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Reciprocal regulation of cyclic GMP content by cyclic GMP-phosphodiesterase and guanylate cyclase in SHR with CsA-induced nephrotoxicity

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1 The effect of the immunosuppressant drug, cyclosporin A (CsA), on the nitric oxide (NO)-cyclic GMP pathway was examined in spontaneous hypertensive rats (SHR).

2 CsA (50 mg kg^{-1}) treatment for 14 days induced typical CsA nephrotoxicity, which was characterized by morphological changes in the glomerulus and proximal tubule as well as an abnormality of creatinine clearance, FENa and BUN.

3 CsA significantly decreased both NOS activity in the kidney and NOx contents in urine, but significantly increased cyclic GMP content in the kidney.

4 A marked change in two kinds of enzyme, which contribute towards the increase in cyclic GMP in tissue, namely, a decrease in cyclic GMP-phosphodiesterase activity and increase in guanylate cyclase activity, was observed in the kidney treated with CsA.

5 In the isolated perfused kidney, a decreased in perfusion pressure induced by SNP in the kidney isolated from CsA group was significantly greater than that of control.

6 There seem to exist a reciprocal mechanism to maintain cyclic GMP content *via* both a decrease in cyclic GMP degradation and an increase in synthesis of cyclic GMP in the kidney treated with CsA. This mechanism is likely to be playing an important role to regulate the homeostasis in the kidney with CsA nephrotoxicity.

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Abbreviations: CsA, cyclosporin A; GC, guanylate cyclase; L-NAME, N-nitro-L-arginine methyl ester NO, nitric oxide; NOS, nitric oxide synthase; PDE, phosphodiesterase

Introduction

Cyclosporin A (CsA), a potent immunosuppressive agent, has become well established as a treatment during solid organ transplantation as well as for immune diseases. However, nephrotoxicity is a serious problem that cannot be ignored during therapy (Myers, 1986). CsA nephrotoxicity is characterized by renal vasoconstriction of the afferent glomerular arteriole followed by a reduced glomerular filtration rate. These renal dysfunctions are mainly due to an imbalance in the release of several vasoactive substances. One is the increased release of vasoconstricting factors such as thromboxane, angiotensin II and endothelin (Meyer-Lehnert *et al*., 1997), and the other is the decrease in vasodilating factors such as prostacyclin and nitric oxide (NO) (Oriji & Keiser, 1998). Investigators have been trying to clarify this mechanism, but as yet have not uncovered a unifying mechanism to account for these diverse complications induced by CsA.

Recent studies have focused on the role of NO in CsA nephrotoxicity, but it is still not well defined. Many studies are reporting that the impaired production of NO is the most possible cause for CsA nephrotoxicity (Diederich *et al*., 1992; Vaziri *et al*., 1998). However, reports demonstrating the preservation of NO synthesis, or even the increase in mRNA levels of NO synthase (NOS) in CsA-treated rat have also been accumulating (Lopez-Ongil *et al*., 1998; Bobadilla *et al*., 1998).

NO has roughly two different aspects in its action, one is favourable for cell viability elicited mainly by constitutive NOS, whereas the other is cytotoxic caused by excessive and sustained amounts of NO produced by inducible NOS (iNOS) (Beckman & Koppenol, 1996). Under physiologically normal conditions, NO proportionally increases cyclic GMP content, but there seems to be a possibility that NOS activity are not always in parallel with cyclic GMP content in some diseases or abnormal conditions. The measurement of cyclic GMP levels in addition to the amount of NO, for example, mRNA levels of NOS or NOx excretion, is ideal when evaluating the functional role of NO in disease conditions. In this sense, there may be a possibility that some studies might fail to assess the role of NO-cyclic GMP correctly. In addition to this, there is little information on the change of the NO-cyclic GMP pathway evaluated inclusively in fully expressed CsA nephrotoxicity.

The aim of the present study was to determine how NO-cyclic GMP contributes to nephrotoxicity induced by CsA. For this purpose, we evaluated not only biochemical

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parameters of the NO-cyclic GMP pathway but also functional change to NO in SHR with nephrotoxicity induced by CsA.

Methods

Animal models

Male SHR weighing 300–350 g were purchased from Charles River Japan. Animals were kept in a climate-controlled, light-regulated space with a 12-h light dark cycle. They were allowed free access to rat chow and water. After 7 days acclimatization, the rats were randomly divided into two groups and treated orally with vehicle or CsA (50 mg kg^{-1}) once a day for 14 days. CsA was dissolved in olive oil.

