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Reduction of ciclosporin and tacrolimus nephrotoxicity by plant polyphenols

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Abstract

The immunosuppressants ciclosporin (cyclosporin A, CsA) and tacrolimus can cause severe nephrotoxicity. Since CsA increases free radical formation, this study investigated whether an extract from *Camellia sinensis*, which contains several polyphenolic free radical scavengers, could prevent nephrotoxicity caused by CsA and tacrolimus. Rats were fed powdered diet containing polyphenolic extract (0–0.1%) starting 3 days before CsA or tacrolimus. Free radicals were trapped with α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and measured using an electron spin resonance spectrometer. Both CsA and tacrolimus decreased glomerular filtration rates (GFR) and caused tubular atrophy, vacuolization and calcification and arteriolar hyalinosis, effects that were blunted by treatment with dietary polyphenols. Moreover, CsA and tacrolimus increased POBN/radical adducts in urine nearly 3.5 fold. Hydroxyl radicals attack dimethyl sulfoxide (DMSO) to produce a methyl radical fragment. Administration of CsA or tacrolimus with ^{12}C-DMSO produced a 6-line spectrum, while CsA or tacrolimus given with ^{13}C-DMSO produced a 12-line ESR spectrum, confirming formation of hydroxyl radicals. 4-Hydroxynonenal (4-HNE), a product of lipid peroxidation, accumulated in proximal and distal tubules after CsA or tacrolimus treatment. ESR changes and 4-HNE formation were largely blocked by polyphenols. Taken together, these results demonstrate that both CsA and tacrolimus stimulate free radical production in the kidney, most likely in tubular cells, and that polyphenols minimize nephrotoxicity by scavenging free radicals.

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Introduction

Ciclosporin (cyclosporin A, CsA), a hydrophobic cyclic peptide of 11 amino acids extracted from the fungus *Tolypocladium inflatum gams*, and tacrolimus, another fungal peptide from *Streptomyces tsukubaensis* (Kino et al 1987), are powerful immunosuppressive agents (Borel et al 1976). These drugs markedly improve transplant graft survival (Margreiter et al 1983; Starzl et al 1989). However, recipients must continue immunosuppressive therapy for the rest of their lives. Although CsA and tacrolimus have different molecular structures, they have a variety of common toxic side effects, the most frequent being nephrotoxicity (Sibley et al 1983; Fung et al 1991). How these drugs cause nephrotoxicity is not well understood. Previous studies showed that CsA treatment caused free radical production in the kidney, probably by mechanisms involving hypoxia–reoxygenation (Zhong et al 1998). Whether tacrolimus causes oxidative stress is unknown, but both CsA and tacrolimus stimulate sympathetic nerve activity (Moss et al 1985; Lyson et al 1993). Therefore, a common link of these drugs may be vasoconstriction in the kidney leading to hypoxia–reoxygenation and renal damage.

Camellia sinenesis (green tea) contains high levels of polyphenols that are efficient free radical and singlet oxygen scavengers. Green tea extracts inhibit lipid peroxidation in a variety of experimental settings and in man (Ruch et al 1989; Hara 1994; Serafini et al 1996; Frankel 1999). Beneficial effects of green tea in decreasing the risk of heart disease and cancer are presumably due to the antioxidant properties of polyphenols (Kada et al 1985; Wang et al 1989; Frankel 1999; Vinson 2000). Thus, this study was designed to test the hypothesis

that green tea polyphenols block free radical formation and prevent the nephrotoxicity of CsA and tacrolimus.

Materials and Methods

Reagents

CsA (Sandimmune oral solution) and tacrolimus (Prograf i.v. solution) were obtained from Novartis (Basel, Switzerland) and Fujisawa Ireland, Ltd (Killorglin, Ireland), respectively. Green tea extract (Taiyo Kagaku Co., Yokkaichi, Mie, Japan) was produced by boiling water extraction of green tea leaves. The extracted solution was filtered, concentrated, purified using ethyl acetate and spray dried. The green tea extract contained 85% polyphenols by weight. Polyphenols in the extract was analysed by high-performance liquid chromatography (HPLC) using a CAPACELLPAK C18 UG120 column and methanol–water–H_3PO_4 (18:82:0.5) isocratic elution at a flow rate of 0.8 mL min^{-1} and detected at 280 nm using a UV detector. Polyphenols in the extract included epigallocatechin gallate (47.2% of total polyphenols), epigallocatechin (11.0%), gallocatechin gallate (11.0%), epicatechin gallate (10.8%), gallocatechin (8.6%), epicatechin (8.4%) and catechin (3.0%). Creatinine assay kits, dipyridyl and α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical. Co. (St Louis, MO). Bathocuproinedisulfonic acid was purchased from Aldrich Chemical. Co. (Milwaukee, WI). Ascorbate oxidase paddles were from Boehringer Mannheim Inc. (Indianapolis, IN). ^{12}C-DMSO (containing 1.1% natural abundance of ^{13}C) and $^{13}C_3$-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 semi-synthetic powdered diet (AIN 76) containing 0.003–0.1% green tea polyphenolic extract for 3 days before CsA or tacrolimus treatment. Diet consumption was 8.2 g per 100 g body weight daily, resulting in a polyphenolic extract intake up to 82 mg kg^{-1} daily when 0.1% polyphenolic extract was added to the diet. This level was chosen because previous studies showed that 0.1% green tea extract in diet blunted the development of renal cell tumours in rats, and 0.2% green tea extract in drinking water (about 100 mg kg^{-1} daily) prevented prostate cancer (Gupta et al 1999; Yoshioka et al 1999). CsA or its vehicle (Cremophor EL 20 mg mL^{-1} in 12.5% dehydrated alcohol) was added to olive oil, and tacrolimus or its vehicle (Cremophor EL; 200 mg mL^{-1} in 80% dehydrated alcohol) was diluted with normal saline. In preliminary experiments, no consistent overt nephrotoxicity was observed at lower doses of CsA (5–15 mg kg^{-1}). Higher doses of CsA are required to cause renal damage in rats than in man, reflecting the lower sensitivity of rats to CsA toxicity (Farthing & Clark 1981). Therefore, in this study, CsA (25 mg kg^{-1}), tacrolimus (2 mg kg^{-1}) or an equivalent volume of vehicles was gavaged daily for 5–21 days. To test if polyphenols could be administered with lipophilic CsA solutions, polyphenols were dissolved in olive oil in some experiments. CsA or its vehicle was added to this mixture resulting in final concentrations of polyphenolic extract in the range 0–2.8%. The mixture without CsA was given to rats by gavage (0.25 mL/100 g), and 3 days later, some rats were given the mixture with CsA (25 mg kg^{-1}) for 5–21 days. All rats were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Glomerular filtration rates

