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Relative roles of endothelial relaxing factors in cyclosporine-induced impairment of cholinergic and β-adrenergic renal vasodilations

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Abstract

Vascular toxicity is a major adverse effect for the immunosuppressant drug cyclosporine A. The present study sought to characterize the relative roles of the endothelium-derived relaxing factors (nitric oxide, endothelium-derived hyperpolarizing factor [EDHF], and prostaglandins) in the cyclosporine-induced impairment of renovascular responsiveness to acetylcholine receptor or β-adrenoceptor activation. Changes evoked by cyclosporine in the responses to either vasorelaxant were evaluated in phenylephrine-preconstricted isolated perfused rat kidneys in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor), tetraethylammonium (K^+ channel blocker), or diclophenac (cyclooxygenase inhibitor). Acetylcholine (0.03–2 nmol) vasodilations were significantly inhibited by prior treatment with L-NAME, tetraethylammonium, or diclophenac, suggesting a role for nitric oxide, EDHF, and prostaglandins in acetylcholine vasodilations. Isoprenaline (0.125–4 μmol) vasodilations were inhibited by L-NAME and tetraethylammonium versus no effect for diclophenac. Cyclosporine (1–4 μM) produced a concentration-related inhibition of vasodilations relaxations produced by either vasodilator. Cyclosporine-induced inhibition of acetylcholine vasodilations was attenuated in tissues pretreated with L-NAME or tetraethylammonium but not diclophenac, implicating nitric oxide and EDHF in cyclosporine–acetylcholine interaction. On the other hand, the inhibition of isoprenaline vasodilations by cyclosporine was virtually abolished by L-NAME. In cyclosporine-treated kidneys, exposure to L-arginine, the substrate of nitric oxide synthesis, fully restored isoprenaline vasodilations to control levels and significantly increased acetylcholine vasodilations. It is concluded that the identity and relative contributions of endothelial factors to renal vasodilatory responses as well as to the inhibition of these responses by cyclosporine largely depend on the vasodilator stimulus.

Keywords: Cyclosporine; Perfused kidney; Endothelium-derived relaxing factor; Acetylcholine; Isoprenaline

1. Introduction

Cyclosporine A is a standard component of the immunosuppressive regimen in both solid organ and bone marrow transplantation and also in the control of several autoimmune diseases (Shulman et al., 1981; Cohen et al., 1984; Kahan, 1989). The major limiting factor of cyclosporine use is the development of nephrotoxicity and/or hypertension (Myers, 1986; Mason, 1990). Several pathophysiological changes that associate cyclosporine therapy have been implicated in its adverse effects such as direct vasoconstriction (Xue et al., 1987), increasing sympathetic and angiotensin activities (Moss et al., 1985; Kon et al., 1995), endothelin release (Kon et al., 1990), and altering the renal metabolism of arachidonic acid favoring a vasoconstrictor prostanoid profile (Perico et al., 1986). Endothelium dysfunction also contributes to the pathogenesis of cyclosporine adverse effects including renal vasoconstriction and subsequent nephrotoxicity (Cairns et al., 1989; Gossmann et al., 2001), microvascular thromboses (Shulman et al., 1981), and hypertension (Gerkens, 1989). Vascular relaxations elicited by acetylcholine (Stephan et al., 1995), bradykinin (Rego et al., 1990), substance P (Mathieu et al., 1997), calcium ionophore A23187 (Rego et al., 1990), and prostaglandin E_1 (Stein et al., 1995) are remarkably impaired by cyclosporine. A recent study from our laboratory has shown that chronic cyclosporine administration impairs endothelium-dependent vasodilations via, at least partly, reducing the availability of testosterone and inhibiting its facilitatory

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effect on cholinergically mediated renal vasodilations (El-Mas et al., 2003a). This conclusion is supported by the observations that: (i) cyclosporine or castration reduced acetylcholine relaxations and plasma testosterone levels; (ii) cyclosporine had no effect on acetylcholine vasodilations in kidneys obtained from castrated rats; and (iii) testosterone replacement of cyclosporine-treated castrated rats restored the physiological plasma levels of the hormone and acetylcholine vasodilations to near-control levels (El-Mas et al., 2003a).

