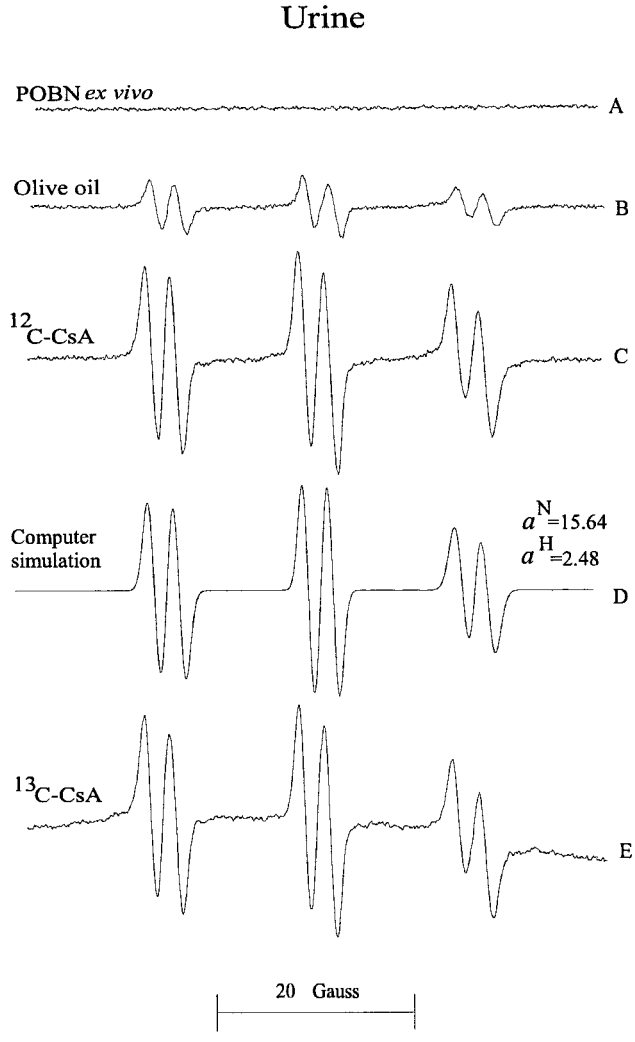

Fig. 3. Effects of [^{12}C]- and [$^{13}C_3$]CsA on ESR spectrum of free radical adducts in urine. After pretreatment with CsA for 5 days, powdered [^{12}C]- or [$^{13}C_3$]CsA was dissolved in 0.1 ml acetone, added to olive oil (0.25 ml/100 g), and bubbled with nitrogen for a half-hour to remove the acetone. Three hours after the last dose of CsA, the spin-trapping reagent 4-POBN (1 g/kg b.wt.) was dissolved in 2 ml normal saline and injected slowly into the tail vein. Urine was collected using metabolic cages for 3 h after injection of 4-POBN. Free radical adducts in urine were detected with a Bruker 200 ESR spectrometer. Typical spectra: A, rat received olive oil and 4-POBN was added ex vivo into urine; B, rat received olive oil and 4-POBN; C, rat received [^{12}C]CsA in olive oil and 4-POBN; D, computer simulation of the radical adduct spectrum of C; E, rat received [^{13}C]CsA in olive oil and 4-POBN.

Results

Effects of CsA, Dietary Glycine, and Renal Denervation on GFRs. GFR, a classical indicator of renal function, was approximately 0.56 ml/min/100 g b.wt. in controls and declined by approximately 65% after 5 days of treatment with CsA (Fig. 2). Dietary glycine largely blunted decreases in GFRs caused by CsA, confirming previous findings from this laboratory (Fig. 2; Thurman et al., 1997; Zhong et al., 1998). This effect appears specific for glycine, because dietary valine (5%) did not block decreases in GFRs caused by CsA. Bilateral renal denervation also attenuated changes in GFRs caused by CsA (Fig. 2).