Rats were placed in metabolic cages, and urine was collected daily. Creatinine levels in urine and sera were determined, and glomerular filtration rates were calculated from the ratio of creatinine in the urine/blood, the volume of urine produced in 24 h and the body weight (Laiken & Fanestil 1985).

Histology and immunohistochemical staining for 4-hydroxynonenal

Rats were anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.), and the left kidney was rinsed with 5 mL normal saline, perfusion-fixed with 10% formaldehyde in phosphate-buffered saline, then removed and placed in 10% formalin for 48 h. Sections were stained with haematoxylin–eosin and analysed microscopically for pathology.

To detect 4-hydroxynonenal (4-HNE), a product of lipid peroxidation (Ferrali et al 1980), some kidney sections were de-paraffinized with xylene and taken through graded alcohol–water mixtures to rehydrate the tissue. Hydrated sections were then exposed to mouse anti-4-HNE monoclonal antibodies at a 1:500 dilution in PBS-Tween for 30 min at room temperature. Peroxidase-conjugated anti-mouse IgG antibody was then applied, and DAB chromagen was added as the peroxidase substrate. After the immunostaining procedure, a light counterstain of Meyer's haematoxylin was applied.

Detection of free radical adducts by electron spin resonance

To assess free radical formation, the spin-trapping reagent α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone (4-POBN; 1 g kg^{-1} body weight) was injected slowly into the tail vein 3 h after the last dose of CsA or 1 h after the last dose of tacrolimus. Administration of 4-POBN at different times after CsA or tacrolimus was based on the fact that CsA peaks in the blood at 3–4 h (Wood et al 1983) while tacrolimus is maximal after about 1.5 h (Undre et al 1999). Urine was collected using metabolic cages for 3 h after injection of 4-POBN. Each mL of urine was collected into 50 μL of a metal chelator solution containing 30 mM bathocuproinedisulfonic acid and 30 mM dipyridyl to prevent ex-vivo free radical formation, and samples were kept on dry ice until analysis. To detect hydroxyl radical formation in-vivo, ^{12}C- or $^{13}C_3$-DMSO (0.8 mL kg^{-1}) was administered by gavage in some experiments 1 h before injection of 4-POBN. Urine samples were placed in an aqueous flat cell, an ascorbate oxidase paddle was inserted, and samples were bubbled with oxygen for 5 min to eliminate interfering ascorbyl free radical and with nitrogen for 5 min to reduce oxygen-dependent line broadening. This treatment completely removed ascorbate free radical from the sample. Free radical adducts were detected with a Bruker Elexsys ESR spectrometer (Bruker

Instrument Inc., Billerica, MA). Instrument settings were: 20-mW microwave power, 0.70-G modulation amplitude, 80-G scan range. Spectral data were analysed for ESR hyperfine coupling constants by computer simulation (Knecht et al 1990). Quantification of free radical adducts was achieved by double integration of ESR spectra using the calculation function of the ESR program (Duling 1994).

Blood levels of ciclosporin and tacrolimus

Blood was collected, at 0, 1, 2, 4, 12, and 24 h after CsA (25 mg kg^{-1}, i.g.) or tacrolimus (2 mg kg^{-1}, i.g.) treatment, from the tail vein into tubes containing 1 mg Na_4EDTA and stored at 0–4°C until analysis. CsA in the blood was determined using a fluorescent polarization immunoassay (Moyer et al 1991). Tacrolimus was measured using a microparticle enzyme immunoassay (Tamura et al 1987).

Statistical analysis

All groups were compared using analysis of variance plus Student–Newman–Keul's post-hoc test. Differences were considered significant at $P < 0.05$.

Results

Polyphenols prevent inhibition of renal function by CsA and tacrolimus

Glomerular filtration rates (GFR), which reflect renal function, were about 0.6 mL/min/100 g body weight in controls and were not altered by dietary polyphenols (Figure 1A). CsA treatment decreased GFR by about 70% after 5 days (Figure 1B). Polyphenolic extract (0.003–0.1% in diet) blunted the decrease of GFR caused by CsA in a dose-dependent manner (Figure 1B). In rats fed 0.1% dietary polyphenols, decreases in GFR from CsA treatment were only about half as large as in rats treated with CsA and fed a control diet (Figure 1B).

To test whether polyphenols could be administered in lipophilic CsA solutions, which would provide a practical route of administration in the clinic, we also added polyphenols to CsA–olive oil solutions in some experiments. Polyphenols dissolved in olive oil also blunted the decrease of GFR caused by CsA in a dose-dependent manner (Figure 1C). In rats treated with 2.8% polyphenols in olive oil, decreases of GFR induced by CsA were blunted maximally from 73% without polyphenols to 43% with polyphenols (Figure 1C). Daily intake of 100 mg kg^{-1} polyphenols in oil (equivalent to 2.8% polyphenols in CsA–oil solution) gave similar protective effects to a dietary intake of 82 mg kg^{-1} polyphenols daily (equivalent to 0.1% polyphenols in the diet). Tacrolimus treatment also decreased GFR by about 60% (Figure 2). Dietary polyphenols (0.1%) again blunted the tacrolimus-induced decrease of GFR (Figure 2).

