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# Cyclosporin A Disrupts Bradykinin Signaling Through Superoxide

Michael Vetter, Zi-Jiang Chen, Geen-Dong Chang, Danian Che, Shiguo Liu, Chung-Ho Chang

**Abstract**—Cyclosporin A (CsA) is used to reduce transplant rejection rates. Chronic use, however, has a destructive toxic effect on the kidney, resulting in hypertension. In this study, we investigated the effects of CsA treatment on the bradykinin/soluble guanylate cyclase signaling cascade and the involvement of superoxide in LLC-PK1 porcine kidney proximal tubule cells. Treatment with 1  $\mu\text{mol/L}$  CsA for 24 hours increased basal cGMP levels by 41%, whereas CsA inhibited bradykinin-stimulated cGMP production by 26%. Western blotting showed increased expression of eNOS, but no other protein in the bradykinin/soluble guanylate cyclase (sGC) pathway was affected. Using lucigenin-dependent chemiluminescence, we found that CsA treatment significantly increased superoxide production. Production of  $\text{O}_2^-$  was not significantly reduced by 10  $\mu\text{mol/L}$  oxypurinol or 30  $\mu\text{mol/L}$  ketoconazole. However, it was inhibited by the NADPH oxidase inhibitor diphenyleneiodonium chloride (10  $\mu\text{mol/L}$ ) as well as the  $\text{O}_2^-$  scavenger superoxide dismutase (SOD) (100 U). On treatment with 50  $\mu\text{mol/L}$  quercetin, 10 mmol/L *N*-acetyl-cysteine, both antioxidants, as well as the  $\text{O}_2^-$  scavenger Tiron (10 mmol/L), concomitant with 1  $\mu\text{mol/L}$  CsA for 24 hours the activation of cGMP production, was restored in combination with a reduction in  $\text{O}_2^-$ . Incubation with 100  $\mu\text{mol/L}$  menadione, a reactive oxygen generator, and 10 nmol/L bradykinin showed similar effects on the level of cGMP as with CsA. CsA treatment was found to increase nitrotyrosine levels. These findings suggest that CsA activates a NADPH oxidase that releases  $\text{O}_2^-$  and disrupts the bradykinin/soluble guanylate cyclase pathway, probably by binding with NO to form peroxynitrite ( $\text{ONOO}^-$ ). (*Hypertension*. 2003;41:1136-1142.)

**Key Words:** cyclosporin ■ bradykinin ■ nitric oxide ■ cyclic GMP ■ antioxidants

Cyclosporin (CsA) is an important immunosuppressant used in improving the chances of whole organ transplant and graft survival.<sup>1,2</sup> However, cyclosporin treatment has been linked to several significant nephrotoxic side effects. The side effects range from afferent arteriolar constriction<sup>3</sup> and a reduction in glomerular filtration rate<sup>4</sup> to interstitial fibrosis<sup>5</sup> and ultimately hypertension. Although the toxic effects are well established, the exact mechanisms that lead to the pathology and hypertension are not agreed on. The proposed mechanisms for the development of hypertension focus on induction of vasoconstrictive pathways as well as obstruction of vasodilative pathways examined in patients, rat models, and endothelial cells. The pathways examined as possibly affected by CsA include the renal sensory nerve endings,<sup>6</sup> the renin-angiotensin system,<sup>7</sup> endothelin-1,<sup>8,9</sup> thromboxane,<sup>9,10</sup> and the renal kallikrein-kinin system.<sup>11,12</sup>

Bradykinin is an important vasodilating peptide involved in the renal kallikrein-kinin system. The bradykinin peptide exerts its effects by binding to its receptor, activating a heterotrimeric G protein complex, phospholipase C, and then nitric oxide synthase (eNOS), which generates nitric oxide

(NO). NO then binds to the heme group of soluble guanylate cyclase (sGC), thereby activating the enzyme to produce cGMP.<sup>13,14</sup> The NO synthase family and the NO radical have been shown to play an important role in many biological processes including maintaining cardiovascular tone<sup>15</sup> and ion balance.<sup>16</sup> Several studies have attempted to link NO<sup>17,18</sup> and the bradykinin pathway<sup>11,12</sup> to CsA-induced hypertension, but no clear answer regarding the involvement of either has been reached.

Besides NO, another free radical, the superoxide anion ( $\text{O}_2^-$ ), also has been shown to play an important role in renal and vascular physiology.<sup>19</sup> It is well accepted that treatment with CsA causes an increase in production of  $\text{O}_2^-$  and that the anion is related to some of the secondary pathological effects of CsA. However, questions still remain as to how the oxygen radical is formed and the physiological result of its production.<sup>20</sup> To further investigate the side effects of cyclosporin, we set out to determine a correlation between the nephrotoxic effect of CsA, the bradykinin/sGC pathway, and the possible involvement of NADPH oxidase produced  $\text{O}_2^-$  in LLC-PK1 porcine proximal tubular cells. Our results demonstrate that

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From the Department of Medicine, Division of Hypertension, Case Western Reserve University (M.V., Z.-J.C., D.C., S.L., C.-H.C.), Cleveland, Ohio; the Department of Medicine, Reproductive Research Center, Shandong Provincial Hospital, Shandong University (Z.-J.C.), Jinan, Peoples Republic of China; and the Graduate Institute of Biological Sciences, National Taiwan University (G.-D.C.), Taipei, Taiwan.

