The Epoxyeicosatrienoic Acid Analog, PVPA, Ameliorates Cyclosporine-Induced Hypertension and Renal Injury in Rats

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ABSTRACT

The introduction of calcineurin inhibitors (CNI) into clinical practice in the late 1970s transformed organ transplantation and led to significant improvement in acute rejection episodes. However, despite their significant clinical utility, the use of these agents is hampered by the development of hypertension and nephrotoxicity, which ultimately lead to end-stage kidney disease and overt cardiovascular outcomes. There are currently no effective agents to treat or prevent these complications. Importantly, CNI-free immunosuppressive regimens lack the overall efficacy of CNI-based treatments and put patients at risk of allograft rejection.

Cytochrome P-450 epoxygenase metabolites of arachidonic acid, epoxyeicosatrienoic acids (EETs) have potent vasodilator and anti-hypertensive properties in addition to many cytoprotective effects, but their effects on CNI-induced nephrotoxicity have not been explored. Here, we show that PVPA, a novel orally active analog of 14,15-epoxyeicosatrienoic acid, effectively prevents the development of hypertension and ameliorates kidney injury in cyclosporine-treated rats. PVPA treatment reduced proteinuria and renal dysfunction induced by cyclosporine. PVPA inhibited inflammatory cell infiltration into the kidney and decreased renal fibrosis. PVPA also reduced tubular epithelial cell apoptosis, attenuated the generation of reactive oxygen species and modulated the unfolded protein response that is associated with endoplasmic reticulum stress. Consistent with the in vivo data, PVPA attenuated cyclosporine-induced apoptosis of NRK-52E cells in vitro. These data indicate that the cytochrome P-450/EET system offers a novel therapeutic strategy to treat or prevent CNI-induced nephrotoxicity.

Keywords: cyclosporine, transplantation, nephrotoxicity, hypertension, epoxyeicosatrienoic acids
INTRODUCTION

Cyclosporine A (CSA) is a calcineurin inhibitor (CNI) whose widespread introduction into clinics in the late 1970s transformed organ transplantation and led to significant improvement in acute rejection episodes. Tacrolimus came into the market subsequently (3, 5, 13, 44). To date, the CNIs continue to be a major component of immunosuppressive regimens in solid organ transplantation worldwide. CNIs are also used in the treatment of many autoimmune conditions (1, 23). However, despite the well-documented improvements in acute rejection rates and short-term graft outcomes, CNI use has not been associated with any significant enhancements in long-term allograft survival. Instead, CNI use is limited by the development of hypertension and nephrotoxicity (2, 28, 29, 31, 32). Indeed, CNI-induced end-stage kidney failure is a major complication and is an ever-increasing problem in non-kidney solid organ recipients (36).

Unfortunately, efficient treatment to prevent or reduce this devastating outcome of CNI therapy is non-existent. Rather, this has led to trials of CNI-free regimens that lack the overall efficacy of CNI-based regimens and actually put patients at risk for allograft rejection (9, 10, 11, 16, 18). Also of note, control of the CNI-induced hypertension per se has not been shown to be adequate in preventing the nephrotoxicity (24, 39). The underlying mechanism of CNI-induced nephrotoxicity remains unclear. CNIs activate the intrarenal renin-angiotensin system and also increase the expression of endothelin-1 leading to afferent arteriolar vasoconstriction and reduction in renal blood flow. CNIs have also been reported to cause oxidative stress which acts variously to cause tissue injury. Inflammatory cell infiltration into the kidney is a common finding and this is initially associated with augmented expression and release of inflammatory cytokines and chemokines in the kidney. The macrophages subsequently, along with other cells, become activated and elaborate large amounts of extracellular matrix components such as
collagen which leads to tubulointerstitial fibrosis. Tubular epithelial cell apoptosis may be due
to the direct effect of the CNI or from local hypoxia due to renal vasoconstriction (8, 17, 34, 40,
49). The foregoing suggests that CNI-induced nephrotoxicity occurs via a multifactorial
mechanism and, therefore, in order to be effective in preventing or treating these CNI-induced
complications, potential therapeutic agents will need to have broad-based modulating effects on
vascular tone, inflammation, oxidative stress and apoptosis. Such an agent would allow patients
to benefit from these useful drugs while preventing overt complications.