Polyphenols decrease renal pathological changes caused by CsA and tacrolimus

Renal histology was examined after treatment with CsA and tacrolimus (Figure 3). The kidneys of rats on control diet

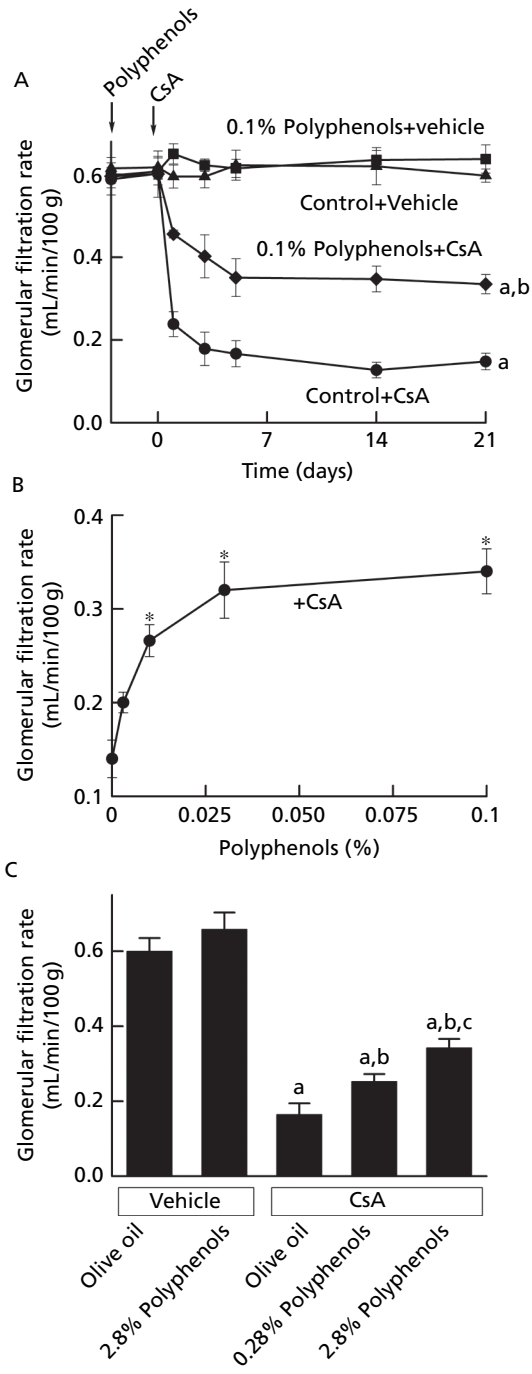

Figure 1 Polyphenols prevent decreases of glomerular filtration rates by ciclosporin (CsA). Rats were fed a semi-synthetic powdered diet containing 0% (control) to 0.1% polyphenols starting 3 days before CsA (25 mg kg^{-1}, i.g. for 3 weeks) (A and B). Some rats were gavaged daily with polyphenols (0–2.8%) dissolved in olive oil (C). Glomerular filtration rates (GFR) were determined, as described in Methods. Values are means ± s.e.m., n = 5 or 6 per group. A. Time courses of GFR changes after 1% dietary polyphenols and CsA; a, $P < 0.05$ vs vehicle; b, $P < 0.05$ vs CsA. B. Dose-dependency of the protective effect of dietary polyphenols. *$P < 0.05$ vs control diet plus CsA. C. Dose-dependency of the protective effect of polyphenols in oil. a, $P < 0.05$ vs olive oil plus vehicle; b, $P < 0.05$ vs olive oil plus CsA and c, $P < 0.05$ vs 0.28% polyphenols plus CsA.

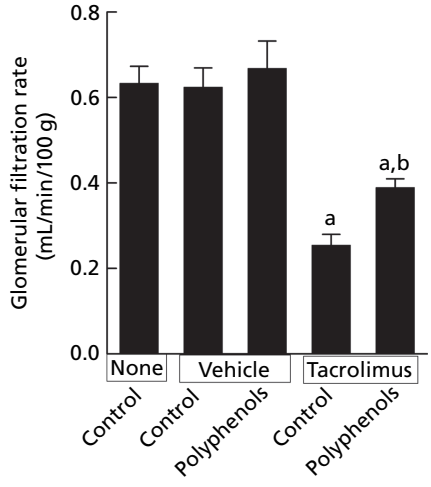

Figure 2 Dietary polyphenols prevent decreases of glomerular filtration rates by tacrolimus. Rats were fed a semi-synthetic AIN-76A powdered diet containing no (control) or 0.1% polyphenols daily starting 3 days before vehicle and tacrolimus treatment (2 mg kg^{-1}, i.g. for 21 days). Values are means ± s.e.m., n = 5 or 6 per group. a, $P < 0.05$ vs control diet plus vehicle; b, $P < 0.05$ vs control diet plus tacrolimus.

(Figure 3A) and polyphenol diet (0.1%, Figure 3B) treated with vehicle exhibited normal histology. CsA caused tubular atrophy (Figure 3C, arrows and Figure 3G), vacuolization (Figure 3I) and calcification (Figure 3J), arteriolar hyalinosis (Figure 3H), and white blood cell infiltration. These pathological effects were minimized by a 0.1% polyphenol-containing diet (Figure 3D). Tacrolimus caused similar pathological changes (Figure 3E); these effects were also minimized by dietary polyphenols (Figure 3F).

Polyphenols decrease CsA- and tacrolimus-induced pentyl and carboxyl radical production

Our previous studies showed that free radicals increased in urine after CsA treatment (Zhong et al 1998). However, whether tacrolimus causes free radical formation is not clear. Therefore, we compared free radical production after tacrolimus with that after CsA treatment. Free radical 4-POBN adducts were minimal in urine from rats receiving vehicle only (Figure 4A). In urine from rats receiving CsA, a multicomponent 6-line ESR spectrum due to 4-POBN radical adducts was detected (Figure 4B) as expected (Zhong et al 1998). Computer simulation of the spectrum revealed two free radical species (Figure 4C). Species I (71%) had hyperfine coupling constants of $a^N = 15.6$ G and $a_\beta^H = 2.34$ G, values which closely match the pentyl/4-POBN radical adduct (Zhong et al 1998). Species II (29%) showed hyperfine coupling constants of $a^N = 15.55$ G and $a_\beta^H = 3.30$ G, which is most likely POBN/$CO_2^{\bullet-}$ (Connor et al 1986). Similarly, a 6-line ESR spectrum, consisting of two radical species, was detected in urine from rats receiving tacrolimus (Figure 4E). The hyperfine coupling constants of the two species in urine from tacrolimus-treated rats were the same as those in the urine from CsA-treated rats. Both tacrolimus and CsA treatment for 5 days increased free radicals by about 3.5 fold (Figure 5A, B). Significantly, increases in free radical production caused by CsA and tacrolimus in the kidney were blocked almost completely by dietary polyphenols (Figure 4D, G, Figure 5). In contrast to the overt increase of free radical adducts in the urine, free radical adducts were barely detectable in the serum after CsA and tacrolimus treatment (data not shown).