Correspondence to Dr Chung-Ho Chang, Department of Medicine, Division of Hypertension, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Room W165, Cleveland, OH 44106. E-mail cxc13@po.cwru.edu

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CsA activates a membrane NADPH oxidase that results in the formation of superoxide and decreases bradykinin/sGC signaling.

## Methods

### cGMP Determination

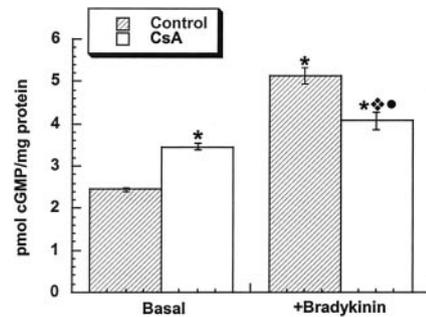
LLC-PK1 cells were grown to confluence in 6-well (35-mm) plates with RPMI medium (pH 7.3) containing 10% horse serum, 5% fetal bovine serum, and 1% Pen/Strep. The cells were washed with 2 mL of serum-free RPMI medium and then incubated with 1  $\mu$ mol/L cyclosporin and/or 50  $\mu$ mol/L quercetin, 10 mmol/L Tiron, and 10 mmol/L NAC for 24 hours at 37°C. The medium was then removed and the cells were incubated with 900  $\mu$ L of RPMI containing 0.5 mmol/L isobutylmethylxanthine for 10 minutes at 37°C; 10 nmol/L bradykinin and/or 100  $\mu$ mol/L menadione was then added to the cells and incubated for 10 minutes. After incubation, the medium was aspirated and 1 mL of cold 10% trichloroacetic acid was added to each well. The cell extracts were scraped, then centrifuged for 15 minutes at 2000g. The supernatants were then extracted with water-saturated ether to remove the trichloroacetic acid. The cGMP levels in the supernatants were then determined by radioimmunoassay as previously described.<sup>21,22</sup>

### Western Blotting

LLC-PK1 cells were grown to confluence in 25-cm<sup>2</sup> flasks in the previously described RPMI media. They were then washed with serum-free RPMI media and treated for 24 hours with 1  $\mu$ mol/L CsA and/or 50  $\mu$ mol/L quercetin, 10 mmol/L Tiron, 10  $\mu$ mol/L DPI, and 10 mmol/L NAC at 37°C. To prepare whole-cell lysates, the cells were lysed by 1% Triton X-100 in 50 mmol/L Tris-HCL buffer (pH 7.6) containing 5 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride, aprotinin (10  $\mu$ g/mL), and leupeptin (10  $\mu$ g/mL). To prepare membrane and cytosolic fractions, the cells were lysed with 25 mmol/L Tris-HCL (pH 7.6), 250 mmol/L sucrose, containing 5 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride, aprotinin (10  $\mu$ g/mL), and leupeptin (10  $\mu$ g/mL). The lysates (20  $\mu$ g) were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 3% bovine serum albumin in 50 mmol/L Tris (pH 8.5), 0.1% sodium azide, and 150 mmol/L NaCl. The PVDF membrane was then incubated with bradykinin-B<sub>2</sub> receptor (Transduction Laboratory, 1:1000), Gq $\alpha$  (Calbiochem, 1:4000), G $\beta$ <sub>1</sub> (Calbiochem, 1:1000), G $\gamma$ <sub>2</sub> (Calbiochem, 1:1000), phospholipase C- $\beta$ <sub>1</sub> (Transduction Laboratory, 1:1000), 26- $\gamma$ <sub>1</sub> (Upstate Biotech, 1:1000), eNOS, iNOS, nNOS (Transduction Laboratory, 1:2500), sGC (raised against amino acids 581 to 595 of the 70-kDa subunit of bovine sGC<sup>22</sup>), p47phox, p67phox (Transduction Laboratory, 1:500), Rac1 (Santa Cruz, 1:1000), or nitrotyrosine (Upstate Biotech, 1:1000) at 4°C for 4 hours. After primary antibody incubation, the PVDF membrane was washed 3 times with saline (50 mmol/L Tris buffer (pH 8.5) containing 150 mmol/L NaCl) plus 0.1% Tween 20. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2500) dilution for 1 hour at room temperature. The immunoreactive proteins were visualized by enhanced chemiluminescence.