Free Radicals in Urine Are Not Derived Directly from CsA. A previous study from this laboratory (Zhong et al., 1998) showed that free radicals in urine increased significantly after CsA treatment; however, the source of these radicals remains unknown. One possibility is that free radicals are derived directly from CsA. To test this hypothesis, rats were given [^{12}C]- or [$^{13}C_3$]CsA (25 mg/kg), and free radicals were captured with the spin-trapping reagent 4-POBN and detected with ESR. Figure 3 depicts a representative ESR spectrum due to free radical adducts in urine from a CsA-treated rat. Only background ESR signals from 4-POBN were detectable in urine from rats receiving olive oil (Fig. 3B); however, a 6-line spectrum due to a 4-POBN radical adduct was detected in urine samples from all animals receiving]^{12}C]CsA (Fig. 3C). Computer simulation of the spectrum (Fig. 3D) revealed a species having hyperfine coupling constants of a^N = 15.64 G and a^H = 2.48 G (Table 1), values typical of carbon-centered 4-POBN radical adducts in aqueous solution. No exact match with radical adducts listed in the National Institute of Environmental Health Sciences database (Li et al., 1988) was obtained, confirming the possibility that the trapped species is a new free radical. After administration of [$^{13}C_3$]CsA, the radical detected in urine also had 6 lines with the same hyperfine coupling constants, indicating that the radical detected is not derived from the ^{13}C-labeled alanine in [$^{13}C_3$]CsA (Fig. 3E). Because CsA is metabolized mainly in the liver and excreted in bile (Thomson et al., 1984), free radical production was also assessed in bile. A 6-line ESR spectrum due to 4-POBN radical adducts was detected in bile samples from animals receiving [^{12}C]CsA. Computer simulation of the spectra was accomplished with one species having coupling constants of a^N = 15.57 G and a_β^H = 2.80 G (Table 1), values reasonably close to those of the radical adduct from urine. Moreover, these values are identical with the coupling constants of authentic POBN-pentyl radical adducts generated from the reaction of 4-POBN with either linoleic or arachidonic acid with lipoxygenase and added to bile (Kadiiska et al., 1998). In bile from [$^{13}C_3$]CsA-treated rats, the ESR spectrum also had 6 lines (data not shown). ESR spectra of extracts of serum, kidney, and liver tissue from [$^{13}C_3$]CsA-treated rats also revealed a 6-line radical/POBN adduct (data not shown).

CsA Causes Hydroxyl Radical Production in the Kidney. A previous study showed that CsA caused hypoxia in the kidney (Zhong et al., 1998). Reperfusion subsequent to hypoxia could theoretically lead to production of the hydroxyl radical, an active and harmful radical which, in turn, could initiate lipid and amino acid radical formation. To investigate whether CsA causes hydroxyl radical formation, DMSO was given to rats along with CsA. It is known that hydroxyl radicals attack DMSO, and that breakdown of the reaction product releases a methyl radical that is readily trapped by 4-POBN. Administration of DMSO significantly increases the sensitivity of detection of hydroxyl radicals with spin-trapping reagents (Burkitt and Mason, 1991). When CsA was administrated with [^{12}C]DMSO, a 6-line ESR spectrum due to 4-POBN radical adducts was detected in urine (Fig. 4C). Computer simulation of the spectrum (Fig. 4D) demonstrated two free radical species. Hyperfine coupling constants of species I (38% of total radicals) were a^N = 15.68 G and a_β^H = 2.61 G, which are identical with the unknown radical found in the urine from CsA-treated rats. Species II (62%) gave hyperfine coupling constants of a^N = 15.96 G and a^H = 2.74 G, values typical of methyl 4-POBN radical adducts in aqueous solution (Table 1). Ex vivo formation of hydroxyl radicals initiated by the Fenton reaction with [^{12}C]DMSO and 4-POBN in urine produced a 6-line radical signal with coupling constants of a^N = 15.96 G and a_β^H = 2.74 G, similar to species II in the urine of CsA/[^{12}C]DMSO-treated rats. These findings are consistent with the hypothesis that species II detected in urine from CsA/[^{12}C]DMSO-treated rats is the methyl radical. In vivo administration of CsA with [^{13}C]DMSO produced a 12-line spectrum (Fig. 4E); one of the species had coupling constants a^N = 15.96 G, a_β^H = 2.74 G, and $a_\beta^{C\text{-}13}$= 4.95 G, which are similar to the coupling constants of the 12-line spectrum in urine in which formation of hydroxyl radicals was initiated ex vivo by the Fenton reaction in the presence of [^{13}C]DMSO and 4-POBN (Fig. 4F). These data clearly illustrate that CsA causes hydroxyl radical formation in the kidney.