Polyphenols decrease CsA- and tacrolimus-induced hydroxyl radical production

CsA treatment leads to formation of hydroxyl radicals, a highly active and toxic radical species, in the kidney (Zhong et al 1998). It is unknown if tacrolimus also causes hydroxyl radical formation. Accordingly, we compared hydroxyl radical formation after CsA and tacrolimus treatment. To detect hydroxyl radical formation in-vivo, DMSO was given to rats along with CsA and tacrolimus. Hydroxyl radicals attack DMSO, producing a methyl radical by the following reaction: $(CH_3)_2SO + {}^{\bullet}OH \rightarrow CH_3SO_2H + {}^{\bullet}CH_3$. The methyl radical can be readily trapped with 4-POBN (Burkitt & Mason 1991). Methyl/4-POBN free radical adducts are sufficiently stable to survive passage into the bladder and subsequent analysis by ESR, whereas hydroxyl/4-POBN free radical adducts are not. Thus, administration of DMSO provides a means of detecting hydroxyl radicals in complex biological systems (Burkitt & Mason 1991).

When CsA was administered with ^{12}C-DMSO, a 6-line ESR spectrum due to 4-POBN radical adducts was detected in urine (Figure 6B). Computer simulation of the spectrum was consistent with 3 free radical species. The first two, species I and II, were the same as those observed when rats were treated with CsA in the absence of ^{12}C-DMSO described above (Figure 5). The third species, species III (39%), gave hyperfine coupling constants of $a^N = 15.96$ G and $a_\beta^H = 2.66$ G, values characteristic of methyl/4-POBN radical adducts in aqueous solution (Zhong et al 1999). These data suggested that, in addition to pentyl and carboxyl radicals, a new radical species (methyl/4-POBN radical adducts) was captured when ^{12}C-DMSO was administered with CsA. Proof that the radical adducts were DMSO-derived was confirmed using ^{13}C-DMSO. Administration of CsA with ^{13}C-DMSO (instead of ^{12}C-DMSO) produced a 12-line spectrum (Figure 6C) due to the additional ^{13}C hyperfine coupling of 4.98 G from the ^{13}C-methyl/4-POBN radical adducts (Figure 6D). The doubling of the number of ESR lines proved the presence of a magnetic C-13 in the radical adducts, which in this case could come only from the ^{13}C-DMSO, thus confirming hydroxyl radical formation.

Similar to CsA, a 6-line ESR spectrum due to 4-POBN radical adducts was also detected in urine after administration of tacrolimus with ^{12}C-DMSO. Likewise, computer simulation of the spectrum was consistent with the same 3 free radical species (pentyl, carboxyl and methyl radical adducts) found in the urine of rats treated with CsA plus ^{12}C-DMSO (Figure 6F). When tacrolimus was given with ^{13}C-DMSO, a 12-line ESR spectrum was again produced (Figure 6G). These data show that, like CsA, tacrolimus causes hydroxyl radical formation.

To determine whether polyphenols decreased highly toxic hydroxyl radicals, CsA plus ^{13}C-DMSO or tacrolimus plus ^{13}C-DMSO were given to rats fed a polyphenol-containing