Twenty-four hour urine samples were obtained 1 day prior to and on day 14 after CsA or vehicle administration using individual metabolic cages. Blood samples were also collected in a tube containing 1000 unit ml^{-1} heparin by subclavian artery puncture under general anaesthesia with ether. They were then immediately centrifuged, and plasma was separated and frozen at −80°C until processed. The right kidney was excised from both groups on day 14 under general anaesthesia with ether, then frozen in liquid nitrogen and kept at −80°C until measurement of parameters. The kidney was homogenized in 1 ml of ice cold 20 mM HEPES buffer containing sucrose 0.32 M, EDTA 0.5 mM, dithiothreitol (DTT) 1 mM and protease inhibitors (leupeptin 3 μM, pepstatin A 1 μM and phenylmethyl sulphonylfluoride 1 mM) at pH 7.2 using a Polytron® homogenizer. The cytosol and particulate fractions were separated by centrifugation at $12{,}500 \times g$ for 60 min, at 4°C.

Plasma and urinary creatinine, blood urea nitrogen (BUN), Na^+ and K^+ levels were measured with an autoanalyser (Hitachi, 80FR). Creatinine clearance and fractional sodium excretion (FENa) were calculated from standard laboratory methods.

Histological studies

Renal histological studies were performed 14 days after CsA or vehicle administration. The left kidney was fixed in phosphate-buffered 10% formalin, processed and then embedded in paraffin before being cut into 5 μm sections using conventional techniques. Sections were stained with periodic acid-Schiff's reagent and haematoxylin-eosin. Whole coloured slides containing at least 100 glomeruli each were analysed. Morphological analyses were blindly performed and analysed using a semiquantitative scale with values from 0 to 4 for each of the following alterations: for tubular vacuolization, 1 = scant, 2 = slight, 3 = moderate, 4 = abundant and diffuse.

Measurement of NOS activity

NOS activity in kidney homogenates was determined as previously described (Lugg *et al*., 1995). The cytosol fraction was passed through Dowex AG50WX-8 (Na^+) resin to remove endogenous arginine, and 50 μl aliquots were incubated for 60 min at 37°C in the presence of L-[^{3}H]-arginine 2 μCi ml^{-1}, NADPH 2 mM, $CaCl_2$ 0.45 mM, and L-arginine 100 μM, calmodulin 10 μg ml^{-1}, with or without 2 mM L-NAME or 5 mM EGTA to determine the levels of Ca^{2+}-dependent and Ca^{2+}-independent NOS activities, respectively. After elimination of the residual L-[^{3}H]-arginine through the resin, [^{3}H]-citrulline was counted by scintillation counter. All values were expressed per mg of soluble protein. Protein concentrations in the cytosol were assayed with the Bio-Rad protein assay reagent® and associates, using bovine serum albumin as a standard. The Ca^{2+}-dependent NOS activity was calculated as the difference between the amount of L-[^{3}H]-citrulline formed in control tubes and the amount formed in the tubes incubated with EGTA. The Ca^{2+}-independent NOS activity was calculated as the difference between the amount of L-[^{3}H]-citrulline formed in tubes incubated with EGTA alone and the amount formed in the tubes incubated with EGTA plus L-NAME.

Measurement of NOx (NO_2 and NO_3) concentration

The concentration of total NO_2 and NO_3 was determined by Griess reaction with sodium nitrite as a standard. For the NOx assay, urine samples were diluted 5–10 fold with distilled water. The reduction of nitrate to nitrite was performed using nitrate reductase. One hundred μl of each sample was incubated for 30 min with 100 μl of nitrate reductase (43 mu ml^{-1}), 100 μl of FAD (35 μM), 100 μl of NADPH (0.28 mM), and 200 μl of potassium phosphate buffer (KH_2PO_4 0.1 M and K_2HPO_4 0.1 M, pH 7.2) at 37°C. After addition of the Greiss reagent (a 1 : 1 mixture of 2% sulphanilamide and 0.2% naphtylenediamine) for 10 min at room temperature, nitrate was measured by spectrophotometry at 540 nm.

Measurement of cyclic GMP content

Cytosol fraction from the kidney was acetylated, and cyclic GMP content was measured using cyclic GMP [^{125}I]-assay system (Amersham International, IL, U.S.A). The sensitivity of this assay was 0.5 fmole. Cyclic GMP content was corrected for protein content.