Reported findings established a key role for nitric oxide and vasorelaxant prostanoids in the cyclosporine-evoked endothelium dysfunction. Stephan et al. (1995) have demonstrated that impairment of acetylcholine renal vasodilations by cyclosporine, administered chronically, relates to the impairment of endothelial nitric oxide and prostaglandin activities. This notion gains support from the observations that replacement of cyclosporine-treated rats with L-arginine, the nitric oxide precursor, or with pentoxyphylline, a stimulator of prostacyclin synthesis, prevents cyclosporine-induced endothelial dysfunction (Berkenboom et al., 1991; Oriji and Keiser, 1998). A linkage between cyclosporine and endothelial nitric oxide activity is also evident from the demonstration that the acetylcholine-stimulated accumulation of cyclic GMP in blood vessels is decreased by cyclosporine (Rego et al., 1990). A reduced nitric oxide plasma levels also associates cyclosporine-induced nephrotoxicity in humans (Gossmann et al., 2001) and supplementation with L-arginine, the nitric oxide precursor, prevents and/or reverses cyclosporine-induced endothelial dysfunction (Bloom et al., 1995; Mathieu et al., 1997; Oriji and Keiser, 1998).

The main objective of the present study was to investigate the roles of three principal relaxing factors of the endothelium [nitric oxide, endothelium-derived hyperpolarizing factor (EDHF), and vasorelaxant prostaglandins] in cyclosporine impairment of renal vasodilatory responses to activation of acetylcholine receptors and β-adrenoceptors by acetylcholine and isoprenaline, respectively. The hypothesis was tested that the nature and relative contribution of these relaxing factors to the cyclosporine–endothelium interaction depends on the type of the vasodilatory stimulus. The current study presents the following three unique aspects. First, it simultaneously determined the relative contribution of three key endothelium-derived relaxing factors in cyclosporine-evoked endothelium dysfunction. In previous studies, only one or a maximum of two factors were considered (Bloom et al., 1995; Mathieu et al., 1997; Oriji and Keiser, 1998; Stephan et al., 1995). Second, it investigated whether cyclosporine adversely affect β-adrenoceptor-mediated vasodilations in the renal vasculature and the endothelial factor(s) involved. Third, the present study characterized the contribution of EDHF to cyclosporine impairment of cholinergically or β-adrenergically mediated renal vasodilations. In contrast to nitric oxide and prostaglandins, the role of EDHF, which hyperpolarizes and relaxes vascular smooth muscle through opening K^+ channels (Feletou and Vanhoutte, 1996), in cyclosporine–endothelium interaction is not clear. To accomplish these goals, in-vitro studies were undertaken in the rat isolated perfused kidney pre-constricted with phenylephrine to determine the acute effects of cyclosporine on the dose–vasodilatory response curves of acetylcholine or isoprenaline in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME), nitric oxide synthase inhibitor, tetraethylammonium, K^+ channel blocker, or diclophenac, cyclooxygenase inhibitor. It is notable that the vasodilator effects of both acetylcholine (Vargas et al., 1994; Stephan et al., 1995) and isoprenaline (Van der Zypp et al., 2000) are endothelium dependent. Some of the data has been previously published in an abstract form (El-Mas et al., 2003b).

2. Methods

Male Wistar rats (230–280 g; High Institute of Public Health, Alexandria, Egypt) were used in the present study. Experiments were performed in accordance with the European Community guidelines for the use of experimental animals and were approved by the institutional ethics committee.

2.1. Rat isolated perfused kidney

The rat kidney was isolated and perfused according to the method described in previous studies including ours (Mohy El-Din and Malik, 1988; Vargas et al., 1994; El-Mas et al., 2003a). Briefly, rats were anesthetized with thiopental sodium (50 mg/kg, i.p.), the abdomen was opened by a midline incision and the left kidney was exposed. The left renal artery was dissected free from its surrounding tissues. Loose ties were made around the renal artery and the abdominal aorta, proximal and distal to the renal artery. A beveled 18-gauge needle connected to a 5-ml syringe filled with heparinized saline (100 U/ml) was used for cannulation. The aorta was ligated, and the left renal artery was cannulated via an incision made in the aorta. The cannula was immediately secured with ligatures and the kidney was flushed with heparinized saline and rapidly excised from its surrounding tissues.