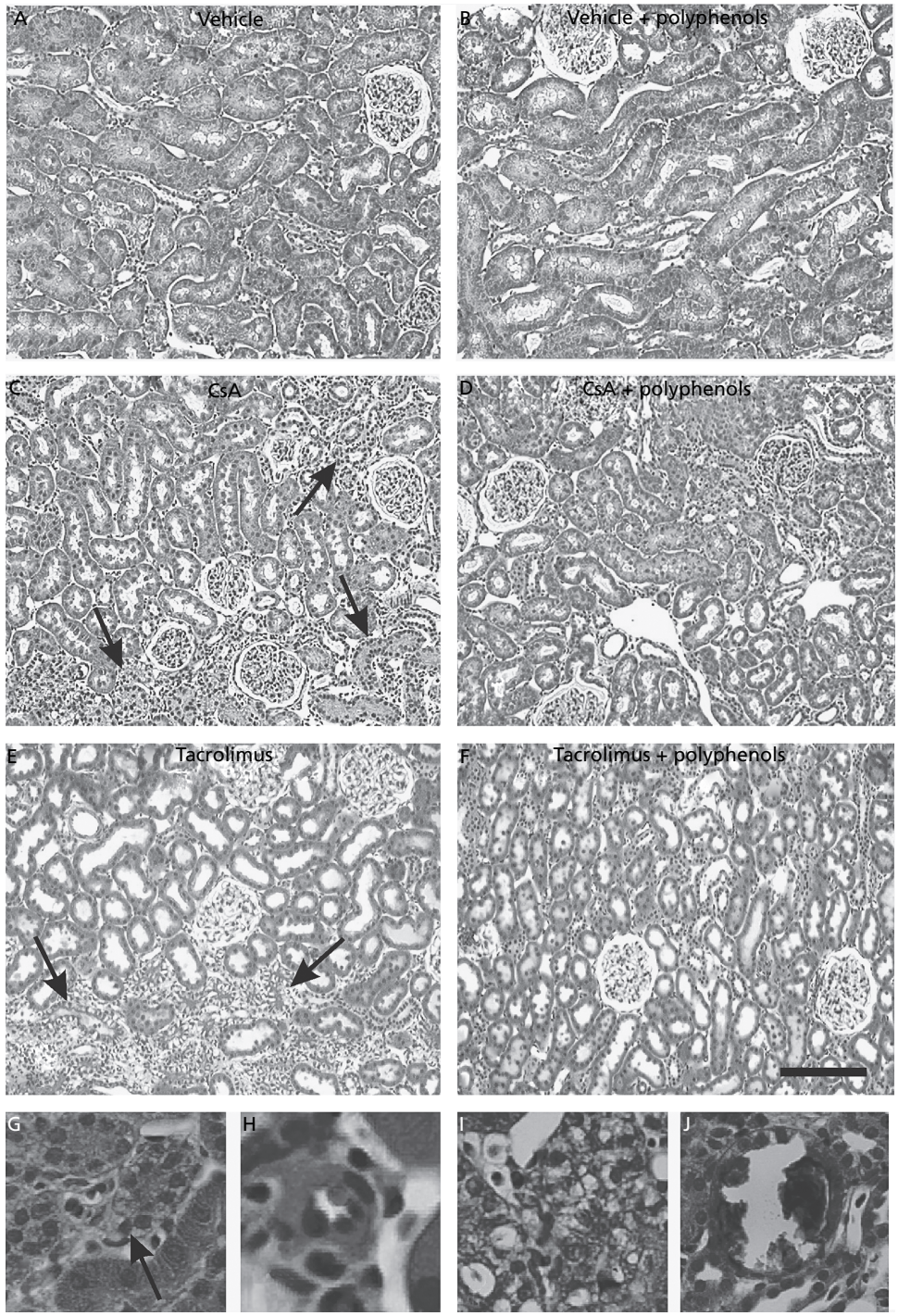

Figure 3 Dietary polyphenols minimize ciclosporin CsA)- and tacrolimus-induced pathological changes in the rat kidney. Conditions were as in Figures 1 and 2. Three weeks after CsA or tacrolimus treatment, kidneys were harvested. Representative microphotographs are shown of control diet plus vehicle (A), 0.1% polyphenols plus vehicle (B), control diet plus CsA (C), polyphenols plus CsA (D), control diet plus tacrolimus (E), polyphenols plus tacrolimus (F), tubular atrophy (G), arteriolar hyalinosis (H), vacuolization (I) and calcification (J). Bar is 100 μm.

diet. In rats treated with polyphenols and CsA plus ^{13}C-DMSO or tacrolimus plus ^{13}C-DMSO, the methyl radical adduct (species III) indicative of hydroxyl radical could not be detected (Figure 6E, I). In addition, the amount of species I and II was also decreased.

Polyphenols decreases CsA and tacrolimus-induced 4-hydroxynonenal adduct formation

4-HNE, a product of lipid peroxidation, was detected immunohistochemically (Figure 7). In kidneys from vehicle-treated controls (Figure 7A) and from rats given dietary