Chloroform extraction of urine from CsA-treated rats resulted in only 10% of the radical adducts partitioning into the

TABLE 1
Hyperfine coupling constants of 4-POBN adducts in urine and bile

Sample Medium	Treatment	%	Radical	Hyperfine Splitting (G)		
				a^N	a_β^H	$a_\beta^{C\text{-}13}$
Urine	CsA/oil[a]	100	Unknown	15.64	2.48	
	CsA/[^{13}C]DMSO[a]	38	Unknown	15.68	2.60	
		62	$\cdot\,^{13}CH_3$	15.96	2.74	4.95
	Fenton system[b] containing DMSO	100	$\cdot\,CH_3$	15.97	2.74	
Bile	CsA/oil[a]	100	Pentyl	15.57	2.80	
	CsA/[^{13}C]DMSO[a]	83	Pentyl	15.57	2.64	
		17	$\cdot\,^{13}CH_3$	15.94	2.74	4.85
	Fenton system[b] containing DMSO	100	$\cdot\,CH_3$	15.93	2.74	

[a] Rats were gavaged with CsA solutions as described in *Materials and Methods.*
[b] 50-μl Fenton/DMSO system was diluted with 500 μl of sample medium.

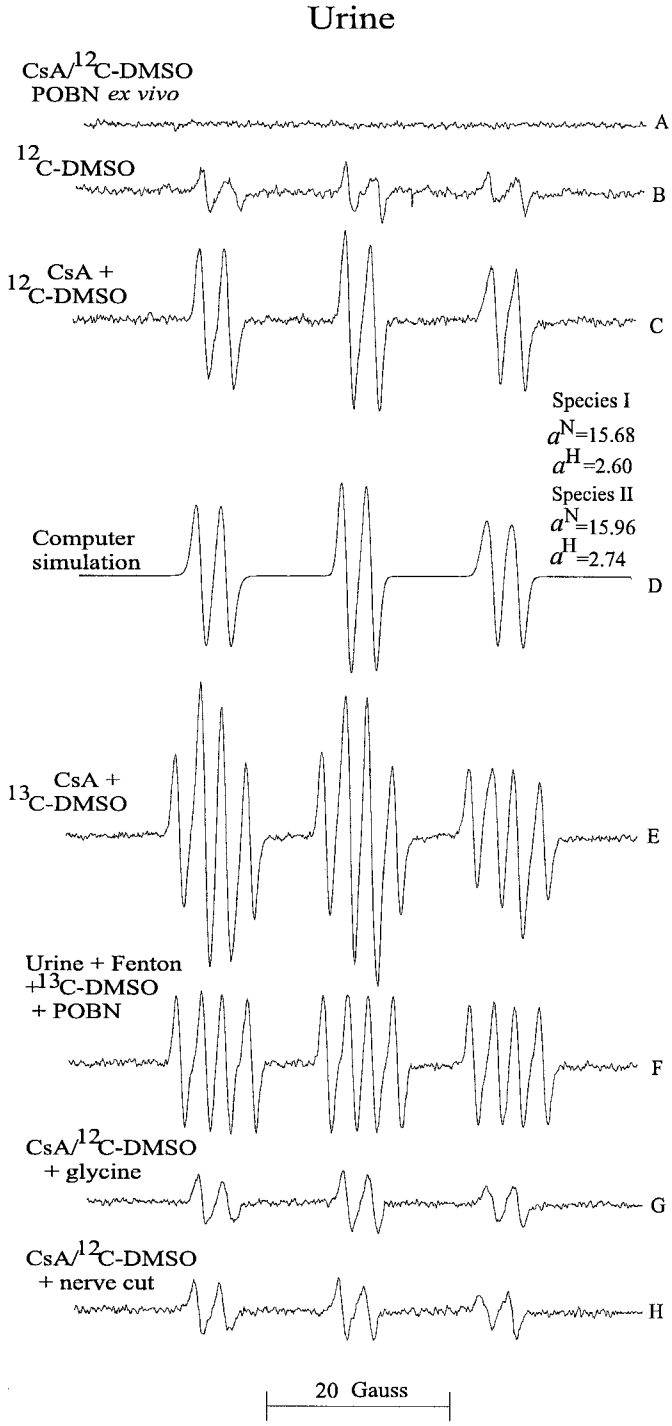

Fig. 4. Effects of [^{12}C]- and [^{13}C]DMSO on ESR spectrum of free radical adducts in urine. After pretreatment with CsA for 5 days, powdered [$^{13}C_3$]CsA was dissolved in 0.2 ml [^{12}C]- or [^{13}C]DMSO and given to the rat by gavage. The spin-trapping reagent 4-POBN (1 g/kg b.wt.) was injected slowly into the tail vein 3 h after the last dose of CsA. Urine was collected using metabolic cages for 3 h after injection of 4-POBN. Free radical adducts in urine were detected with a Bruker 200 ESR spectrometer. Typical spectra: A, rat received CsA and [^{12}C]DMSO, and 4-POBN was added ex vivo into urine; B, rat received [^{12}C]DMSO and 4-POBN; C, rat received CsA, [^{12}C]DMSO, and 4-POBN; D, computer simulation of the radical adduct spectrum of C; E, rat received CsA in [^{13}C]DMSO and 4-POBN; F, Fenton system containing [^{13}C]DMSO and 4-POBN was added to urine from untreated rats; G, rat was fed a glycine diet and received CsA in [^{12}C]DMSO and 4-POBN; and H, renal nerve of the rat was severed and the rat received CsA in [^{12}C]DMSO and 4-POBN.