Measurement of cyclic GMP-PDE activity

Cyclic GMP-PDE activity of the cytosol fractions from the kidney was determined using a modification of the two step radioisotope procedure (Thompson & Appleman, 1971). The reaction mixture (250 μl total volume) contained the cytosol fraction, [^{3}H]-cyclic GMP 0.1 μM, $MgCl_2$ 30 mM, DTT 1 mM in Tris-HCl (pH 8.0) buffer 50 mM, with or without IBMX 50 mM, EDTA 50 mM or $CaCl_2$ 40 mM plus 10 units ml^{-1} calmodulin. The reaction was initiated by addition of radiolabelled substrate and incubated in a water bath at 30°C for 10 min, then stopped by boiling at 100°C for 1 min. Reaction mixtures were incubated with 2 mg ml^{-1} snake venom to hydrolyze GMP to guanine for 10 min at 30°C. After addition of anion exchanger, the reaction mixture was vortex-mixed and centrifuged at $300 \times g$ for 10 min. The resulting supernatant was transferred to Lumaplate®, and the [^{3}H]-5-mononucleotide formed by hydrolysis of cyclic nucleotide determined by Topcounter®. The calcium calmodulin (Ca-CaM)-dependent cyclic GMP-PDE activity was calculated as the difference between the amount of [^{3}H]-5-

mononucleotide formed in tubes with $CaCl_2$ plus calmodulin and the amount formed in the tubes incubated with EDTA. The calcium calmodulin-independent cyclic GMP-PDE activity (cyclic GMP-specific cyclic GMP-PDE activity) was calculated as the difference between the amount of [^{3}H]-5-mononucleotide formed in tubes incubated with EDTA alone and the amount formed in the tubes incubated with EDTA plus IBMX.

Measurement of soluble GC activity and particulate GC activity

The cytosol fraction from the kidney was fractionated further by centrifuging at $100{,}000 \times g$ for 60 min at 4°C. The resulting supernatant was collected and used as the cytosolic fraction (for soluble GC). The pellet was resuspended in membrane-resuspension containing Tris-HCl (pH 7.6) 50 mM and EDTA 1 mM and used as the membrane fraction (for particulate GC). Soluble GC activity was measured by the method of Kimura *et al*. (1975) with slight modifications. Reaction mixture containing Tris-HCl (pH 7.6) 50 mM, IBMX 0.5 mM, creatine phosphate 3.5 mM, 2.5 units tube^{-1} creatine phosphokinase and 50 μl of the cytosolic fraction were preincubated for 10 min at 37°C. The assay was initiated by addition of 4 mM $MgCl_2$ and 1 mM GTP in a final volume of 100 μl, and incubated for 15 min at 37°C. The reaction was terminated by the addition of 0.9 ml of 50 mM sodium acetate buffer (pH 4.0) to prevent non-enzymatic formation of cyclic GMP, and heated at 90°C for 3 min. Cyclic GMP was determined by the radio-immunoassay kit (cyclic GMP[^{125}I]-assay system®, Amersham International, IL, U.S.A.). Particulate GC activity was measured by the same procedure as for the soluble GC except that 1 mM ATP was added to the working solution. The activity was corrected for protein content.

Isolated perfused kidney

Rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}). A polyethylene catheter (PE50) was placed in the left renal artery. Immediately after the insertion of the catheter, 500 u of heparin sodium were injected. The kidney was excised and immediately mounted in the perfusion system. The kidney was perfused at a constant flow rate of 6 ml min^{-1}, using a peristaltic pump. The perfusate was Krebs solution of the following composition in mM: NaCl 118; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 10; $CaCl_2$ 2.5 with 95% O_2-5% CO_2 and maintained at 37°C. The kidney was allowed to equilibrate in the perfusion chamber for 30 min to achieve a constant resting tone before the experiment was started. Vasodilator response to sodium nitroprusside (SNP) was measured as change in perfusion pressure (mmHg) with a pressure transducer (Nihonkohden, Japan) in so-called 'high tone' preparation that was preconstricted by addition to the perfusing Krebs solution of 10 μM L-phenylephrine (PhE). The dilatation to SNP was expressed as a per cent of the constriction by PhE.

Drugs

CsA was produced by Fujisawa Pharmaceutical Co., Ltd., Japan. L-NAME, EGTA, EDTA, DTT, leupeptin, pepstatin A, phenylmethyl sulphonylfluoride, L-arginine, NADPH, calmoduline, ATP, GTP, snake venom, IBMX, creatine phosphate, creatine phosphokinase, FAD, PhE, SNP, nitrate reductase and sodium acetate were purchased from Sigma Chemical Co., Ltd. [^{3}H]-cyclic GMP, L-[^{3}H]-arginine were purchased from NEN Life Science Products, Inc. All other chemicals were of the purest commercially available grade.

Statistical analysis

Data are presented as means ± s.e.mean. Unless stated otherwise, all the experimental groups had eight animals. Difference between groups was analysed using unpaired Student's *t*-test.