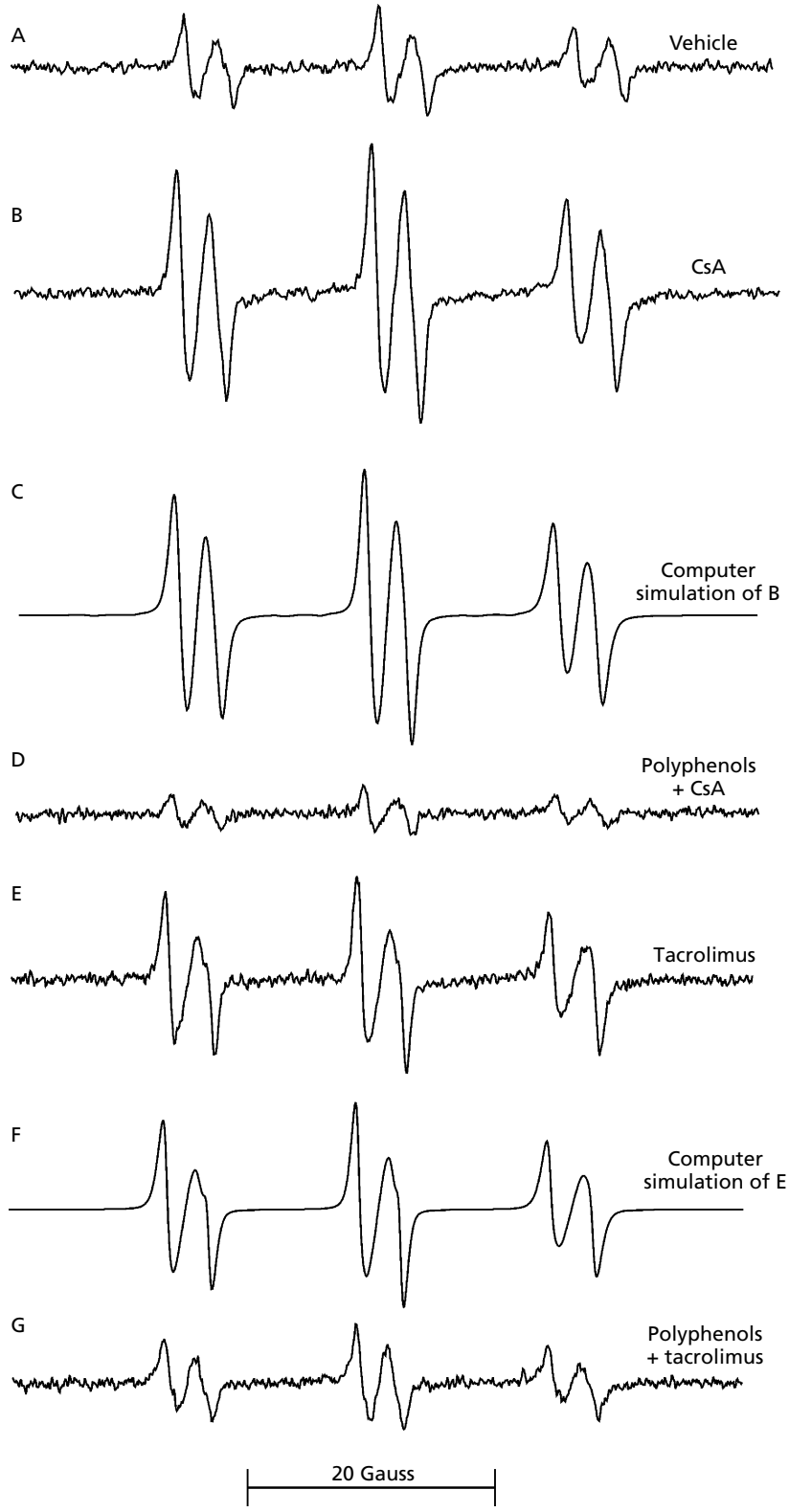

Figure 4 Electron paramagnetic resonance spectra of free radical adducts in rat urine after ciclosporin (CsA) and tacrolimus treatment. Conditions were as in Figure 3, except that CsA and tacrolimus treatments were for 5 days. Free radical 4-POBN adducts in urine were detected with a ESR spectrometer. Typical spectra are shown of control diet plus vehicle (A), control diet plus CsA (B), computer simulation of the radical adduct spectrum of B (C), 0.1% dietary polyphenols plus CsA (D), control diet plus tacrolimus (E), computer simulation of radical adduct spectrum of E (F) and 0.1% dietary polyphenols plus tacrolimus (G).

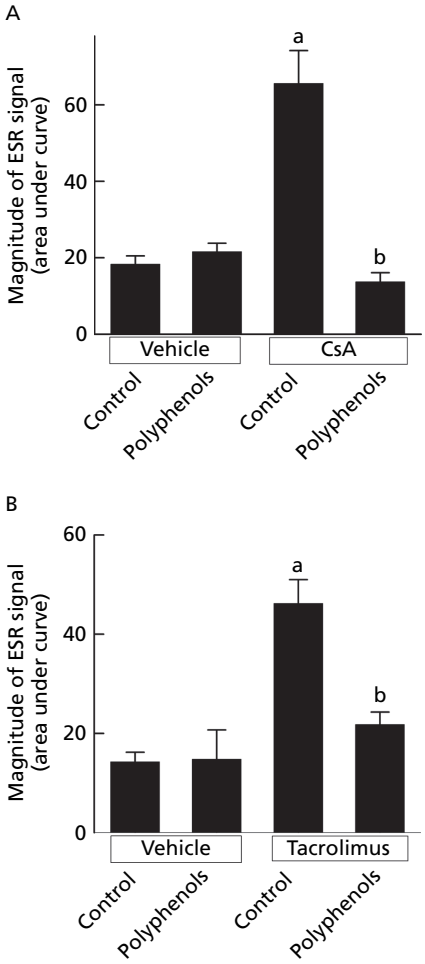

Figure 5 Dietary polyphenols decrease ciclosporin (CsA)- and tacrolimus-induced free radical production. Conditions were as in Figure 4. Free radical adducts were quantified by double integration of ESR spectra and normalized to urine volume produced in 3 h. A. CsA. B. tacrolimus. Values are means ± s.e.m., n = 4 or 5 per group. a, $P < 0.05$ vs control diet plus vehicle; b, $P < 0.05$ vs control diet plus CsA or tacrolimus.

polyphenols and vehicle (Figure 7B), 4-HNE adducts were undetectable. After treatment with CsA, 4-HNE staining occurred extensively in the outer medulla and cortex. 4-HNE immunoreactivity was present in both proximal and distal tubules but was minimal in glomeruli (Figure 7C). Similar patterns of 4-HNE staining were observed in kidneys from tacrolimus-treated rats (Figure 7E). Dietary polyphenols largely prevented 4-HNE adduct formation after CsA and tacrolimus (Figure 7D, F).

Polyphenols did not affect blood levels of ciclosporin and tacrolimus

The peak CsA level in the blood was $2342 \pm 172\ \text{ng mL}^{-1}$ (mean ± s.e.m.) in rats fed a control diet and $2357 \pm 332\ \text{ng mL}^{-1}$ in rats fed a polyphenol-containing diet ($P > 0.5$). Average peak tacrolimus was $3.27 \pm 0.5\ \text{ng mL}^{-1}$ in rats fed a control diet and $3.6 \pm 0.6\ \text{ng mL}^{-1}$ in rats fed a polyphenol-containing diet ($P > 0.5$). Therefore, polyphenols did not alter blood levels of either CsA or tacrolimus after acute treatment.

Discussion

CsA and tacrolimus increase free radical production in the kidney

CsA and tacrolimus are two widely used immunosuppressants in transplantation medicine (Margreiter et al 1983; Kino et al 1987; Starzl et al 1989). Although the molecular structures of these drugs are different, they share common nephrotoxicity in more than 30% of patients (Sibley et al 1983; Fung et al 1991). Both drugs decrease GFR and cause degenerative changes of proximal tubules and hypertrophy of the juxtaglomerular apparatus (Su et al 1995). In agreement with this clinical experience, decreases in GFR and similar pathological changes were observed with both drugs in the rats studied here (Figures 1–3).