### NADPH/Superoxide Assay

LLC-PK1 cells were grown to confluence in 6-well plates and treated with 1  $\mu$ mol/L cyclosporin and/or 10  $\mu$ mol/L DPI, 100 U SOD, 100  $\mu$ mol/L menadione, 30  $\mu$ mol/L ketoconazole, 10  $\mu$ mol/L oxypurinol, 10 mmol/L Tiron, 10 mmol/L NAC, or 50  $\mu$ mol/L quercetin for 24 hours at 37°C. The cells were then washed twice with ice-cold PBS. The cells were then scraped and suspended in 500  $\mu$ L of homogenization buffer containing 20 mmol/L K<sub>2</sub>HPO<sub>4</sub>, 1 mmol/L EGTA, aprotinin (10  $\mu$ g/mL), leupeptin (10  $\mu$ g/mL), and 1 mmol/L phenylmethylsulfonyl fluoride. The suspension was homogenized with 50 strokes in a Dounce homogenizer on ice. The activity of NADPH oxidase was then determined by lucigenin-dependent chemiluminescence (LDC), as previously described.<sup>23,24</sup> Briefly, 900  $\mu$ L of an assay buffer containing 50 mmol/L NaHPO<sub>4</sub>, 1 mmol/L



**Figure 1.** Effect of CsA treatment on bradykinin-stimulated cGMP production in LLC-PK1 cells as determined by radioimmunoassay; 24-hour treatment with 1  $\mu$ mol/L CsA alone increased cGMP. Addition of bradykinin (10 nmol/L) to untreated cells increased basal cGMP. CsA pretreatment decreased bradykinin-stimulated cGMP production. Experiment was performed in triplicate with similar results. Error bars represent deviation from the mean of 4 replicates. \* $P \leq 0.001$  vs basal control,  $P \leq 0.002$  vs basal CsA, ● $P \leq 0.0005$  vs bradykinin control.

EGTA, 150 mmol/L sucrose, 5  $\mu$ mol/L lucigenin (Sigma), and 100  $\mu$ mol/L NADPH was added to 100  $\mu$ L of the cell homogenate. Chemiluminescent photoemission was determined in relative light units (RLU) with the use of a Lumat LB 9501 Luminometer.

### Statistics

Densitometry of all Western blots was performed with UN-SCAN-IT software from Silk Scientific. All densitometry data were then normalized with  $\beta$ -actin staining. All statistical analysis was done with the Student *t* test. A probability value of  $<0.05$  was assumed to be significant.

## Results

### Bradykinin/sGC Signaling Pathway Is Impaired by Cyclosporin Treatment

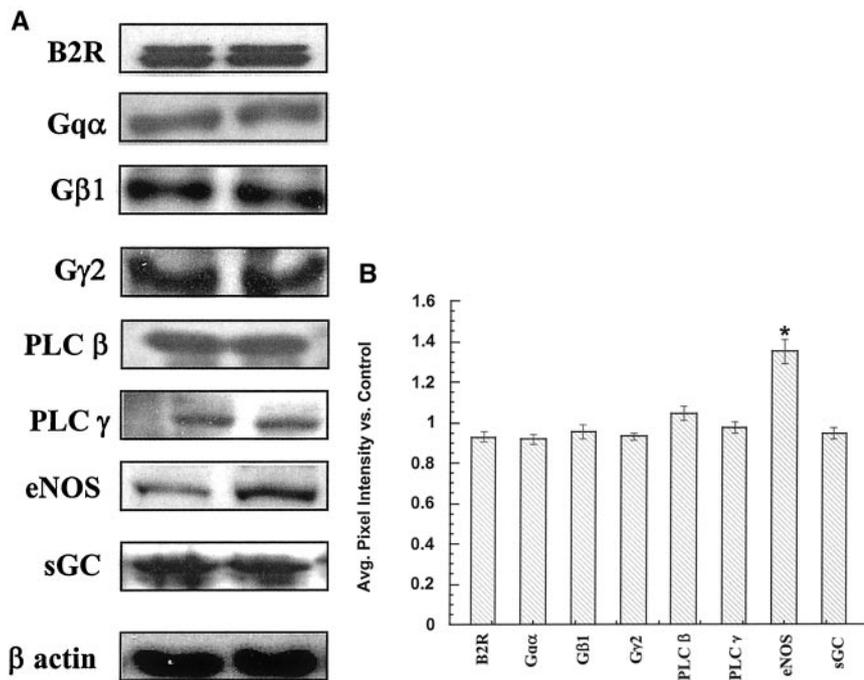
To determine the effects of CsA treatment on the bradykinin/sGC pathway, we measured the production of bradykinin-stimulated cGMP in LLC-PK1 cells. Addition of 10 nmol/L bradykinin for 10 minutes to the cells increased the cGMP  $\approx 2$ -fold (Figure 1). Incubation with 1  $\mu$ mol/L CsA for 24 hours increased the cGMP production by 1.5-fold. However, 24-hour pretreatment of the cells with 1  $\mu$ mol/L CsA significantly reduced the activation of sGC by bradykinin.

### Impairment of Bradykinin/sGC Signaling Is Not Due to Change in Protein Expression

We next investigated the possibility of CsA-stimulated expression differences in the bradykinin pathway component proteins. Western blotting showed that the expression of bradykinin-B<sub>2</sub> receptor, Gq $\alpha$ , G $\beta$ <sub>1</sub>, G $\gamma$ <sub>2</sub>, phospholipase C ( $\beta$  and  $\gamma$ ), and sGC was not affected by 24-hour treatment with 1  $\mu$ mol/L CsA (Figure 2A). In contrast, eNOS expression was significantly increased (Figure 2B). These results indicate that CsA does not inhibit the sGC signaling by decreasing protein expression.