Arachidonic acid is metabolized by key enzyme systems to small molecule mediators with
diverse physiologic and pathophysiologic effects. Specifically, the cytochrome P-450
epoxigenases (mostly CYP2C and CYP2J) metabolize arachidonic acid to four bioactive
regioisomeric epoxieicosatrienoic acids (EETs); 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET
(6, 52). EETs are mostly produced in the endothelium and are expressed in many tissues
including the kidney, heart, lung and liver. EETs mediate many autocrine and paracrine
functions, including the regulation of vascular tone and tubular sodium and water absorption in
the kidney. EETs have anti-hypertensive action in vivo (4, 19, 51). Also, previous studies have
demonstrated that EETs have anti-inflammatory, anti-fibrotic, and anti-apoptotic effects (14, 21,
22, 35, 42, 48). In support of the above, we and others recently reported that EETs attenuated
cisplatin-induced nephrotoxicity by reducing oxidative stress, inflammation, ER stress, and
apoptosis, without affecting the chemotherapeutic effects of cisplatin (20, 26, 41). In this study,
we examined the protective role of the novel orally active EET analog, PVPA, on an
experimental model of CSA-induced nephrotoxicity. We demonstrate that PVPA administration
robustly prevented CSA-induced hypertension and ameliorated histological damage and kidney
dysfunction. The mechanism of action seems to be through the suppression of inflammation, oxidative stress, endoplasmic reticulum stress and apoptosis.

MATERIALS AND METHODS

Materials

All chemicals and assay kits were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. The novel, orally active 14,15-epoxyeicosatrienoic acid analog, PVPA [N-isopropyl-N-(5-((3-(5-N-isopropylheptanamido)pentyl)-2-(pivaloylimino)-2,3-dihydrobenzo[d]thiazol-4-yl)oxy)pentyl)heptanamide] was designed and synthesized in the laboratory of Dr. Falck (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA). PVPA was given in drinking water and its concentration was adjusted in a way that the daily intake (dose) was 10 mg/kg body weight. The administered dose was chosen on the basis of the drug's pharmacokinetic properties and is similar to the dose of similar agents used in recent in vivo experiments (20).

Animals, dosing and experimental design

Male Sprague-Dawley rats weighing 180-200 g (Envigo, Indianapolis, IN, USA) were used. Animals were kept in a temperature-controlled environment with a 12-h light-dark cycle and were allowed free access to a low-salt diet (0.02% sodium, Teklad Global 2918, Madison, WI, USA) and to tap water, except in the PVPA treatment group where the drug was put in the drinking water. CSA (supplied at 50 mg/ml in Cremophor EL plus 32.5% v/v alcohol by Novartis Pharma, Basel, Switzerland) was diluted in normal saline to 15mg/ml. Rats were acclimatized for a minimum of 1 week before experimentation. All animals received humane
care in compliance with the National Research Council’s Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. The animals were placed into 3 groups. Group 1 (Control group) received daily s.c. injection of Cremophor EL only. Group 2 (CSA group) received daily s.c. injection of CSA only (20mg/kg). Group 3 (PVPA group) received daily s.c. injection of CSA (20mg/kg) plus PVPA (10 mg/kg/d) in drinking water. PVPA was initiated 5 days before the start of CSA treatment. All the animals were treated for 28 days. At 1 day before euthanasia, rats were maintained in individual metabolic cages and urine was collected over a 24-hour period.

Non-invasive Blood Pressure Measurements
Non-invasive blood pressure measurements were performed by tail plethysmography (IITC Life Sciences Inc. Woodland Hills, CA, USA). Rats were acclimatized to the apparatus during 3 sessions over 5-7 days. Following acclimatization, weekly blood pressure measurement was done during the course of the study. Systolic blood pressure (SBP) was measured exactly at the same period of the day (9–10 am) on every occasion. The average values for SBP were obtained from ten sequential cuff inflation-deflation cycles.

Biochemical analysis
The levels of blood urea nitrogen (BUN) (BioAssay Systems, Hayward, CA, USA), urinary protein and creatinine (Cayman Chemical Company, Ann Arbor, MI, USA) were measured spectrophotometrically using commercial kits. Urinary albumin was measured using ELISA kits (Exocell, Philadelphia, PA, USA). Kidney cortex and medulla homogenate was prepared with a
lysis buffer (50 mM HEPES, pH 7.4, with 5 mM CHAPS and 5 mM DTT), centrifuged at 10,000 g for 10 min and then the resulting supernatant was used for the assay. Caspase 3 activity in the kidney homogenate was determined using a commercial fluorimetric assay kit (Sigma-Aldrich, St. Louis, MO, USA). The caspase 3 fluorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. The caspase 3 activity is expressed as nmol of AMC/min/μL. Kidney tissue protein content was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Lipid peroxidation in the renal medulla and cortical homogenate was measured as thiobarbituric acid reactive substances (TBARS) by a fluorometric assay (Cayman Chemicals, Ann Arbor, MI, USA).