Results

Physiological studies

Body weight and parameters of renal functions are summarized in Table 1. Body weight in the CsA group was significantly lower than that of the control group. The CsA group lost 50.9 g whereas the control group gained 5.5 g during the 14 days of study. Plasma creatinine and BUN were significantly higher in the CsA group. The CsA group had a significant reduction in GFR as measured by creatinine clearance and a reduction in protein excretion. Urine volume did not change in the CsA group. Plasma sodium levels were significantly lower in the CsA group than in the control group, whereas potassium levels were slightly higher in the CsA group. Urinary excretion of sodium did not differ between the two groups, whereas urinary excretion of potassium markedly decreased in the CsA group. EFNa significantly increased in the CsA group.

Histological studies

A light microscopy image of the renal cortex of CsA-treated rat showed hypertrophy of the afferent arteriole and Bowman's capsule, abundant and focal proximal tubular vacuolization, distension of the distal tubule and glomerular thromboses. A loss of the brush border in proximal tubules, extracellular matrix deposition, and moderate arteriolar thickening were also observed. The tubular vacuolization index score of the CsA group was 3, whereas the control group was 0. Apoptotic cells identified by their dense nuclei and loss of cytoplasm (Thomas *et al*., 1998) were found in the renal tubule. These apoptotic cells were not found in the control rat.

Nitric oxide synthase activity in kidney

NOS activity was measured as NO formation determined by the conversion of [^{3}H]-arginine to [^{3}H]-citrulline. Calcium independency, a feature of inducible NOS (iNOS), was ascertained by using EGTA, a chelater of calcium. iNOS activity in the CsA group was about half that of the control group, and was statistically significant (control: 603.52 ± 80.19 fmol min^{-1} mg protein^{-1} vs CsA group: 284.52 ± 30.29 fmol min^{-1} mg protein^{-1}, Figure 1A). Con-

Table 1 Systemic parameters before and after treatment of CsA

	Control group		*CsA group*	
	Initial	*Final*	*Initial*	*Final*
Body weight (g)	331.2±13.5	336.7±9.9	319.9±3.7	269.0±6.3
pCr (mg dl^{-1})	0.23±0.02	0.34±0.04	0.26±0.02	0.78±0.05**
BUN (mg dl^{-1})	17.8±0.9	16.5±0.5	18.1±1.1	66.5±6.0**
cCr (mg min^{-1})	7.88±0.70	6.11±0.47	7.25±0.56	2.11±0.20**
Urine protein (mg ml^{-1} kg^{-1})	69.3±7.3	88.0±4.7	65.2±8.0	41.6±6.2**
Urine volume (ml)	17.5±4.0	23.8±5.7	18.2±2.0	19.3±2.8
pNa (mEq dl^{-1})	145.4±0.5	144.0±0.7	146.4±0.4	138.8±1.3*
pK (mEq dl^{-1})	3.19±0.04	3.90±0.08	3.19±0.09	4.19±0.15
uNa (mEq dl^{-1})	2.91±0.28	2.99±0.50	2.91±0.45	2.91±0.50
uK (mEq dl^{-1})	5.25±0.44	6.62±0.51	5.94±0.51	4.20±0.46*
FENa (%)	0.18±0.01	0.25±0.05	0.19±0.03	0.72±0.09**

Values are expressed as mean±s.e.mean. *$P<0.01$, **$P<0.001$ versus control group (Final). pCr, plasma creatinine; BUN, blood urea nitrogen; cCr, creatinine clearance; pNa, plasma sodium; pK, plasma potassium; uNa, urinary sodium; uK, urinary potassium; FENa, fractional sodium excretion.

stitutive NOS activity in CsA group was slightly higher, but not statistically significant (control: 62.74±15.77 fmol min^{-1} mg $protein^{-1}$ vs CsA group: 90.87±37.93 fmol min^{-1} mg $protein^{-1}$, Figure 1B).

Urinary NOx

As NO is labile, measurement of the stable metabolites, NO_2 and NO_3, has been employed as an index of production. NO production was assessed by the metabolites NO_2 and NO_3 in urine. A correction for urinary creatinine was performed to avoid fluctuations related to reduced GFR. Urinary excretion of NOx in CsA group significantly decreased compared with that of the control group (control: 3.26±0.74 μmol mg $creatinine^{-1}$ vs CsA group: 0.96±0.20 μmol mg $creatinine^{-1}$, Figure 2).

Cyclic GMP content in kidney

The cyclic GMP content in the CsA group was 1.6 fold higher than that of the control group, and was statistically significant (control: 4.33±0.43 fmol mg $protein^{-1}$ vs CsA group: 7.02±0.63 fmol mg $protein^{-1}$, Figure 3).