organic phase (Fig. 5, A and B). This finding implies that the free radical trapped by 4-POBN is either very polar or carries a charge. In contrast, chloroform extraction of urine samples containing 4-POBN/$\cdot CH_3$ radical adducts formed either in vivo (Fig. 5, C and D) or in vitro (Fig. 5, F and G) resulted in either 60 or 70% of the radical adducts partitioning into the organic phase. Therefore, the in vitro case involving the Fenton reaction demonstrates partitioning behavior of a 4-POBN radical adduct formed from a nonpolar free radical ($\cdot CH_3$). Thus, these extraction experiments clearly differentiate between the highly polar CsA-dependent 4-POBN radical adduct and those formed from a nonpolar free radical such as $\cdot CH_3$.

Free Radical Production Due To CsA Is Blocked by Dietary Glycine and Renal Denervation. Free radical adducts were barely detectable in urine from rats treated with [^{12}C]DMSO but increased about 5-fold after treatment with CsA/[^{12}C]DMSO (Fig. 6). In contrast, different weak radical species based on coupling constants were detected in serum extracts; however, they were not altered by CsA. Therefore, an increase in radicals in the urine is most likely produced in the kidney, not transferred from other organs via the blood. Significantly, increases in free radical production caused by CsA in the kidney were blocked totally by dietary glycine or severing the renal nerve (Fig. 6), including both the hydroxyl-derived methyl radical adduct and the unknown radical adduct.

Effects of CsA and Glycine on Efferent Renal Nerve Activity and Mean Arterial Blood Pressure. Because increased renal nerve activity could theoretically cause vasoconstriction, hypoxia-reoxygenation phenomenon and radical production, the effects of CsA and glycine on renal nerve firing was studied. The absolute levels of efferent renal nerve activity were not different between rats fed control or glycine diets. In rats fed a control casein-containing diet, efferent renal nerve activity increased gradually after the infusion of CsA; the mean percentage of increase in nerve activity in the first 10-min infusion period was 50% and reached a new steady state of 100% in 20 min (Fig. 7A). This finding was consistent with a previous report (Moss et al., 1985). Dietary glycine totally blocked increases in efferent renal nerve activity caused by CsA (ANOVA, $P < .05$; Fig. 7B).

Hydroxyl-Derived Methyl Radical Production Is Minimal in Liver. Free radicals were undetectable when 4-POBN was added to bile from CsA/[^{12}C]DMSO-treated rats (Fig. 8A) and were barely detectable in bile from [^{12}C]DMSO-treated rats with 4-PONB in vivo. In rats treated with CsA/[^{13}C]DMSO, two radical species were detected in the bile. Species I had coupling constants of a^N = 15.75 G and $a_\beta{}^H$ = 2.64 G, similar to those found in the bile from CsA-treated rats (Fig. 8, D and E and Table 1) and corresponding to the unknown radical adduct observed in the urine (Table 1). Because these values are identical with the coupling constants of authentic POBN-pentyl radical adducts generated from the reaction of 4-POBN with arachidonic acid with lipoxygenase and added to bile (Kadiiska et al., 1998), it is concluded that species I is most likely a POBN-pentyl radical. The second species gave hyperfine coupling constants of a^N = 15.94 G, $a_\beta{}^H$ = 2.74 G, and $a_\beta{}^{C13}$ = 4.85 G, which closely resembled those of [^{13}C]DMSO-derived radical adducts prepared using a Fenton system (Fig. 8F). The coupling constants of species II were similar to those of the methyl-

POBN adduct detected in urine (a^N = 15.93 G and a_β^H = 2.74 G); however, it only contributed about 15% to the ESR signal in bile. Because administration of CsA with [^{13}C]DMSO produced a 12-line signal in the bile (Fig. 8E), it is concluded that the hydroxyl radical is formed in the liver; however, it appears that this radical makes up very little of the total radical adduct spectrum (15%).