**Immunohistochemistry and TUNEL assay**

Formalin-fixed kidneys were embedded in paraffin and used for immunohistochemical studies. After routine deparaffinization, heat-induced epitope retrieval was done using a citrate-based buffer. Endogenous peroxidase was quenched by incubation with 3% H₂O₂ in phosphate-buffered saline (PBS; pH 7.4) for 5 min. The sections were permeabilized using 0.1% (wt/vol) Triton X-100 in PBS for 15 min. Nonspecific binding was minimized by incubating sections with 5% normal serum from the species in which the secondary antibody was raised (diluted in 5% bovine serum albumin/PBS) for 60 min at room temperature. Endogenous biotin and avidin binding sites were blocked by sequential incubation with avidin and biotin for 15 min (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used to detect GRP78/BiP, C/EBP homologous protein (CHOP) and CD68 were goat anti-rat GRP78 (Santa Cruz Biotechnology,
Santa Cruz, CA, USA), rabbit anti-rat CHOP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-rat CD68 (Bio-Rad, Hercules, CA, USA), respectively. Biotinylated secondary antibodies were used for development with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kits; Vector Laboratory, Burlingame, CA, USA). The slides were counterstained with hematoxylin or methyl green and photographed. Apoptosis was examined by TUNEL staining with ApopTag® Plus Peroxidase In Situ Apoptosis Kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. Quantification of CD68- and TUNEL-positive cells was done by counting the positive cells in 15 randomly selected, non-overlapping fields (×200 magnification) in the outer medulla and cortex. Quantification of GRP78 and CHOP staining were assessed at ×200 magnification using an image analyzing software, NIS Elements AR version 3.0 (Nikon Instruments Inc., Melville, NY, USA). To minimize observer bias, the analysis was performed in a blinded fashion without knowledge of the treatment group from which the tissues originated.

Histologic analysis

Formalin (10%)-fixed kidney samples were embedded in paraffin. Sections (4 μm) were prepared and stained with hematoxylin and eosin and Mason’s trichrome. The slides were scanned and visualized using a Hamamatsu NanoZoomer HT digital scanner/ NDP.view2 viewing software (Hamamatsu Photonics). The hematoxylin and eosin stained kidneys were reviewed and assessed for tubular vacuolization. The scoring was done semiquantitatively by counting the number of fields with three or more tubules affected by isometric vacuolization from a total of 20 randomly selected non-overlapping fields (×200 magnification) observed in the renal cortex. The trichrome-stained slides were assessed for the degree of tubulointerstitial...
fibrosis using NIS Elements AR version 3.0 software (Nikon Instruments Inc.). The renal tissue areas positive for collagen staining were expressed as the percentage area fraction relative to total area analyzed. All examinations and scoring were done in a blinded manner.

Quantitative Real-Time PCR

Total RNA was prepared from kidney tissues stored in RNAlater stabilization solution using a TRIzol Plus RNA Purification Kit and then reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer’s instructions (Thermo Fisher Scientific Inc, USA). Quantitative amplification of the cDNA was performed on an ABI-Prism 7900HT Sequence Detection system and evaluated using SDS and RQ manager softwares (versions 2.3 and 1.2; Applied Biosystems, Thermo Fisher Scientific Inc, USA). Results were normalized to GAPDH content by the comparative CT method and relative mRNA levels are expressed as fold change compared with the vehicle-treated control animals. The following primer and probe sets (Integrated DNA Technologies, Coralville, Iowa, USA) were used: GAPDH forward: GTAACCAGGCGTCCGATAC, reverse: TCTCTGEDGCTCCTCCCTGTTC, and probe: /56-FAM/CACACCAGGC/ZEN/CTTCACCATCTTGTCT/3IABkFQ/ ; Collagen 1a1 forward: CATTGTGTATGCAGCTGACTTC, reverse: CGCAAAGAGTCTACATGTCTAGG, and probe: /56-FAM/CCGGAGGTC/ZEN/CACAAAGCTGAACA/3IABkFQ/.

Cell Cultures

The well-characterized normal rat proximal tubular epithelial cell line, NRK-52E, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (with 4.5 g/l glucose; D5671, Sigma-Aldrich, USA)
supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate and 4 mM L-glutamine in 5% CO2 at 37 °C.

**MTT Assay**

NRK52E cells were seeded into 96-well plates in a volume of 200 µl per well (40,000 cells/ml) and allowed to grow to 80% confluence. After washing once with serum-free media, cells were incubated in 200 µl per well of serum-free media containing the indicated amount of PVPA for 24 hours. Cell viability was determined by addition of 20 µl of MTT at a concentration of 5 mg/ml in PBS. After incubation for 1 hour, the medium was removed and 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm using a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

**TUNEL staining of NRK-52E cells**

NRK-52E cells were seeded into Lab-Tek II Chamber Slides (8 chambers; Thermo Fisher Scientific Inc., USA) at a density of 25,000 cells per chamber in 0.5 ml complete media and allowed to grow to 80% confluence. After washing once with serum-free media, cells were incubated in 0.5 mL per chamber of serum-free media with or without 10 µM PVPA for 1 hour. Cells were then treated with 5 µM CSA or an equivalent amount of vehicle. After 24 hours, cells were washed once with PBS before fixation in 4% paraformaldehyde in PBS for 10 min at room temperature. Apoptosis was examined by TUNEL staining with ApopTag® Plus Peroxidase In Situ Apoptosis Kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. Quantification of TUNEL-positive cells was done by counting the positive cells in
10 randomly selected, non-overlapping fields (×200 magnification) per chamber. 6 replicate chambers per condition were counted.