Cyclic GMP-PDE activity in kidney

Ca-CaM dependent cyclic GMP-PDE activity in the kidney was the same as cyclic GMP-specific cyclic GMP-PDE activity. Cyclic GMP specific cyclic GMP-PDE activity in the CsA group was 28 fold lower than that from the control group (control: 29.44±10.1 fmol hydrolyzed cyclic GMP min^{-1} mg $protein^{-1}$ vs CsA group: 1.05±0.47 fmol hydrolyzed cyclic GMP min^{-1} mg $protein^{-1}$, Figure 4). The Ca-CaM dependent PDE activity in the CsA group was more than 100 fold lower than that of the control group (control: 25.85±8.47 fmol hydrolyzed cyclic GMP min^{-1} mg $protein^{-1}$ vs CsA group: 0.04±0.04 fmol hydrolyzed cyclic GMP min^{-1} mg $protein^{-1}$, Figure 4). All these changes were statistically significant.

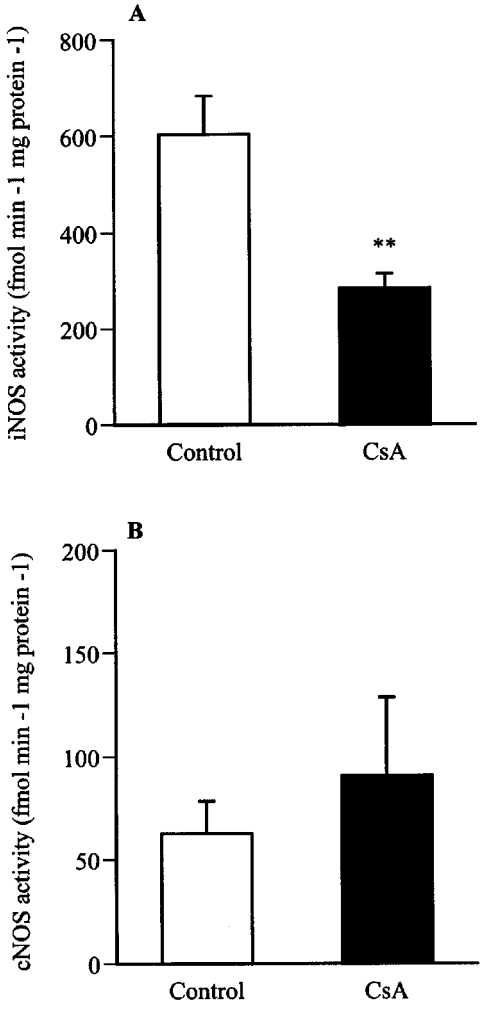

Figure 1 Inducible NOS (iNOS) activity (A) and constitutive NOS (cNOS) activity (B) in kidney isolated from control group and CsA group. **$P<0.01$ versus control group.

Guanylate cyclase activity in kidney

Soluble guanylate cyclase (sGC) activity was about 2 fold higher than particulate guanylate cyclase (pGC) activity in both the control and CsA group. As shown in Figure 5A, sGC activity in the kidney from the CsA-treated rat was 2.4 fold higher compared with control, and was statistically significant (control: 2.06±0.14 fmol mg $protein^{-1}$ vs CsA

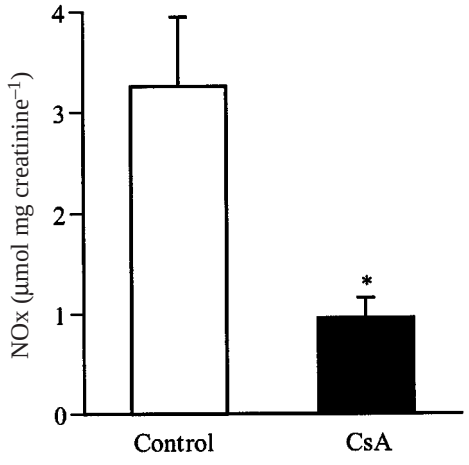

Figure 2 Urinary excretion of NOx (NO_2 and NO_3) in control group and CsA group. $^*P<0.05$ versus control group.

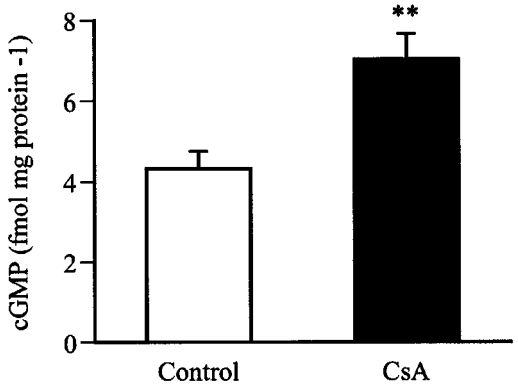

Figure 3 Cyclic GMP content in kidney isolated from control group and CsA group. $^{**}P<0.01$ versus control group.