### Cyclosporin Treatment Activates NADPH Oxidase to Release Superoxide

CsA has been shown to produce O<sub>2</sub><sup>-</sup> in several in vivo and in vitro studies. Therefore, we examined the possible role of NADPH oxidase and the production of O<sub>2</sub><sup>-</sup> in blocking the



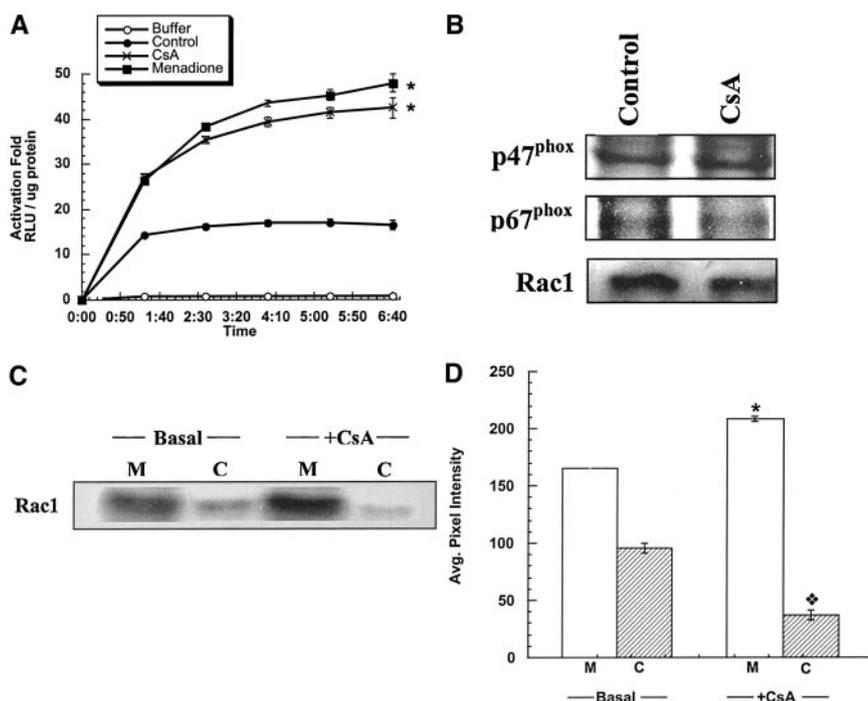
**Figure 2.** Effect of CsA treatment on bradykinin signaling cascade proteins. A, Western blot analysis of bradykinin- $B_2$  receptor,  $Gq\alpha$ ,  $G\beta_1$ ,  $G\gamma_2$ , phospholipase  $C\gamma$  and  $\beta$ , eNOS, and sGC on control and 1  $\mu\text{mol/L}$  CsA-treated LLC-PK1 cells. Lysates were subjected to SDS gel electrophoresis and Western blotting with respective antibodies. No significant expression difference was seen except for an increase in eNOS in CsA-treated cells. B, Densitometry analysis is given in the bar graph as average pixel intensity relative to respective control bands after normalization to  $\beta$ -actin. Error bars represent standard deviation from the mean of 3 experiments. \* $P \leq 0.01$ .

bradykinin pathway. We found that CsA treatment significantly increased the production of  $O_2^-$ . Protein from experimental samples was added to assay buffer without the NADPH nucleotide, labeled "buffer," to show that the  $O_2^-$  release was from an oxidase dependent on the NADPH nucleotide. Menadione, an exogenous source of  $O_2^-$ , was added to demonstrate the effectiveness of the assay to detect  $O_2^-$  (Figure 3A). Western blotting was performed to verify the expression of the component proteins of the NADPH oxidase system, p47phox, p67phox, and Rac1 in LLC-PK1 cells (Figure 3B). No significant difference was found in their

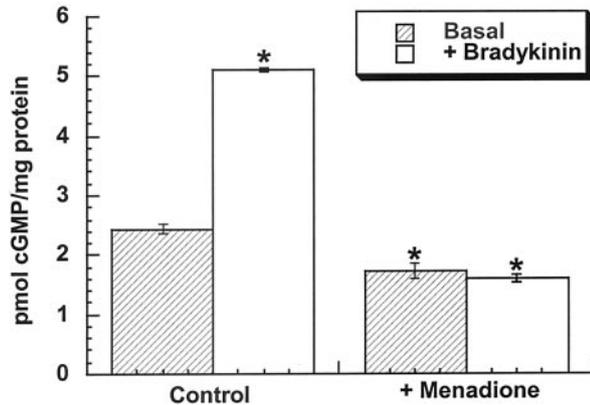
expression levels (data not shown). To further confirm activation of the NADPH oxidase by CsA, Western blotting on membrane and cytosolic fractions was performed (Figure 3C). We observed that treatment with CsA resulted in translocation of the Rac1 protein from the cytosol to the membrane (Figure 3D).