Statistical analysis
Results are reported as means ± SEM. In vitro experiments were performed at least twice and statistical significance between two measurements was determined by the two-tailed unpaired Student’s t-test. Differences between multiple groups were determined by ANOVA, using GraphPad Prism® Version 4.0 software (GraphPad Software Inc, La Jolla, CA, USA). P values of ≤ 0.05 were considered statistically significant.

RESULTS
PVPA Prevents the Development of Hypertension in CSA Treated Rats
Hypertension is a common complication in patients who receive calcineurin inhibitor treatment and this has been associated with increased cardiovascular morbidity and mortality, as well as graft loss in renal transplant recipients (7, 27, 38). To investigate whether PVPA affected the development of hypertension in an experimental model of CSA-induced nephrotoxicity, we measured SBP in conscious male Sprague Dawley rats by the non-invasive tail-cuff method. Baseline SBP was not different between the three groups of rats. Consistent with the known effect of CSA on blood pressure, the animals developed robust hypertension by day 7 of CSA treatment with an increase in SBP of 33±4 mmHg (P < 0.001), followed by a steady rise in SBP throughout the remainder of experimentation. By the end of the 28-day study period, the mean SBP in the CSA-only group was significantly elevated compared with the PVPA group (+Δ32
mmHg; \( P < 0.001 \); Figure 1). Indeed, the final mean SBP in the PVPA group remained very close
to the baseline value and was not statistically different from that of the control rats that did not
receive CSA treatment \((\Delta 9 \text{ mmHg}; P = 0.17)\). These results indicate that PVPA had a profound
protective effect on the development of CSA-induced hypertension. The final body weight of the
CSA-only animals was not statistically different from that of the PVPA group \((316 \pm 7 \text{ vs } 313 \pm
4 \text{ g}; P = 0.71)\) and hence the observed differences in SBP are not due to differences in body
weight.

**PVPA Attenuates Proteinuria and Renal Dysfunction in CSA Treated Rats**

Prolonged treatment with CSA leads to the development of irreversible parenchymal changes in
the kidney which progresses to chronic kidney disease and is a major risk factor for the
development of end-stage kidney failure in solid organ transplant recipients. We assessed
albuminuria and proteinuria as markers of chronic renal damage. Albuminuria was 3.5-fold
higher in the CSA treated animals at the end of the 28-day treatment period compared with the
control group which only received a vehicle \((P < 0.001)\). Administration of PVPA significantly
reduced the CSA-induced albuminuria by about 90% \((P < 0.001; \text{ Figure 2A})\). Similarly, the
increased proteinuria induced by CSA treatment was significantly reduced by co-administration
of PVPA \((P < 0.05; \text{ Figure 2B})\). Importantly, at the end of the study, no differences in urinary
sodium and potassium excretion rates were noted in the CSA-only group versus the PVPA group
\((\text{Sodium: } 0.60 \pm 0.08 \text{ vs } 0.79 \pm 0.08 \text{ mmol/day, } P = 0.14; \text{ Potassium, } 1.68 \pm 0.12 \text{ vs } 1.74 \pm 0.18
\text{ mmol/day, } P = 0.8)\). This indicates that salt intake was similar between the CSA-only and PVPA
rats. Altogether, these data imply that the reduction in albuminuria and proteinuria in the PVPA
group are not due to differences in salt consumption or natriuresis. Consistent with the
albuminuria/proteinuria results, BUN, a measure of renal function was increased more than two-fold in the CSA-only group at the end of the study compared with the vehicle-treated rats while the increase was significantly attenuated in the PVPA group by about 30% \((P < 0.001); \) Figure 2C).