The kidney was transferred into a jacketed glass chamber maintained at 37 °C and continuously perfused with Krebs' solution (NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11 mM) maintained at 37 °C and gassed with 95% O_2 and 5% CO_2. Kidney perfusion was adjusted at a constant flow rate of 5 ml/min using a peristaltic pump (Model P3-Pharmacia Fine Chemicals®). The pump delivered a pulsatile flow, and an open circuit was used so that the venous effluent was allowed to drain freely. The kidney perfusion pressure was continuously monitored by means of a Gould-Statham pressure transducer distal to the pump and

recorded on a Grass polygraph (model 7D). Inasmuch as the renal flow was kept constant, changes in perfusion pressure were indicative of alterations in renal vascular resistance. An equilibration period of 30 min was allowed at the beginning of the experiment to ensure stabilization of the kidney perfusion pressure (Vargas et al., 1994; El-Mas et al., 2003a). To study the vasodilatory effects of acetylcholine or isoprenaline, the renal vascular tone was elevated by a continuous infusion of the α_1-adrenoceptor agonist phenylephrine (10 μM) as described in our previous study (El-Mas et al., 2003a).

2.2. Protocols and experimental groups

2.2.1. Roles of endothelium-derived relaxing factors in acetylcholine or isoprenaline vasodilations

A total of eight groups of rats ($n=6-8$ each) were employed in this experiment to investigate the roles of the endothelium-derived relaxing factors (nitric oxide, EDHF, and prostaglandins) in the vasodilatory responses elicited by acetylcholine or isoprenaline in the rat isolated perfused kidney. Under conditions of sustained elevations in renovascular tone induced by phenylephrine, dose–response curves to bolus injections of acetylcholine (0.03–2 nmol) or isoprenaline (0.125–4 μmol) at 5-min intervals were established. A single vasodilator (acetylcholine or isoprenaline) was tested in each preparation. Generally, a given dose of either vasodilator was injected when the response to the previous dose disappeared and the vascular tone returned to the pretreatment levels. Dose–response curves of acetylcholine or isoprenaline were established before and 20 min after the infusion of: (i) L-NAME 100 μM, (ii) tetraethylammonium 3 mM, (iii) diclophenac 7 μM, or (iv) a combination of the three drugs. In order to standardize the vasodilatory responses to acetylcholine or isoprenaline, a bolus dose of papaverine (50 nmol) was injected into each preparation and the effects of acetylcholine or isoprenaline were expressed as a percentage of the papaverine vasodilation (Heuzé-Joubert et al., 1992; El-Mas et al., 2003a). Bolus doses of all drugs were administered at a constant volume of 100 μl. The injected volume caused a small and transient increase in renal perfusion pressure, which preceded the drug-evoked response. A control injection of saline was given to each preparation to verify that the responses to drug injections were not artifacts. The relative contributions of endothelium-derived relaxing factors to acetylcholine or isoprenaline vasodilations were determined by computing the percentage reductions evoked by L-NAME, tetraethylammonium, or diclophenac in the responses produced by the highest dose of acetylcholine or isoprenaline.

2.2.2. Concentration-dependent effect of cyclosporine on vasodilatory responses to acetylcholine or isoprenaline

Two groups of rats were used in this experiment to investigate the effect of cyclosporine (1–4 μM) on renal vasodilations evoked by acetylcholine (0.03–2 nmol, $n=7$) or isoprenaline (0.125–4 μmol, $n=7$). In each preparation, responses to each vasodilator were measured before and after consecutive infusion of cyclosporine 1, 2, and 4 μM. An infusion period of 20 min was allowed for each concentration of cyclosporine before testing the vasodilator activity. In preliminary control experiments, four consecutive dose–response curves of acetylcholine or isoprenaline were constructed in four preparations and exhibited comparable vasodilatory responses, thus eliminating time as a factor that may alter the responses. The vasodilatory effects produced by the highest dose of acetylcholine ($98.5 \pm 2.1\%$, $97.6 \pm 2.0\%$, $96.0 \pm 1.6\%$, and $95.1 \pm 1.7\%$) or isoprenaline ($81.3 \pm 4.3\%$, $80.0 \pm 4.4\%$, $78.9 \pm 4.3\%$, and $77.1 \pm 4.6\%$) were similar in the four consecutive control curves.