Mechanisms of nephrotoxicity of CsA and tacrolimus are not fully understood. A variety of mechanisms, including activation of the renin–angiotensin system, increased release of endothelin-1, dysregulation of NO synthase, upregulation of transforming growth factor-β1 and apoptosis have been implicated in different studies (Duggin et al 1986; Kon et al 1990; Mitamura et al 1994). Both drugs bind peptidyl-prolyl cis-trans isomerases, regulating calcineurin activity, although the binding proteins for CsA and tacrolimus are different (cyclophilin and FK 506 binding proteins, respectively) (Harding et al 1989). Accordingly, CsA and tacrolimus are proposed to cause renal toxicity by inhibiting calcineurin phosphatase activity (Su et al 1995). Alternatively, CsA nephrotoxicity may be due to preglomerular vasoconstriction (English et al 1987; Mehring et al 1992), leading to hypoxia–reperfusion injury. CsA increases release of potent vasoconstrictors (Duggin et al 1986; Kon et al 1990) and activates renal sympathetic nerves (Moss et al 1985). Binding of 2-pimonidazole, a hypoxia marker, increases after CsA treatment (Zhong et al 1998). Tacrolimus, like CsA, also increases thromboxane, endothelin and renin synthesis (Moutabarrik et al 1992; Mitamura et al 1994; Su et al 1995), stimulates sympathetic nerve activity (Lyson et al 1993) and decreases renal blood flow (Andoh et al 1996). Tacrolimus also increases monocyte $p22^{phox}$ in kidney transplant patients with hypertension, a component of superoxide-generating NADPH oxidase (Calo et al 2002). This study provides direct evidence of increased free radical production, including highly toxic hydroxyl radicals, not only after CsA but also after tacrolimus (Figures 4–6). Both drugs increased oxidative stress in the kidney as evidenced by increased 4-HNE staining in both proximal and distal tubular cells (Figure 7), indicating that tubular cells are probably the predominant cellular sources of free radicals. Previous studies showed that CsA increases expression of pro-apoptotic genes and causes renal cell apoptosis (Chung et al 2005; Shihab et al 2005). It is possible that oxidative stress also contributes to apoptotic cell death caused by CsA.

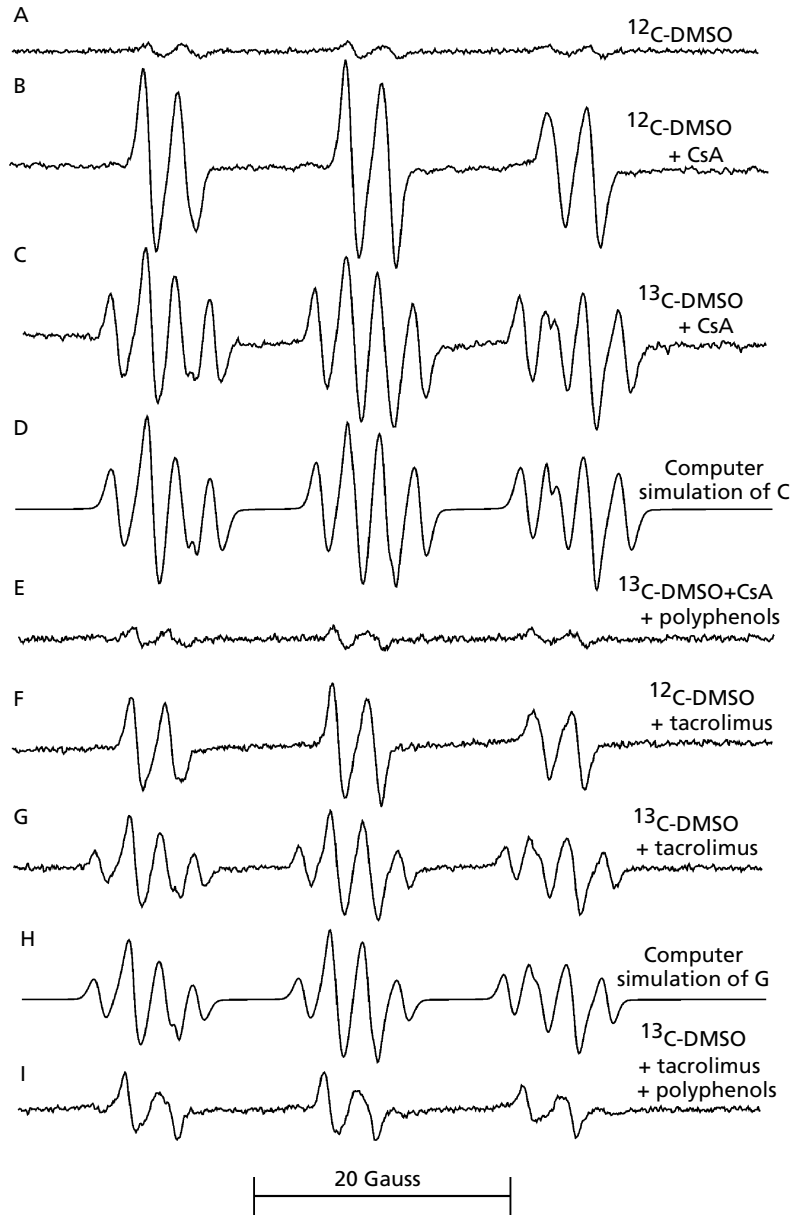

Figure 6 Dietary polyphenols decrease ciclosporin (CsA)- and tacrolimus-induced hydroxyl radical formation. Conditions were as in Figure 4 except that ^{12}C- or $^{13}C_3$-DMSO (0.2 mL/250 g) was gavaged 1 h before 4-POBN injection. Typical spectra are shown of control diet plus vehicle (A), control diet plus CsA and ^{12}C-DMSO (B), control diet plus CsA and ^{13}C-DMSO (C), computer simulation of the radical adduct spectrum of C (D), dietary polyphenol plus CsA and ^{13}C-DMSO (E), control diet plus tacrolimus and ^{12}C-DMSO (F), control diet plus tacrolimus and ^{13}C-DMSO (G), computer simulation of radical adduct spectrum of G (H) and dietary polyphenol plus tacrolimus and ^{13}C-DMSO (I).