**PVPA Ameliorates Renal Morphological Changes Induced by CSA**

To assess histologic features of CSA-induced tubular and interstitial damage, kidney sections stained with hematoxylin and eosin (H&E) and Masson’s trichrome were examined. Prolonged CSA treatment resulted in tubular injury with swelling and isometric vacuolization which was evident on the H&E-stained slides. Co-treatment with PVPA significantly reduced the extent and severity of this injury (Figure 3A, B). Chronic damage from CSA results in deposition of extracellular matrix, including collagen within kidney parenchyma that ultimately leads to chronic kidney disease and end-stage kidney failure. CSA treatment resulted in 1.8-2.8 fold increase in Masson’s trichrome staining in the cortex and medulla, consistent with enhanced interstitial fibrosis. PVPA administration led to significant improvement in the CSA-induced interstitial fibrosis, reducing the extent of collagen deposition by 25-45% (blue area, Figure 3C, D). We further assessed the fibrosis by measurement of the gene expression level of collagen 1a1. As depicted in Figure 3E, CSA treatment induced collagen 1a1 mRNA expression. In contrast, the increased expression was significantly attenuated by PVPA co-treatment.

**PVPA Reduces CSA-Induced Renal Oxidative Stress**

Reactive oxygen species (ROS) play key roles in normal renal physiology, regulating many different processes including gluconeogenesis, electrolyte transport, hemodynamics and gene
expression (15, 43, 46). Excessive elaboration of ROS during pathologic states, however, leads to oxidative stress which is associated with cellular apoptosis, tissue inflammation and fibrosis. Oxidative stress has been shown to be an important mediator of CSA-induced nephrotoxicity. We evaluated whether PVPA administration modifies oxidative stress during CSA treatment. The level of thiobarbituric acid-reactive substances (TBARS), a measure of ROS levels, was significantly increased by 60-80% in the cortex and medulla after CSA treatment compared with vehicle treatment. Co-administration of PVPA significantly suppressed the ROS generation in the medulla by about 30% compared with the CSA only group. In the cortex, PVPA co-administration reduced TBARs levels by about 20%, although this did not reach statistical significance (Figure 4).

PVPA Reduces Renal Inflammation during CSA Treatment

CSA-induced nephropathy, like other causes of chronic kidney disease, is associated with tubulointerstitial inflammatory response and macrophage influx which contributes to the establishment of the renal damage and tubulointerstitial fibrosis. To ascertain the effect of PVPA on the CSA-induced inflammatory response, we performed immunohistochemical staining using an antibody against CD68, a marker of macrophages. As shown in Figure 5, the number of infiltrating macrophages into the kidney increased more than 6-fold in CSA-treated rats compared with the control animals. The co-administration of PVPA resulted in a significant reduction in macrophage infiltration by 50%.

PVPA Reduces Renal Tubular Epithelial Cell Apoptosis during CSA Treatment
Tubular epithelial cell (TEC) apoptosis could be a direct effect of the CNI or may occur as a result local hypoxia due to renal vasoconstriction. Apoptosis represents a key feature in the pathogenesis of CSA-induced nephrotoxicity (45, 47). We assessed the protective effects of the PVPA on CSA-induced renal tubular epithelial cell apoptosis by using the terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining method. The number of TECs containing TUNEL-positive nuclei increased markedly after 28 days of CSA treatment when compared with the vehicle treated animals. Administration of PVPA significantly ameliorated tubular cell apoptosis induced by CSA (P< 0.01, Figure 6A, B). Apoptosis or programmed cell death involves the activation of a group of proteases called “caspases”. Caspase 3 is the final effector caspase that mediates apoptotic cell death. We, therefore, examined the effect of PVPA on caspase 3 activity in the kidney. We found that caspase 3 activity increased by more than 2-fold with CSA treatment. Co-administration of PVPA reduced the caspase 3 activity by more than 50% in the medulla of CSA treated rats (Figure 6C).

**PVPA Ameliorates ER Stress in the Kidney during CSA Treatment**

Recent evidence indicates that CSA causes endoplasmic reticulum (ER) stress which has been implicated in the pathophysiology of various renal diseases. Excessive ER stress leads to overexpression of the transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP), which mediates ER-stress induced apoptosis in addition to induction of inflammation (33). In order to elucidate the effect of PVPA treatment on this process, we examined the expression of the ER stress marker GRP78 (glucose regulated protein 78) and also CHOP in kidney tissues using immunohistochemical analysis. We found more than a 2-fold increase in
GRP78 expression in both the cortex and medulla consistent with increased ER stress following CSA treatment compared with control rats. The co-administration of PVPA to CSA-treated rats downregulated GRP78 expression levels by more than 50% (Figure 7A, B). Similarly, the expression level of the pro-apoptotic transcription factor CHOP was upregulated by 1.6-2.8 fold in the cortex and medulla in CSA-treated rats and co-treatment with PVPA significantly attenuated this increase by about 80% (Figure 7B, C). These results are in support of the other findings showing attenuation of inflammation and apoptosis after co-administration of PVPA and suggest that the protective effect of PVPA during CSA treatment is mediated, to some extent, by modulation of ER stress.