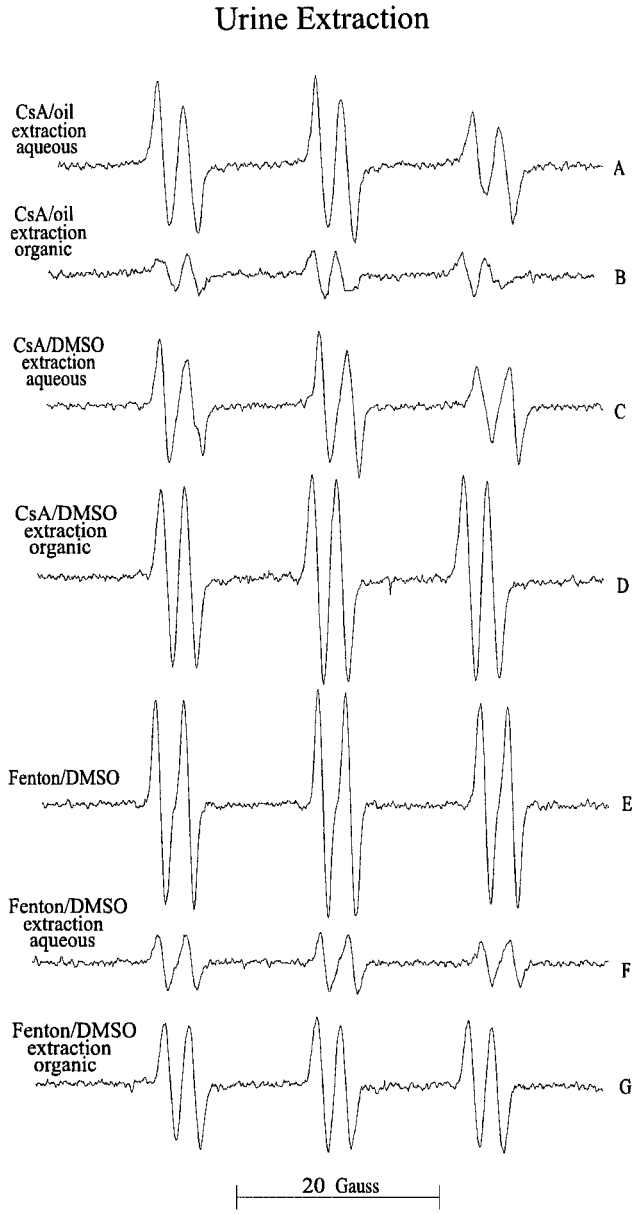

Fig. 5. ESR spectrum of free radical adducts in urine extracts. Powdered [$^{13}C_3$]CsA was dissolved in either olive oil or [^{12}C]DMSO. Urine was collected and extracted with chloroform as described in *Materials and Methods*. Typical spectra: A, rat received [$^{13}C_3$]CsA in olive oil and 4-POBN, the aqueous phase of urine extracts; B, rat received [$^{13}C_3$]CsA in olive oil and 4-POBN, the organic phase of urine extracts; C, rat received [$^{13}C_3$]CsA in [^{12}C]DMSO and 4-POBN, the aqueous phase of urine extracts; D, rat received [$^{13}C_3$]CsA in [^{12}C]DMSO and 4-POBN, the organic phase of urine extracts; E, Fenton system containing [^{12}C]DMSO and 4-POBN was added to urine from untreated rats before extraction; F, Fenton system containing [^{12}C]DMSO and 4-POBN was added to urine from untreated rats, the aqueous phase of urine extracts; G, Fenton system containing [^{12}C]DMSO and 4-POBN was added to urine from untreated rats, the organic phase of urine extracts.

Discussion

Free Radicals Are Not Derived Directly from CsA. A previous study showed that a free radical adduct due to CsA was detected in urine (Zhong et al., 1998), with coupling constants not similar to any known free radicals. How CsA causes free radical production is unknown; a possibility exists that CsA or its metabolites form radicals directly. CsA increased malondialdehyde, a product of lipid oxidation, in isolated hepatic microsomes (Inselmann et al., 1990). This suggests that CsA produces free radicals that attack lipid components, resulting in lipid peroxidation. Therefore, it is possible that metabolism of CsA by renal cytochrome P-450 could directly lead to free radicals. To investigate this possibility, [^{12}C]- or [$^{13}C_3$]CsA was given to rats. If the radical was derived directly from CsA and its attachment to the spin trap involved one of the ^{13}C's β to the nitroxide, the nucleus of ^{13}C that exhibited two spin angular momentum states in the magnetic field would create two additional spin states for the unpaired electron. The latter effect would cause the original 6-line ESR spectrum to become a 12-line spectrum (Thurman et al., 1991). However, ESR spectra of radical/4-POBN adducts in urine only exhibited 6 lines after administration of either [^{12}C]- or [$^{13}C_3$]CsA (Fig. 3). Similarly, 12-line peaks were not detected in bile, serum, kidney, or liver tissue extracts after [$^{13}C_3$]CsA administration. Therefore, it appears that free radials are probably not derived directly from the