Polyphenols decrease nephrotoxicity caused by CsA and tacrolimus

Polyphenols decreased the nephrotoxicity of CsA and tacrolimus (Figures 1–3), consistent with a recent report showing protection by green tea extracts against CsA nephrotoxicity (Mohamadin et al 2005). Polyphenols decrease nephrotoxicity of CsA and tacrolimus possibly by alteration of the absorption or metabolism of these drugs. However, up to 2% green tea extract in drinking water for 60 days did not change the activity of cytochrome P450 3A, the major enzyme responsible for the metabolism of CsA and tacrolimus (Sohn et al 1994). In addition, this study showed that blood levels of CsA and tacrolimus after acute treatment were not different in rats fed control or polyphenol-enriched diets, indicating that polyphenols did not decrease nephrotoxicity by affecting the pharmacokinetics of these drugs. However, whether polyphenols alter blood levels of CsA or tacrolimus after chronic treatment was not determined. Alternatively, polyphenols may decrease nephrotoxicity by decreasing oxidative stress. Since free radicals initiate lipid peroxidation chain reactions, scavenging radicals by polyphenols would

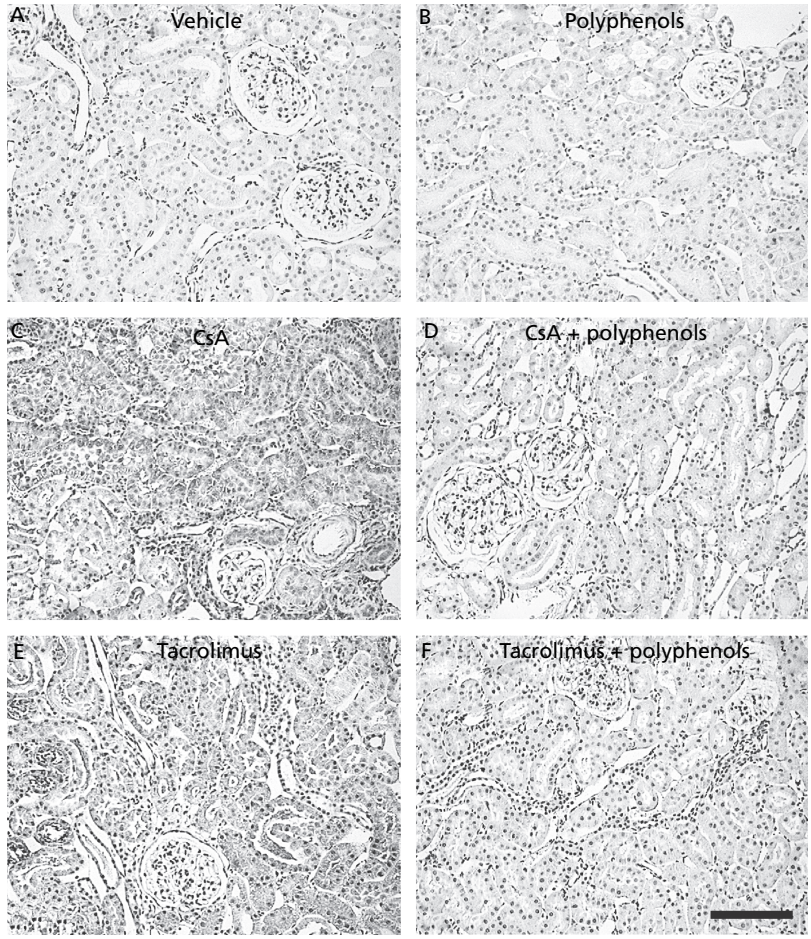

Figure 7 Dietary polyphenols decrease ciclosporin (CsA)- and tacrolimus-induced 4-hydroxynonenal formation. Conditions were as in Figure 3. Sections were stained for 4-hydroxynonenal adducts as described in Methods, and show control diet plus vehicle (A), 0.1% dietary polyphenols plus vehicle (B), control diet plus CsA (C), 0.1% dietary polyphenols plus CsA (D), control diet plus tacrolimus (E) and 0.1% dietary polyphenols plus tacrolimus (F). Bar is 50 μm.

not only detoxify these initiating radicals but also block downstream reactions. Green tea polyphenols inhibit lipid peroxidation induced by chemicals, such as bromotrichloromethane, iron, ethanol, H_2O_2 and CCl_4, in experimental animals (Hara 1994; Miyagawa et al 1997; Lin et al 1998; Yoshino & Murakami 1998). Polyphenols also decrease blood malondialdehyde and lipid hydroperoxide levels and increase total antioxidant capacity in animals and man (Serafini et al 1996; Shen et al 1998; Vinson & Dabbagh 1998). Considerable epidemiological and experimental evidence shows that green tea polyphenols decrease oxidation of LDL and increase the ratio of HDL/LDL, a change associated with decreased risk of heart disease (Muramatsu et al 1986; Serafini et al 1996; Vinson & Dabbagh 1998). Beneficial effects of green tea are considered most likely due to the antioxidant properties of its polyphenols.

In this study, polyphenols prevented increases of free radicals, especially highly toxic hydroxyl radicals in urine caused by CsA and tacrolimus (Figures 4–6). These effects were associated with improvement of renal function and pathological changes after CsA and tacrolimus treatment (Figures 1–3). Thus, polyphenols decrease renal toxicity of these drugs, at least in part, by decreasing free radicals. However, whether single polyphenol species can protect against renal toxicity of CsA and tacrolimus remains to be studied. Our previous studies showed that epicatechin, a component of *C. sinensis* polyphenols, has similar protective effects on liver ischemia–reperfusion injury as green tea extracts containing multiple polyphenol components, suggesting that the protective effects of green tea extracts is due to polyphenolic components (Zhong et al 2002).

Both dietary and oil solutions of polyphenols protected against nephrotoxicity caused by CsA (Figures 1 and 3). Daily dietary intake of 82 mg kg^{-1} polyphenols (equivalent to 0.1% polyphenols in diet) and 100 mg kg^{-1} polyphenols in oil (equivalent to 2.8% polyphenols in CsA solution) had similar protective effects (Figure 1). Therefore, these two methods of administering polyphenols could be applied clinically to decrease nephrotoxicity of CsA if they are also proven to be effective in clinical trials. Green tea extract is now commercially available, permitting relatively high intake of polyphenols without relying on drinking tea. In addition, green tea

extract could be added directly into lipophilic CsA or tacrolimus solutions, thus providing a practical route of clinical administration.

Conclusion

This study shows that two major immunosuppressants, CsA and tacrolimus, increase renal production of free radicals, including highly toxic hydroxyl radicals. Green tea polyphenols partially blunt CsA and tacrolimus nephrotoxicity and decrease free radical formation. Thus, renal protection by green tea extracts is likely due to scavenging of free radicals by polyphenols.

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