**PVPA Reduces CSA-Induced Apoptosis In Vitro**

In the present study, we demonstrated anti-hypertensive and kidney protective effects of PVPA in the CSA treated animals, and such kidney protective effects of PVPA can be attributed to its anti-hypertensive action. To investigate if the kidney protective effects of PVPA in the CSA treated animals also resulted by its direct cytoprotective effects, we conducted *in vitro* studies using NRK-52E cells. First, to confirm the safety of PVPA on kidney cells, NRK-52E cells were incubated with various concentrations of PVPA and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PVPA had no deleterious effect on the proliferation or viability of the cells over a wide range of pharmacologically relevant dosing (1-20 μM; Figure 8A). As in the in vivo system, we found that incubation of NRK-52E cells with CSA resulted in a significant increase in the number of apoptotic cells as determined by TUNEL staining, when compared with vehicle treatment. Co-treatment of the cells with PVPA significantly reduced apoptosis in the cells (Figure 8B, C). These results
suggest that PVPA ameliorates CSA-induced nephrotoxicity at least in part through direct
cytoprotective effects on epithelial cells.

DISCUSSION

Calcineurin inhibitors (CNI) are a major component of the immunosuppressive regimen used in
solid organ transplant recipients and are also used to treat many other immune-mediated
diseases. By inhibiting calcineurin, the CNIs exert their immunosuppressive effects by
preventing the dephosphorylation and nuclear translocation of NFAT and thus reducing the
expression of IL-2 which is important for T-cell proliferation and activation. Unfortunately,
despite their significant clinical utility, CNI use is hampered by the development of
hypertension, nephrotoxicity and cardiovascular morbidity. The nephrotoxicity is universal and
occurs over time. The irreversible kidney changes noted following chronic CNI use include renal
arteriolar hyalinosis, glomerular sclerosis, tubular atrophy and tubulointerstitial fibrosis that are
associated with progressive renal dysfunction (30). Attempts at developing immunosuppressive
protocols that avoid, withdraw, or minimize CNI use in solid organ transplantation have mostly
been unsuccessful due to increased risk of acute rejection and therefore the potential for chronic
graft failure (9, 10, 11, 16, 18). Consequently, effective therapy to prevent or treat CNI-induced
nephrotoxicity is urgently needed. Accumulating evidence indicates that the cytochrome P-450/
EET system has many organ protective properties. In the present study, we investigated the
protective role of the novel orally active epoxyeicosatrienoic acid analog, PVPA, on an
experimental model of CSA-induced hypertension and nephrotoxicity. Our results show that
exogenous administration of PVPA effectively prevented CSA-induced hypertension and
ameliorated interstitial fibrosis and kidney dysfunction. We demonstrate that PVPA
administration inhibited inflammatory cell infiltration into the kidney, indicating that PVPA has anti-inflammatory effects in this model. Also, PVPA reduced oxidative stress, modulated the unfolded protein response that is associated with endoplasmic reticulum stress and importantly, attenuated tubular epithelial cell apoptosis. Overall, our data suggests that PVPA decreased CSA-induced renal injury through lowering of blood pressure, and also through its direct cytoprotective effects on epithelial cells. To our knowledge, this study is the first to show a role for EETs in this model and the findings suggest that the cytochrome P-450/EET system may offer a novel multi-mechanism therapeutic strategy to prevent CNI-induced nephrotoxicity.

The mechanism of CNI-induced hypertension and nephrotoxicity is complex and remains incompletely understood. CNIs may cause hypertension by enhancing salt and water retention through the activation of sodium channels including the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) and epithelial sodium channel (ENaC) (25, 50). CNIs have also been reported to stimulate the intra-renal renin-angiotensin system (RAS) in addition to promoting increased levels of endothelin 1, both of which could contribute to the development of hypertension, tissue hypoxia and renal fibrosis. Also, CNI administration is associated with increased production of reactive oxygen species (ROS) and there is extensive evidence supporting the involvement of oxidative stress in the pathogenesis of CNI-induced hypertension and nephrotoxicity. Among others, oxidative stress due to excess production of ROS can lead to reduction in nitric oxide levels resulting in endothelial dysfunction and vasoconstriction and may play a role in the development of hypertension and nephrotoxicity. Furthermore, chronic CNI administration leads to tubular atrophy, at least in part through TEC apoptosis. CNI-induced TEC apoptosis could be a direct effect of the CNI or from indirect causes. Another common finding with CNI treatment is that of increased inflammatory response within the kidney. Numerous studies have demonstrated that
CNI treatment leads to increased production of inflammatory cytokines, chemokines and
adhesion molecules and this is associated with enhanced inflammatory cell infiltration into the
kidney (30). The infiltrating cells are a source of ROS and may also cause chronic damage
through the deposition of excessive amounts of extracellular matrix that results in renal fibrosis.
Recent studies have identified endoplasmic reticulum (ER) stress as a potential mechanism for
CNI-induced nephrotoxicity (40). Chronic treatment with CNIs has been shown to cause ER
stress, an ER dysfunctional state characterized by the accumulation of unfolded (or misfolded)
proteins. The adaptive cellular response to ER stress involves the activation of the unfolded
protein response (UPR), aimed at restoring homeostasis and maintaining functional integrity of
the cells. During excessive or persistent ER stress, the UPR can activate inflammatory and
apoptotic pathways leading to cell death (33).