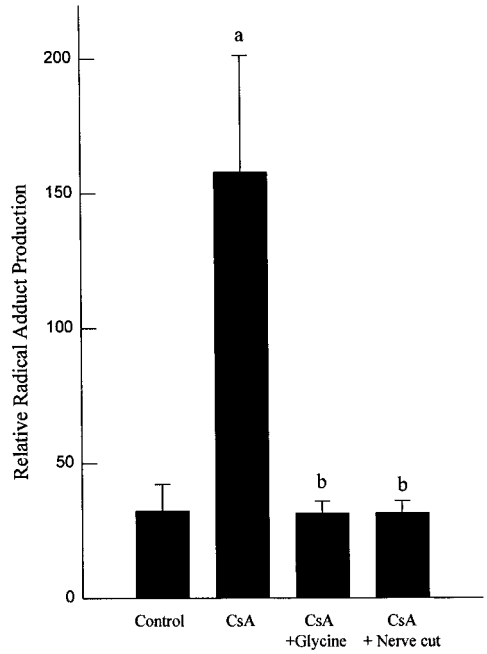

Fig. 6. Effects of glycine and renal denervation on free radical production caused by CsA. After a 5-day pretreatment with CsA, powdered [$^{13}C_3$]CsA (25 mg/kg) was dissolved in 0.2 ml DMSO and administered by gavage. Three hours after the last dose of CsA, the spin-trapping reagent 4-POBN (1 g/kg b.wt.) was injected slowly into the tail vein. Urine was collected using metabolic cages for 3 h after injection of 4-POBN and was kept on dry ice until analysis. Free radical adducts in urine were detected with a Bruker 200 ESR spectrometer. The relative radical adduct concentration was measured by double integration of ESR spectra at identical gains. Relative radical adduct production was calculated by multiplying the relative radical adduct concentration by the volume of urine collected during the 3-h sampling interval. Values are means ± S.E.M. (ANOVA, n = 5–7 in each group). a, $p < .05$ compared with control; b, $p < .05$ compared with the CsA group.

CsA molecule. Further support for this unknown radical adduct not being derived from CsA comes from its partitioning predominantly into the aqueous phase in the chloroform extraction. Such behavior would be unlikely from a radical adduct containing a CsA or its fragment, which would be nonpolar like the parent molecule.

CsA Caused Hydroxyl Radical Formation in the Kidney In Vivo. Alternatively, CsA could cause hypoxia-reoxygenation. Indeed, pimonidazole binding, which measures hypoxia in the cell, was increased nearly 3-fold by CsA in the kidney (Zhong et al., 1998). Free radicals were also increased dramatically in urine after CsA treatment (Zhong et al., 1998) (Fig. 6). These data suggest that hypoxia-reoxygenation is involved in CsA nephrotoxicity. The oxidative stress caused by iron overload leads to formation of highly active hydroxyl radicals (Burkitt and Mason, 1991). However, hydroxyl adducts of spin traps are very unstable, which limits their detection in vivo. Hydroxyl radical can cause the release of a methyl radical when it attacks DMSO, and the methyl radical is readily captured by spin-trapping reagents to give a very stable carbon-centered radical adduct, thus significantly increasing the possibility of hydroxyl radical detection (Burkitt and Mason, 1991). In these studies, when CsA was administered along with [^{12}C]DMSO, two radical adducts were detected in the urine; one had hyperfine coupling constants similar to those of methyl/4-POBN radical adducts formed in a Fenton system containing DMSO and measured in urine (Fig. 4 and Table 1). In addition, administration of CsA with [^{13}C]DMSO produced a 12-line ESR signal (Fig. 4). These data provide definitive evidence that hydroxyl radicals were indeed produced after CsA treatment in vivo, most likely as a consequence of hypoxia-reoxygenation. Importantly, hydroxyl-derived methyl radicals were produced in large quantity in the kidney (>60% of total radicals, Table 1), the major target of CsA toxicity, but only minimally in the liver (Table 1). Therefore, it is concluded that local production of a hydroxyl radical, a highly active and detrimental radical, plays an important role in nephrotoxicity caused by CsA.