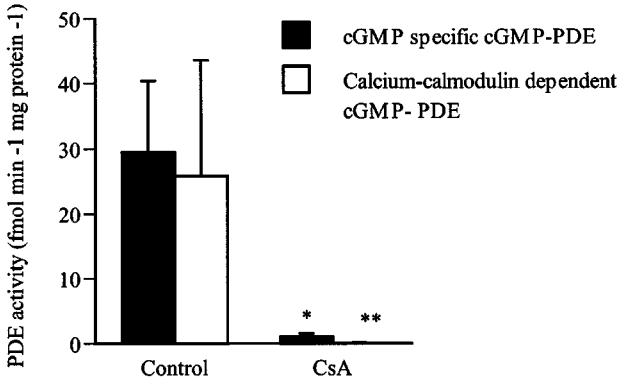

Figure 4 Cyclic GMP-specific cyclic GMP-phosphodiesterase (PDE) activity and calcium-calmodulin dependent cyclic GMP-PDE activity in kidney isolated from control group and CsA group. $^*P<0.05$, $^{**}P<0.01$ versus control group.

group: 4.91 ± 0.80 fmol mg protein^{-1}). As shown in Figure 5B, pGC activity in the kidney from the CsA-treated rat was about 2.3 fold higher compared with control, and was statistically significant (control: 1.11 ± 0.09 fmol mg protein^{-1} vs CsA group: 2.53 ± 0.49 fmol mg protein^{-1}).

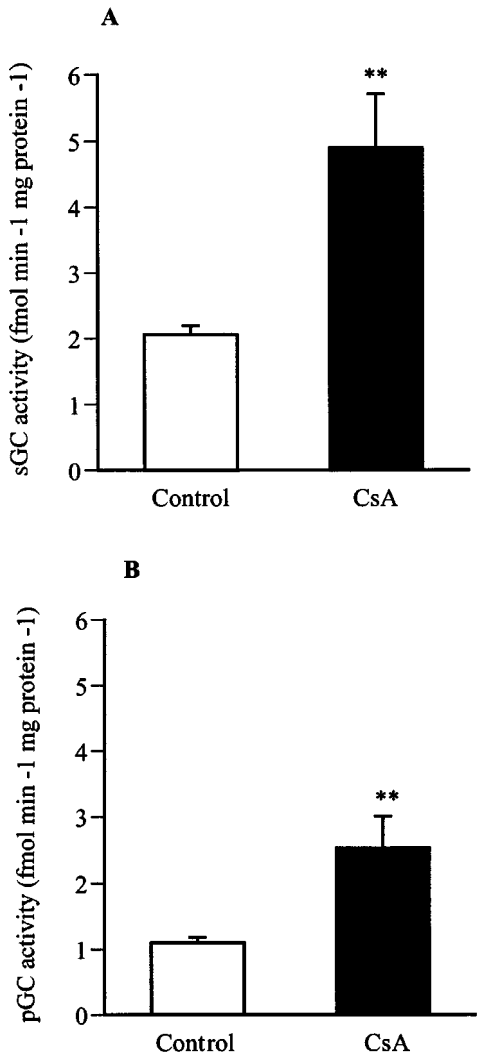

Figure 5 Soluble guanylate cyclase (sGC) activity (A) and particulate guanylate cyclase (pGC) activity in kidney isolated from control group and CsA group. $^*P<0.01$ versus control group.

Isolated perfused kidney

There was no significant difference in the perfusion pressure induced by PhE between the two groups (control: 224.5 ± 9.6 mmHg vs CsA group: 207.7 ± 10.6 mmHg). This vasoconstrictor response remained stable throughout the experiment. SNP dose-dependently caused the decrease in perfused pressure in both groups. As shown in Figure 6, a decrease in perfusion pressure induced by SNP in the kidney isolated from the CsA group was significantly greater than that of the control group.

Discussion

Our results show that there was a significant increase in cyclic GMP content despite a marked decrease in NOS activity in the SHR kidney with typical CsA nephrotoxicy. In addition, there is reciprocal mechanism to keep cyclic GMP content *via* decrease in cyclic GMP-PDE activity as well as an increase in sGC and pGC activity in CsA-treated rat kidney.