### Menadione Treatment Mimics the Effects of Cyclosporin on cGMP

To determine the correlation between  $O_2^-$  and the reduction of cGMP production, we investigated the effects of addition



**Figure 3.** Activation of NADPH oxidase by CsA treatment. A, Treatment of LLC-PK1 cells with CsA increases the amount of NADPH-dependent  $O_2^-$  released as determined by lucigenin-dependent chemiluminescence. Menadione (100  $\mu\text{mol/L}$ ), an exogenous superoxide producer, confirms superoxide detection within the system. Data shown are representative of experiments performed in triplicate. Error bar represents deviation from mean time point replicates. \* $P \leq 0.0001$  vs control. B, Western blotting of NADPH oxidase component proteins p47phox, p67phox, and Rac1. C, Western blotting of membrane (M) and cytosolic (C) fractions shows CsA-induced translocation of Rac1 component protein toward membrane. D, Densitometry analysis of Rac1 translocation. Data are expressed as average pixel intensity normalized to  $\beta$ -actin. Error bars represent standard deviation from the mean of 3 experiments. \* $P \leq 0.0002$  vs basal membrane,  $P \leq 0.0001$  vs basal cytosol.

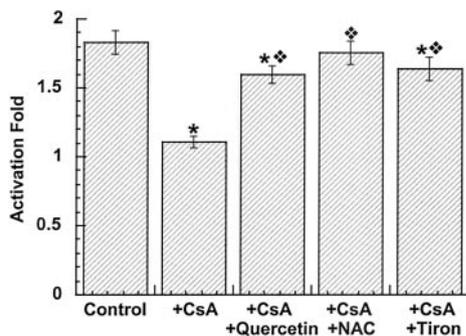


**Figure 4.** Menadione blocks bradykinin/sGC signaling in LLC-PK1 cells. Bradykinin (10 nmol/L) increased the amount of cGMP in control cells. Addition of 100  $\mu$ mol/L menadione decreased both basal and bradykinin-stimulated cGMP production. The experiment was performed in triplicate with similar results. Error bars represent deviation from the mean of 4 replicates. \* $P \leq 0.001$  vs basal control.

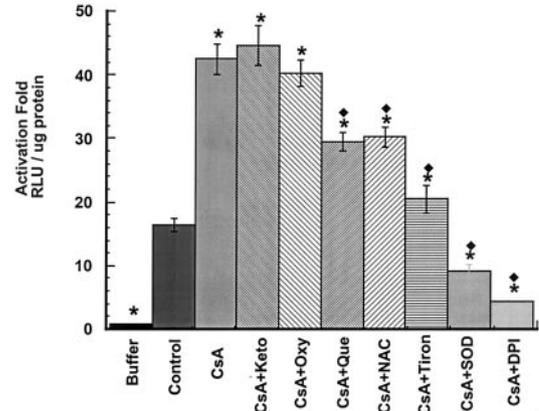
of exogenous  $O_2^-$ ; 10 nmol/L of bradykinin alone increased cGMP by 2-fold. Incubation of the cells with 100  $\mu$ mol/L menadione decreased basal cGMP production by  $\approx 29\%$ . Menadione also decreased the bradykinin-stimulated cGMP production to a level not significantly different from the basal menadione-treated cells (Figure 4). This demonstrates the ability of superoxide to disrupt the bradykinin signaling pathway.

### Cyclosporin Effect on Bradykinin-Stimulated cGMP Production Is Reversible With Antioxidants, Quercetin and NAC, and the Superoxide Scavenger Tiron

To further confirm the involvement of  $O_2^-$  in the disruption of the pathway, we examined the effects of antioxidants and superoxide scavengers. Figure 5 shows that treatment of LLC-PK1 cells with 10 nmol/L bradykinin gives a 1.8-fold increase in activation of sGC as compared with untreated



**Figure 5.** Dual treatment of LLC-PK1 cells with CsA and quercetin, NAC, and Tiron restores some of the bradykinin-induced cGMP lost in CsA treatment. Control cells treated with bradykinin (10 nmol/L) alone showed almost 2-fold sGC activation over nontreated cells. CsA treatment blocked activation. Treatment with quercetin (50  $\mu$ mol/L), NAC (10 mmol/L), or Tiron (10 mmol/L) restored nearly half the activation that was lost. Experiment was performed in triplicate with similar results. Error bars represent deviation from the mean of 4 replicates. \* $P \leq 0.03$  vs control,  $P \leq 0.0001$  vs CsA.



**Figure 6.** Superoxide production can be reduced with antioxidants and  $O_2^-$  scavengers. SOD, NAC, quercetin, Tiron, and DPI inhibit  $O_2^-$ ; oxypurinol and ketoconazole do not. Treatment with SOD (100 U) quercetin (50  $\mu$ mol/L), NAC (10 mmol/L), or Tiron (10 mmol/L), along with CsA (1  $\mu$ mol/L), blocks production of  $O_2^-$ . DPI (10  $\mu$ mol/L), an NADPH oxidase inhibitor, also blocks the production of  $O_2^-$ . CYP450 inhibitor ketoconazole (30  $\mu$ mol/L) and xanthine oxidase inhibitor oxypurinol (10  $\mu$ mol/L) did not show significant reduction of  $O_2^-$ . Data shown are representative of experiments performed in triplicate. Error bar represents deviation from mean of time point replicates. \* $P \leq 0.05$  vs control,  $\diamond P \leq 0.002$  vs CsA.