In the rat model of CNI-induced hypertension and nephrotoxicity, we noted a profound
increase in SBP as early as 7 days after initiation of CSA followed by a sustained elevation in the
SBP till the end of the experiment. In comparison, the animals that received
PVPA in addition to CSA maintained their SBP close to the baseline value and at the end of the
treatment period had SBP that was not statistically different from the control animals. The
mechanism of the anti-hypertensive effect of PVPA in this model was not examined, but most
likely involves vasodilatation of the renal microvessels and also through inhibition of sodium
channels (4,19). The mean weight of the CSA only group at the end of the study was not
different from the PVPA group, implying that the observed differences in blood pressure were
not driven by weight differences. Renal fibrosis is a hallmark of chronic damage by CNI
treatment, and, like other causes of CKD, it is the final common pathway through which CNI-
induced renal injury leads to end-stage kidney failure. In this study, we found that CSA caused
tubulointerstitial fibrosis and this was associated with renal dysfunction as shown by the increased proteinuria and BUN levels. The CSA-induced collagen deposition and renal dysfunction were significantly ameliorated in animals treated with PVPA. Macrophage infiltration into the kidney was increased by CSA and this was attenuated by co-administration of PVPA. The reduction in macrophage infiltration due to PVPA treatment could explain the improved fibrosis and renal dysfunction noted in our study. We also observed increased expression of TBARs, a marker of oxidative stress, extensive tubular injury as shown by tubular vacuolization and also increased apoptosis in the kidneys of animals treated with cyclosporine. All these are precursors to the development of fibrosis during chronic administration of cyclosporine (30), and these changes were all ameliorated by co-administration of PVPA. Following 28 days of CSA administration to the animals, we found evidence of ER stress in tubular epithelial cells as depicted by the marked increase in expression of the master chaperone, GRP 78, and also the transcription factor, CHOP, which is associated with apoptotic cell death. Co-treatment of the animals with PVPA attenuated ER stress. These findings are consistent with our recent report showing the modulating effects of EET analogs on cisplatin-induced ER stress (20). Future studies will elucidate the molecular mechanisms through which PVPA modulates ER stress.

Under physiologic conditions, the cytochrome P-450 epoxygenases metabolize arachidonic acid to bioactive EETs in various tissues and these compounds act in autocrine and paracrine fashion, mediating vasodilatory and other effects. Recent studies have provided evidence that EETs activate cell-surface receptors to increase the levels of cyclic adenosine monophosphate (cAMP) that activates smooth muscle cell large-conductance calcium-activated K⁺ channels resulting in vasodilation. Specifically, the kidney expresses high amounts of epoxygenase
activity and produces 14,15-EET as the major epoxide. The renal EETs are expressed in both the microvessels and tubules and they have been shown to control hemodynamic and epithelial transport functions in the kidney. During pathophysiological states, there is impaired epoxyxygenase generation of EETs (20) and this may contribute to reduction in renal blood flow and glomerular filtration. The current availability of exogenous EETs implies that we can explore this pathway further to understand the physiologic basis for certain diseases, and possibly treat or prevent the occurrence of specific diseases. Accumulating evidence indicates that in addition to its vasodilator properties and, hence, ability to alleviate hypertension, EETs are able to protect tissues through other mechanisms including anti-inflammatory, antiapoptotic and antioxidative effects. Indeed, studies by our group and others have shown that EETs provide organ protection in a number of preclinical models of human diseases, including diabetes, hypertension, ischemic cardiac injury and cisplatin-induced nephrotoxicity (12, 20, 37, 51). Our current findings are in total agreement with the above and demonstrate the renoprotective effect of PVPA on CSA-induced hypertension and nephrotoxicity. The beneficial effect of PVPA in this model is likely due to the suppression of inflammation, oxidative stress and apoptosis through the modulation of ER stress. The protective effects of PVPA were also seen in the in vitro system where PVPA reduced cyclosporine-induced apoptosis in rat proximal tubular epithelial cells.

In conclusion, the 14,15-epoxyeicosatrienoic acid analog, PVPA, effectively prevented CSA-induced hypertension and reduced interstitial fibrosis and kidney dysfunction by ameliorating inflammation, oxidative stress, ER stress and apoptosis. The results of these experiments have important implications not only for CNI-induced nephropathy, but also for the treatment and
prevention of other kidney diseases that are mediated by apoptosis, inflammation and oxidative stress.