CsA Causes Radical Production by Increasing Renal Nerve Activity. As mentioned above, CsA causes vasoconstriction (Murray et al., 1985; English et al., 1987; Barros et al., 1987; Mehring et al., 1992), tissue hypoxia in the kidney (Zhong et al., 1998), and free radical formation. These find-

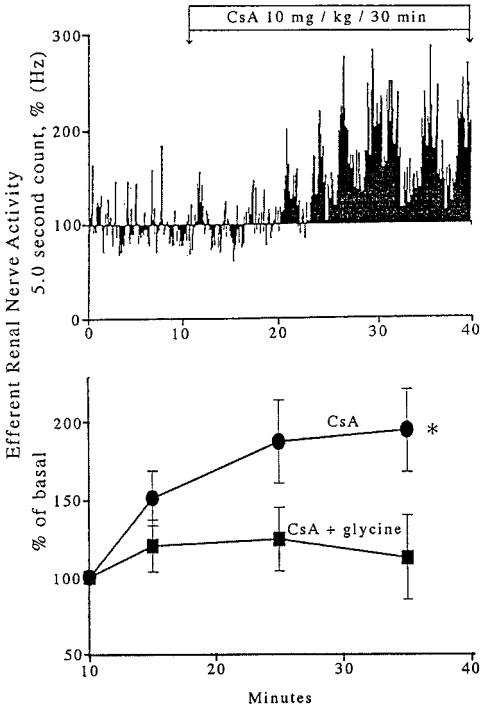

Fig. 7. Effects of CsA and glycine on efferent renal nerve activity. Rats were fed control or glycine-containing diets as described in *Materials and Methods*. Renal nerves were isolated carefully, severed near the kidney, and placed on a bipolar hook electrode to record the efferent nerve activity. The electrical signal was magnified with a differential amplifier, passed through a band-pass filter with frequency cutoffs at 100 and 1000 Hz, and displayed on an oscilloscope. Impulses exceeding the background noise level were counted by nerve activity monitors. Basal levels of renal efferent nerve activity were recorded in two to four 20-min periods until stable nerve activity was established. CsA (Sandimmune i.v. solution) was added to the maintenance infusion of normal saline (0.67 mg/ml) and infused over 30 min to provide a total dose of 10 mg/kg b.wt. Nerve activity was monitored throughout the experiment and expressed as mean frequency (Hz) and the percentage of change from the control period was assessed over three successive 10-min intervals. A, typical recording of efferent nerve activity; B, mean efferent nerve activity as percentage of basal levels (ANOVA, $n = 4–5$ in each group). *$p < .05$ compared with controls.