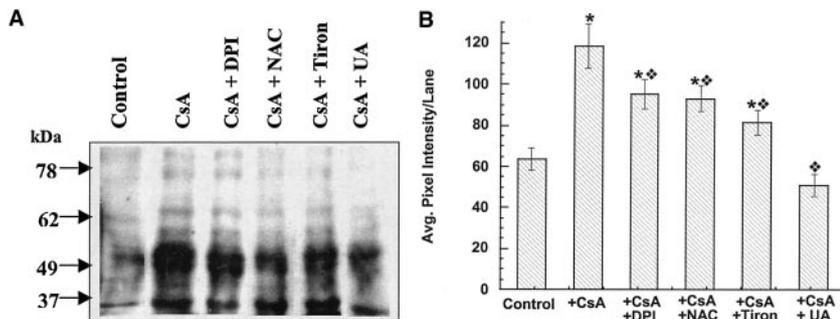
cells (Figure 5). Preincubation with 1  $\mu$ mol/L CsA decreased the bradykinin-stimulated activity of cGMP production to only a 1.1-fold increase in activation. Coincubation of the cells with 1  $\mu$ mol/L CsA and 50  $\mu$ mol/L quercetin, 10 mmol/L NAC, or 10 mmol/L Tiron returned the bradykinin-stimulated sGC activation to 1.6-, 1.75-, and 1.65-fold that of untreated cells, respectively.

### Superoxide Scavengers, Antioxidants, and Oxidase Inhibitors Reduce CsA-Induced Superoxide Release

Because the cGMP production was restored by treatment with quercetin, NAC, and Tiron, we investigated whether the same chemicals would effect the detection of superoxide (Figure 6). We observed that concomitant treatment with 1  $\mu$ mol/L CsA and 50  $\mu$ mol/L quercetin, 10 mmol/L NAC, or 10 mmol/L Tiron decreased the amount of  $O_2^-$ . The significant reduction in luminescence on treatment with SOD further confirmed that the luminescence observed is due to the detection of  $O_2^-$ . Basal treatment with the chemicals alone showed a decrease or no significant difference in superoxide production (data not shown). To determine the exact oxidase involved, we utilized several oxidase inhibitors. Neither ketoconazole (30  $\mu$ mol/L), an inhibitor of the cytochrome P450 system, nor oxypurinol (10  $\mu$ mol/L), a xanthine oxidase inhibitor, was able to significantly reduce the release of  $O_2^-$ . We then examined the effect of the NADPH oxidase inhibitor DPI. We found that 10 minutes of treatment with 10  $\mu$ mol/L DPI significantly decreased the  $O_2^-$  production by CsA.

### CsA Treatment Increases Nitrotyrosine Residues

Because superoxide and nitric oxide are known to bind to form peroxynitrite, which in turn nitrosylates tyrosine residues, we examined the levels of nitrotyrosine in control and



**Figure 7.** CsA treatment increases peroxy-nitrite formation. A, Western blotting with nitrotyrosine antibodies shows increase in level of nitrosylated tyrosine residues after CsA treatment in LLC-PK1 cells. Effect was reversed by NAC (10 mmol/L), Tiron (10 mmol/L), DPI (10  $\mu$ mol/L), and the peroxy-nitrite scavenger uric acid (1 mmol/L). B, Densitometry analysis of each lane shows difference in nitrotyrosine staining. Data are expressed as average pixel intensity normalized to  $\beta$ -actin. Error bars represent standard deviation from the mean of 3 experiments. \* $P \leq 0.001$  vs control,  $P \leq 0.01$  vs CsA.

CsA-treated cells. Western blotting showed increased levels of nitrotyrosine after CsA treatment (Figure 7A). Densitometry analysis showed that these levels were reduced by the antioxidants quercetin and NAC, the superoxide scavenger Tiron, the NADPH oxidase inhibitor DPI, and the peroxy-nitrite scavenger uric acid (Figure 7B).

### Discussion

CsA has long been used as an immunosuppressant after organ graft to reduce the chances of graft rejection. However, the clinical use of CsA has consistently led to severe side effects, most significantly nephrotoxicity and hypertension. Though the pathology of CsA treatment is evident, the mechanisms that lead to its development are still unclear and disputed. In the current study, we investigated the role of NADPH oxidase in the adverse effects of CsA treatment on porcine kidney proximal tubule cells. We discovered that in these cells, chronic CsA treatment disrupts the bradykinin/sGC pathway through a NADPH dependent oxidase production of superoxide.