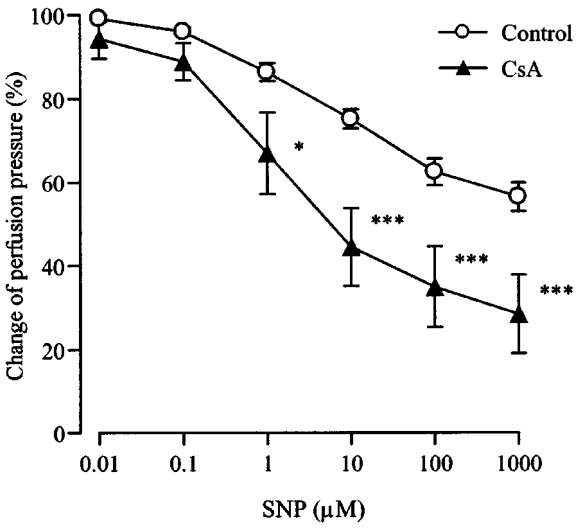

Figure 6 Concentration response curves to sodium nitroprusside (SNP) in isolated perfused kidney obtained from control and CsA group. $*P<0.05$, $***P<0.001$ versus control group.

CsA nephrotoxicity is characterized in two sites of injury: the glomerulus and the proximal tubule (Ader & Rostaing, 1998). In this study, SHR was selected as an experimental animal because CsA nephrotoxicity was induced more distinctly than in the SD strain (Ryffel *et al*., 1986). Typical functional disturbances of glomerulus in CsA-treated SHR were characterized by an increase in BUN, increase in plasma creatinine levels and a decrease in GFR measured by creatinine clearance. FENa, an index of tubular function, was significantly increased in CsA-treated rat, showing impaired tubulointerstitial function. In histological analysis, typical morphological changes such as proximal tubular vacuolization were also observed in all CsA-treated rats. These morphological and functional changes show that typical CsA nephrotoxicity has been adequately established in the experimental model.

There is a lot of evidence to show that NO, one of the most important paracrine modulators, controls renal functions (Blum *et al*., 1998; Braam, 1999). NO is produced by at least three different NOS isoforms, so called inducible NOS; iNOS and constitutive NOS; nNOS and eNOS (Furusu *et al*., 1998). Generally, iNOS is not constitutively expressed, but abundantly expressed in renal tissue such as the medullary thick ascending limb of the loop of Henle, glomeruli as well as intertubular and afferent arteries under basal conditions (Kone & Baylis, 1997). The constitutive expression of iNOS in the kidney suggests it possesses a homeostatic role. In the present study, iNOS activity in CsA-treated rat declined to approximately half of non-treated rat, supporting the hypothesis that decreased iNOS is a main cause of CsA nephrotoxicity (Vaziri *et al*., 1998). At present, there is no evidence to show the direct relationship between the decrease in iNOS activity and CsA nephrotoxicity, but one possible explanation is that decreased iNOS activity would disturb overall renal functions such as regional renal blood flow, renal auto-regulation, glomerular filtration, renin secretion and salt excretion.

The moderate increase in constitutive NOS activity found in our study is consistent with other experimental evidence (Bobadilla *et al*., 1998). A possible reason why constitutive NOS activity increased in CsA-treated rat kidney is that nNOS, expressed mainly in macula densa cells, is directly involved in the control of the tubuloglomerular feedback system (Wilcox, 1998), and decreased distal tubular load of NaCl caused by CsA may stimulate nNOS gene formation. With respect to eNOS, some evidence shows that there may be a promoter region of the eNOS gene, which contains sequence elements responsive to CsA (Navarro-Antolin *et al*., 2000), and indeed the increase in expression of eNOS was shown in the kidney after 7 days treatment with CsA in rat (Bobadilla *et al*., 1998). Further research is required to clarify the various mechanisms for regulating constitutive NOS by CsA.

In order to confirm the decrease in iNOS activity, excretion of NOx in urine was measured. As was speculated, the urinary excretion of NO metabolites drastically decreased. This observation supports our experimental evidence of the significant decrease in renal iNOS activity in CsA-treated rat kidney. Reports concerning NO levels in kidney treated with CsA are controversial, with some showing increases (Assis *et al*., 1997), others decreases (Vaziri *et al*., 1998), and some no change (Pollock *et al*., 1998). The reason for these differences is unclear, but they may depend on the dosage or experimental design such as treatment period and/or strain. Nevertheless, we found decreases in excretion levels of NO as well as iNOS activity in CsA treated rats.