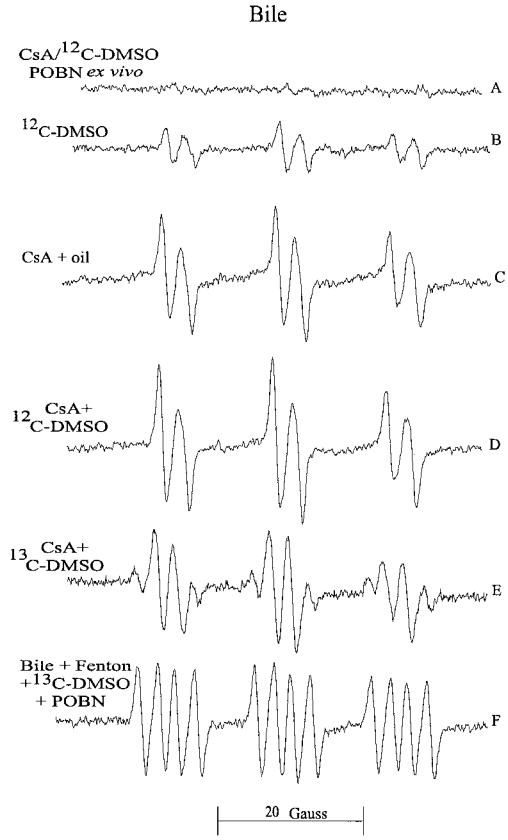

Fig. 8. Effects of [^{12}C]- and [^{13}C]DMSO on ESR spectrum of free radical adducts in bile. After pretreatment with CsA for 5 days, powdered [^{12}C]- or [$^{13}C_3$]CsA was dissolved in 0.2 ml [^{12}C]- or [^{13}C]DMSO and given to the rat by gavage. The spin-trapping reagent 4-POBN (1 g/kg b.wt.) was injected slowly into the tail vein 3 h after the last dose of CsA. Bile was collected via a polyethylene tube placed in the common bile duct into 50 μl of 30 mM desferal for 1 h after injection of 4-POBN. Free radical adducts in bile were detected with a Bruker 200 ESR spectrometer. Typical spectra: A, rat received DMSO and 4-POBN was added ex vivo into bile; B, rat received [^{12}C]DMSO and 4-POBN; C, rat received CsA in olive oil and 4-POBN; D, rat received CsA in [^{12}C]DMSO and 4-POBN; E, rat received CsA in [^{13}C]DMSO and 4-POBN; F, Fenton system containing [^{13}C]DMSO and 4-POBN was added to bile from untreated rats.

ings could be related to hypoxia-reoxygenation in the kidney. The mechanism by which CsA causes vasoconstriction is unclear; one possibility is that it increases sympathetic nerve activity. Consistent with this hypothesis, CsA increases renal nerve firing (Moss et al., 1985), and nephrotoxicity of CsA is diminished in denervated kidneys (Fig. 2) (Murray et al., 1985). Alternatively, CsA could directly stimulate vascular smooth muscle or mesangial cell contraction, processes that are dependent on influx of calcium. Indeed, CsA has been shown to increase intracellular calcium in these cells (Meyer-Lehnert and Schrier, 1988; Lo Russo et al., 1996) and cause vasoconstriction directly in isolated arterial rings and renal arterioles (Xue et al., 1987; Lanese and Conger, 1993; Lanese et al., 1994). CsA also stimulates the release of many vasoactive mediators, such as angiotensin II (Murray et al., 1985), thromboxanes (Rogers et al., 1988), and endothelins (Kon et al., 1990), which could contribute to vasoconstriction. In this study, efferent renal nerve activity was increased significantly after CsA infusion (Fig. 7), and severing the renal nerve totally blocked free radical production due to CsA (Figs. 4 and 6). Taken together, it is concluded that CsA causes hypoxia-reoxygenation and toxic free radical formation in the kidney, at least in part, by increasing renal nerve activity. This increased renal nerve stimulation is most likely an important early event in CsA-induced renal injury.

Glycine Prevents CsA-Induced Free Radical Production by Blocking Renal Nerve Stimulation. A previous study has demonstrated that dietary glycine prevents CsA-induced alterations in renal function and pathological changes that include proximal tubular dilatation, cell necrosis, and infiltration of macrophages (Thurman et al., 1997). In addition, glycine prevented hypoxia and free radical formation due to CsA treatment (Zhong et al., 1998). How glycine prevents CsA-induced free radical formation is unclear. Glycine did not alter blood levels or the pharmacokinetics of cyclosporin in rats (Zhong et al., 1998). Alternatively, glycine could prevent free radical formation by blocking hypoxia-reoxygenation caused by CsA. Dietary glycine, which prevented pathological changes associated with chronic CsA treatment (Thurman et al., 1997), significantly diminished pimonidazole adduct formation (an indicator of renal hypoxia) and free radical production in urine (Zhong et al., 1998). Therefore, it is likely that glycine works by blocking hypoxia-reoxygenation caused by CsA. Indeed, glycine is an inhibitory amino acid that hyperpolarizies the nerve cell membrane and inhibits spinal reflex activity, including renal nerve responses (Ito and Cherubini, 1991). Here, it blocked increases in efferent renal nerve activity caused by CsA (Fig. 7). A recent report has indicated that the sympathoexcitatory action of CsA is caused by an interaction with the central baroreflex mechanism, which becomes reset to a higher level during i.v. infusions of CsA (Ryuzaki et al., 1997). Thus, a direct inhibitory effect of glycine on the actions of CsA within the brainstem is possible because glycine is a prominent neurotransmitter in the reflex control of cardiovascular activity, and microinjections of glycine into the nucleus of the solitary tract lowered heart rate and blood pressure (Talman and Robertson, 1989). Moreover, perfusion with glycine increased renal blood flow (Heyman et al., 1992) and dietary treatment with glycine prevented changes in glomerular size and volume and hypoxia caused by CsA (Zhong et al., 1998). Therefore, it is concluded that glycine prevents CsA nephrotoxicity, at least in part, by minimizing neurogenic vasoconstriction by its inhibitory action on renal nerves. This action prevents tissue hypoxia and production of toxic radicals. If glycine treatment could be shown to work in clinical trials in humans, it could be a useful agent to reduce the renal toxicity of this class of compounds.

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