Bradykinin, and the kallikrein-kinin system in general, is an important peptide signaling system involved in maintaining vascular tone.<sup>25</sup> Bradykinin exerts its effects by activating eNOS to produce NO, which binds and activates sGC.<sup>13,14</sup> Though the involvement of the bradykinin system in CsA-induced nephrotoxicity has been discussed in the literature, the conclusions to be drawn are unclear. Wang et al<sup>11</sup> suggest that there is an increase in expression of bradykinin ( $B_2$ ) receptor mRNA in renal tissue to compensate for CsA-induced hypertension, whereas Bompert et al<sup>12</sup> concluded that CsA reduces the expression of bradykinin ( $B_2$ ) receptor mRNA in the renal cortex, leading to or aggravating the renal effects of CsA treatment. In our current study, in the proximal tubule, at a protein level, we found no expression difference in the bradykinin- $B_2$  receptor. These discrepancies could simply be due to the different tissues and cells used in the various studies. Our data, however, do show that chronic treatment with CsA significantly reduces the amount of bradykinin-stimulated cGMP, signifying a disruption of the signaling cascade downstream of the receptor within the proximal tubule.

The bradykinin-signaling pathway from the receptor to sGC involves several proteins. It is possible that the diminished bradykinin signaling after CsA treatment may be due to altered expression of one of these component proteins. However, Western blotting showed that of the component proteins, only eNOS expression was altered. Our data and

other studies<sup>26</sup> demonstrated that CsA can increase eNOS expression. We found no expression difference of iNOS or nNOS (data not shown). Consistent with the eNOS data, we also observed an increase in cGMP with CsA treatment, which would lead to the conclusion that there is an increase in NO production. Other groups have also shown this increase in NO. In bovine aortic endothelial cells, using cell permeable fluorescent dyes, Navarro-Antolin et al<sup>27</sup> were able to detect NO production and showed that CsA increases intracellular NO. However, Lima et al<sup>18</sup> detected a decreased nitrite level from the media of cultured LLC-PK1 cells after 72 hours of CsA treatment. This study, as well as others, uses an indirect extracellular method to determine the production level of NO that may have been altered or quenched by other biochemical or physiological processes. In contrast, our study used the activation of the NO target enzyme sGC as a determination of NO production. Therefore, CsA may increase the expression of eNOS and thereby the release of NO in basal conditions, but the free radical messenger is somehow quenched before reaching sGC in a bradykinin-stimulated situation.

Superoxide ( $O_2^-$ ) has been described to be a prominent component of many signaling pathways as well as a disease risk factor.<sup>28,29</sup> Using a lucigenin-based chemiluminescent assay, we were able to show that CsA treatment increased  $O_2^-$  production. We were able to further confirm that the increase in luminescence is caused by  $O_2^-$  by showing that treatment with SOD and the oxygen radical scavenger Tiron decreases the amount of luminescence. Our data also show that the release of  $O_2^-$  is dependent on the presence of the NADPH nucleotide, furthering the conclusion that the  $O_2^-$  is produced by a NADPH oxidase. NADPH oxidase systems are membrane oxidases that have been well described in immune and vascular cells.<sup>29</sup> Recently, the component proteins for a NADPH oxidase system, which include p47phox, p67phox, and Rac1, have been described in the kidney.<sup>30,31</sup> We found that the p47, p67, and Rac1 subunits are expressed in LLC-PK1 cells. Besides showing the expression of the subunits, we were able to demonstrate that CsA treatment triggered translocation of the Rac1 protein from the cytosol to the membrane, signifying activation of the oxidase. To further confirm the involvement of the NADPH oxidase system in the CsA-induced production of  $O_2^-$ , we used the NADPH oxidase inhibitor DPI. We found that DPI significantly blocked the release of  $O_2^-$ .

To further isolate NADPH oxidase as the system producing the  $O_2^-$ , we examined other  $O_2^-$ -producing pathways. Xanthine oxidase is another cellular system that is known to

produce O<sub>2</sub><sup>-</sup>. By using the xanthine oxidase inhibitor oxy-purinol, we were able to show that xanthine oxidase does not account for the production of O<sub>2</sub><sup>-</sup> in our system. It has also been proposed by several groups that the release of O<sub>2</sub><sup>-</sup> is brought on by the cytochrome P450 (CYP) metabolism of CsA.<sup>32</sup> By using an inhibitor of the CYP pathway, ketoconazole, we demonstrated that the observed CsA-induced O<sub>2</sub><sup>-</sup> production is not caused by CYP metabolism of CsA. This conclusion is supported by several factors, primarily that CsA is metabolized by the CYP3A4 isoform of CYP; however, it has been shown that within the kidney, CYP3A5 rather than CYP3A4 is the major CYP isoform.<sup>33</sup> In fact, in CsA-treated renal microsomes, the ability to metabolize CsA was 30 times lower than that in similarly treated hepatic microsomes.<sup>34</sup> Also, in electron spin resonance studies performed on rats treated with CsA, it was discovered that the oxygen radicals found in the urine were not resulting from metabolites of CsA, though those found in the bile did resemble CsA metabolites.<sup>35,36</sup>

The decrease in NO-stimulated sGC activity resulting from CsA treatment can then be explained by its interaction with the NADPH oxidase produced O<sub>2</sub><sup>-</sup>. NO, *in vivo*, is known to quickly bind to O<sub>2</sub><sup>-</sup> to form the strong oxidant peroxynitrite (ONOO<sup>-</sup>). NO has such a high affinity for the superoxide anion that it has been shown to compete with SOD for binding of O<sub>2</sub><sup>-</sup>.<sup>15</sup> It has even recently been shown that CsA treatment can induce the formation of peroxynitrite, dependent on the concentration of superoxide anion.<sup>37</sup> Peroxynitrite has been shown to interact with proteins and produce nitrotyrosine residues.<sup>15</sup> Through the use of a nitrotyrosine antibody, we are able to clearly show that CsA treatment significantly increases the amount of nitrotyrosine (see Figure 7). Our data also show that the formation of nitrotyrosine is inhibited by the addition of the NADPH oxidase inhibitor DPI, the antioxidant NAC, as well as the oxygen radical scavenger Tiron and the peroxynitrite scavenger uric acid. Therefore, it is likely that the release of O<sub>2</sub><sup>-</sup> by CsA treatment quenches NO through peroxynitrite formation, thereby disrupting the bradykinin/sGC signaling.