To clarify the physiological role of NO precisely, measurement of cyclic GMP, a second messenger of NO, is recommended. A surprising finding in this study was that cyclic GMP content in kidney treated with CsA was significantly higher than that of control rats. Cyclic GMP content in the kidney of SHR treated with CsA should be lower because a significant decrease in both iNOS activity and excretion of NOx was observed in our study. There are two possible explanations for this increase in cyclic GMP content in the kidney of CsA-treated rat: the first is diminished degradation of cyclic GMP; the second is increase in synthesis of cyclic GMP from NO. The first possibility was confirmed by our experimental result, which showed a significant decrease in activity of two kinds of PDEs, Ca-CaM dependent PDE and cyclic GMP specific cyclic GMP-PDE. Cyclic GMP-PDEs with decreased activity insufficiently hydrolyze cyclic GMP, which consequently results in accumulation of cyclic GMP in kidney. Intracellular cyclic GMP levels are also controlled by efflux of cyclic GMP *via* an active transport process in addition to the enzymatic breakdown by cyclic GMP-PDEs, but this efflux mechanism is considered to be quantitatively of minor importance in comparison to enzymatic breakdown (Mercapide *et al*., 1999). Further study should be conducted to elucidate the mechanism of decrease in cyclic GMP-PDE activity in CsA nephrotoxicity. The second possibility was also confirmed by our experimental result, showing more than a 2.4 fold increase in sGC activity in CsA-treated rat. To the best of our knowledge, there are no reports showing the direct relationship between changes in sGC activity and CsA nephrotoxicity. However, recent studies have shown that CsA increases hypoxia in rat kidney (Zhong *et al*., 1998). In addition to this, an interesting phenomenon reported in the lung is that sGC activity was upregulated more than 5 fold in the hypoxic condition (Li *et al*., 1999). These two reports

indirectly support our experimental data, which show an increase in sGC activity in CsA-treated rat. There is another possibility that increased activity of sGC might be secondary caused by the reduction in NO concentration within the kidney in this study. Supersensitivity of sGC to NO has been well characterized in other NO deficiency states. Brandes *et al*. (2000) clearly showed that a decrease in NO level in endothelial NO synthase deficient mice caused an enhanced sensitivity of sGC activity in the aorta.

In order to ascertain the marked change in these two kinds of enzyme, which may contribute towards the increase in cyclic GMP in kidney from CsA group, we evaluated the effect of SNP, NO donor, on the perfusion pressure in isolated kidney. As we expected, a significant increase in the responsiveness to SNP was observed in CsA group, and these functional data support the change in these biological parameters in NO-cyclic GMP pathway, in other words, increased sensitivity of renal tissue to NO.

Increase in pGC activity seems to partially contribute to the increase in cyclic GMP in CsA kidney, although basal pGC activity was about half of sGC activity. NO deficient condition might also cause the upregulation of pGC activity in this study, because it has been reported that atrial natriuretic peptide (ANP)-pGC signalling is also upregulated in endothelial NO synthase deficient mice (Hussain *et al*., 2001).

We showed the increase in cyclic GMP content in CsA-treated rat kidney, which is probably due to the combination of increase in GC activity and decrease in cyclic GMP-PDE activity. The following observations suggest a beneficial effect of an increase in cyclic GMP content on CsA nephrotoxicity. Cyclic GMP possesses vasodilation and cytoprotection by causing calcium extrusion through Ca^{2+} pumps and Ca^{2+}-activated K^+ channels (Lincoln *et al*., 1994). Several articles reported that CsA causes increases in intracellular calcium content and calcium influx (Gallego *et al*., 1994; Bokemeyer *et al*., 1993; Lo Russo *et al*., 1996; Avdonin *et al*., 1999) as well. There may be a possibility that the increase in free calcium in the kidney by CsA itself promotes the progression of renal disease by modulating renal haemodynamics, and subsequently exerts cell proliferation during renal atherosclerotic processes. The increase in cyclic GMP content, therefore, is anticipated to retard the progression of CsA nephrotoxicity including glomerulosclerosis and tubular dysfunction. Another action of cyclic GMP is anti-apoptosis, and this seems to be favourable for CsA nephrotoxicity. Similarly, as previously found with kidney of CsA-treated animals (Thomas *et al*., 1998), apoptotic cells were observed in our present study. Other indirect evidence showing the beneficial effects of increased cyclic GMP is as follows: administration of L-arginine and ANF improved CsA induced nephrotoxicity (Andoh *et al*., 1997; Lang *et al*., 1992), and conversely L-NAME, a NOS inhibitor, deteriorated the nephrotoxicity further (Assis *et al*., 1997). All these experiments suggest an important role of cyclic GMP for impaired renal function induced by CsA.

In conclusion, our present study shows an increase in cyclic GMP content in spite of a decrease in NOS activity and excretion of NOx in kidney with fully expressed CsA nephrotoxicity. This increase in cyclic GMP seems to be brought on by both increase in sGC and pGC activity and decrease in cyclic GMP-PDEs activity. This reciprocal mechanism is probably playing an important role in keeping the homeostasis in the kidney with CsA nephrotoxicity.

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