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Disclosure
All the authors declared no competing interests.

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**FIGURE LEGENDS**

**Figure 1:** PVPA Prevents the Development of Hypertension in CSA Treated Rats
A rat model of CSA-induced nephrotoxicity was established by daily s.c injection of CSA for a total of 28 days. The three groups of rats studied were treated with either vehicle only, CSA only or CSA plus PVPA. SBP was measured by the tail-cuff method at weekly intervals. Values are mean ± SEM; n= 5-8 per group. *P< 0.001 vs PVPA group.

**Figure 2: PVPA Attenuates Proteinuria and Renal Dysfunction in CSA Treated Rats**
A 24-hour urine sample was collected at the end of the treatment period and was used to measure (A) albumin excretion rate and (B) protein excretion rate. (C) Blood urea nitrogen (BUN) was measured at the end of the treatment. Data are represented as the mean ± SEM; n= 5-8 per group. *P < 0.05; **P < 0.001 vs CSA group.

**Figure 3: PVPA Ameliorates Morphological Changes Induced by CSA**
(A) Representative hematoxylin and eosin staining of kidney sections from the three groups of rats showing areas of isometric vacuolization. (B) Semiquantitative score of vacuolization in the kidney sections. (C) Representative Masson’s trichrome-stained sections from the three groups of rats showing areas of fibrosis (blue areas). (D) Semiquantitative score of renal fibrosis as depicted by collagen deposition. (E) Real-time RT-PCR quantification of collagen 1 expression in kidney sections. Original magnification of slides, ×400. Data are represented as the mean ± SEM; n= 5-8 per group. *P < 0.05 vs CSA group.

**Figure 4: PVPA Reduces CSA-Induced Oxidative Stress**
Kidney thiobarbituric acid-reactive substances (TBARS) levels in the three groups of rats. Data are expressed as means ± SEM; n= 5-8 per group. *P< 0.05 vs CSA group.

**Figure 5: PVPA Inhibits Inflammation during CSA Treatment**
(A) Representative CD68-stained sections of kidney tissues from the three groups of rats (Original magnification of slides, ×400). Arrows show infiltrated CD-68 positive cells. (B) Semiquantitative score of CD68-positive cells in kidney sections. Data are represented as the mean ± SEM; n= 5-8 per group. *P < 0.05 vs CSA group.

**Figure 6: PVPA Reduces Renal Tubular Epithelial Cell Apoptosis During CSA Treatment**
(A) Representative light photomicrographs of kidney sections stained by the TUNEL method at the completion of 28 days of treatment (Original magnification of slides, ×400). Arrows show TUNEL-positive cells. (B) Semiquantitative score of TUNEL-positive cells in kidney sections. (C) Caspase 3 activity in kidney tissues. Data are represented as the mean ± SEM; n= 5-8 per group. *P < 0.05 vs CSA group.

**Figure 7: PVPA Ameliorates ER Stress in the Kidney during CSA Treatment**
(A) Representative light photomicrographs of kidney sections showing immunopositive staining for GRP78. (B) Semiquantitative score of GRP78 immunostaining in kidney sections. (C) Representative light photomicrographs of kidney sections showing immunopositive staining for CHOP. (D) Semiquantitative score of CHOP immunostaining in kidney sections. Data are represented as the mean ± SEM; n= 5-8 per group. *P < 0.05 vs CSA group. (Original magnification of slides, ×400)

**Figure 8: PVPA Reduces CSA-Induced Apoptosis In Vitro**
(A) NRK-52E cells were incubated with various concentrations of PVPA for 24 h. PVPA did not affect the proliferation or viability of the cells as assessed by MTT assay. (B) Representative light photomicrographs of NRK-52E cells stained by the TUNEL method after 24 hours of treatment with vehicle (control) or 5 µM CSA ± pretreatment with 10 µM PVPA (Original magnification of slides, ×400). (C) Semiquantitative score of TUNEL-positive cells. *$P < 0.05$ vs CSA (cells not pretreated with PVPA).
Figure 1

Systolic Blood Pressure (mmHg)

- Control
- CSA
- PVPA

Day

-7 0 7 14 21 28
Figure 2
Figure 3
Cortical TBARS ($\mu$M/mg of protein)

- Control
- CSA
- PVPA

Medullary TBARS ($\mu$M/mg of protein)

- Control
- CSA
- PVPA

Figure 4
Figure 5
Figure 6

A: Control, CSA, PVPA

B: TUNEL-Positive Nuclei per HPF

C: Medullary Caspase 3 activity (RFU/mg Protein)
Figure 7
Figure 8