Examining the effects of altering the balance of the 2 radicals can further this conclusion. Several studies have shown that addition of L-arginine (L-arg) to CsA-treated systems can block some of the nephrotoxic effects. Yang et al<sup>38</sup> showed in whole rats that cotreatment with CsA and L-arg reduced the amount of fibrosis and tubular injury while increasing the GFR and NO level. Addition of exogenous L-arg provides abundant substrate for eNOS. Since O<sub>2</sub><sup>-</sup> is the limiting reactant in the formation of peroxynitrite, extra L-arg allows the system to produce more NO than O<sub>2</sub><sup>-</sup> and allows the signaling cascade to continue. It is also possible to reverse the peroxynitrite formation by reducing the O<sub>2</sub><sup>-</sup>. Quercetin is known to be a strong antioxidant that can scavenge free radicals *in vivo*, and it has recently been reported that quercetin can decrease oxidative stress caused by CsA treatment.<sup>39</sup> In our study, we found that concomitant treatment of the cells with quercetin or NAC and CsA restored some of the bradykinin signaling as well as decreasing the release of CsA-induced O<sub>2</sub><sup>-</sup> and subsequently peroxynitrite. We can then conclude that the addition of quercetin and NAC

quenches some CsA-induced superoxide, allowing the NO to bind to sGC to form cGMP. We have also shown that the addition of exogenous O<sub>2</sub><sup>-</sup> can mimic the blocking effects of CsA treatment. Cellular reduction of menadione causes the release of significant amounts of O<sub>2</sub><sup>-</sup>. We showed that addition of menadione to basal and bradykinin-challenged cells blocks cGMP production. Since O<sub>2</sub><sup>-</sup> has been shown to be the limiting factor in the formation of peroxynitrite, saturating the system with O<sub>2</sub><sup>-</sup> blocks almost all NO signaling to the sGC enzyme. The ability of menadione to block cGMP production and antioxidants to restore it emphasizes the importance of O<sub>2</sub><sup>-</sup> in the CsA disruption of the bradykinin/sGC pathway.

The pathology of CsA has mostly been examined in the vasculature and smooth muscle. However, in our study and others, CsA treatment has also been shown to have direct effects on the tubules of the kidney. Other studies with LLC-PK1 cells have shown that CsA causes a loss of cell-cell adhesion<sup>40</sup> as well as inducing growth arrest and cell death.<sup>41</sup> In the conditions of the current study, no significant loss of viability was observed. Our study proposes that within the proximal tubule, some the pathology resulting from CsA treatment might be due to a blocking of signaling by superoxide production. The bradykinin-sGC pathway has several important physiological functions in the maintenance of blood pressure. Though its most significant role is in regulating vascular tone, it also has been shown to have direct effects on ion balance within the kidney. Several studies have shown that a disruption of bradykinin signaling inhibits the kidney's ability to excrete excess sodium.<sup>42</sup> More specifically, NO is an important aspect of the bradykinin pathway. The disruption of the bradykinin signaling and the evidence of peroxynitrite formation suggest that NO has been quenched. A reduction of NO within the proximal tubule has been shown to disrupt the activity of Na<sup>+</sup>/ATPase and Na<sup>+</sup>/H<sup>+</sup> exchangers and ultimately affect the permeability of the proximal tubule membrane.<sup>17</sup> Similar disruptions of ion balance mechanisms have been observed by peroxynitrite formation.<sup>43</sup> Through the use of LLC-PK1 proximal tubule cells, we were able to show that CsA treatment increases the production of O<sub>2</sub><sup>-</sup>, which in turn blocks bradykinin signaling and quenches NO through the production of peroxynitrite. Though further study is needed to determine the direct importance of these components in blood pressure and ion maintenance within the proximal tubule, the ability of CsA to disrupt their physiological function suggests several pathways from which CsA pathology might arise.

### Perspectives

Since its physiological functions were described, CsA has been widely used clinically. Despite its ability to improve survival rates and reduce rejections after organ transplants, it has been shown to have a wide range of pathological side effects. The current study shows that chronic treatment with CsA blocks bradykinin signaling in the proximal tubule through NADPH oxidase produced superoxide and the subsequent formation of peroxynitrite. These signaling pathways and radicals play many important roles in the vasculature as well as kidney tubules. The demonstration of the disruption of

the balances in these signaling mechanisms by CsA describes them as a new candidate to be considered in the many pathways that lead to CsA pathology.

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