2.2.3. Roles of relaxing factors in cyclosporine-induced endothelium dysfunction

Six groups of rats ($n=6-7$ each) were employed in this experiment to investigate whether relaxing factors of the endothelium differentially contribute to cyclosporine impairment of acetylcholine and isoprenaline renal vasodilations. In each preparation, three consecutive dose–response curves for acetylcholine (0.03–2 nmol) or isoprenaline (0.125–4 μmol) were established: (i) control responses; (ii) responses 20 min after infusion of L-NAME (100 μM), tetraethylammonium (3 mM), or diclophenac (7 μM) to inhibit nitric oxide synthase, K^+ channels, and cyclooxygenase, respectively; and (iii) responses 20 min after infusion of cyclosporine (2 μM). Each preparation received a single inhibitor (L-NAME, tetraethylammonium, or diclophenac) followed by cyclosporine. The percentage reductions in the responses to acetylcholine (2 nmol) or isoprenaline (4 μmol) evoked by cyclosporine alone (in the presence of the inhibitors) were computed by comparing responses obtained in the presence of a particular inhibitor (second dose–response curve) to those produced in the presence of the inhibitor plus cyclosporine combination (third dose–response curve).

2.2.4. Effect of L-arginine on cyclosporine impairment of renal vasodilations

Two groups of rats were used in this experiment to investigate whether L-arginine supplementation reverses cyclosporine-induced attenuation of vasodilatory responses to acetylcholine ($n=8$) or isoprenaline ($n=7$). Three consecutive dose–response curves of acetylcholine or isoprenaline were established before, 20 min after cyclosporine (2 μM) infusion, and 20 min after L-arginine (100 μM) infusion.

2.2.5. Interaction between nitric oxide and EDHF

This experiment determined whether the modulatory effects of nitric oxide and EDHF on renal endothelial function are mutually facilitated. The individual inhibitory effect of L-NAME or tetraethylammonium on acetylcholine vasodilations was compared to the inhibition caused by each

inhibitor in the presence of the other inhibitor. Two groups of rats were used ($n=5$ each). In the first group, renal vasodilator responses to acetylcholine were established (i) before, (ii) 20 min after L-NAME, and (ii) 20 min after L-NAME plus tetraethylammonium. In the second group of rats, acetylcholine vasodilations were established (i) before, (ii) 20 min after tetraethylammonium, and (iii) 20 min after tetraethylammonium plus L-NAME.

2.3. Drugs

Phenylephrine hydrochloride, N^G-nitro-L-arginine methyl ester, L-arginine, tetraethylammonium, isoprenaline hydrochloride (Sigma, St. Louis, MO, USA), diclophenac (Novartis Pharma, Basel, Switzerland), papaverine hydrochloride (Recordati, Milano, Italy), acetylcholine chloride (Roche), thiopental sodium (Thiopental, Biochemie, Vienna, Austria) were purchased from commercial vendors. Cyclosporine was a gift from Novartis Pharma. Cyclosporine was dissolved in a mixture of ethanol/distilled water (1:1) and a volume of 0.2 ml was mixed with the Krebs' solution (Xue et al., 1987).

2.4. Statistical analysis

Values are expressed as mean ± S.E.M. The vasodilatory responses to acetylcholine and isoprenaline were measured as the maximum decreases in the renal perfusion pressure in tissues preconstricted with phenylephrine, and expressed as a percentage of the vasodilation caused by papaverine 50 nmol (El-Mas et al., 2003a). The analysis of variance (ANOVA) followed by a Newman–Keuls post-hoc analysis was used for multiple comparisons with the level of significance set at $P<0.05$.

3. Results

3.1. Roles of endothelium-derived relaxing factors in acetylcholine or isoprenaline vasodilations

The effects of inhibition of nitric oxide synthase, K^+ channels, or cyclooxygenase evoked by L-NAME, tetraethylammonium, and diclophenac, respectively, on the vasodilatory responses to acetylcholine or isoprenaline in the rat isolated perfused kidneys preconstricted with phenylephrine are shown in Fig. 1. The average basal renal perfusion pressure was 59.2 ± 2.5 mm Hg. The infusion of phenylephrine into the renal vasculature produced an abrupt increase in the perfusion pressure, which was gradually declined and stabilized within 20 min at a higher level (+125 to 150 mm Hg) for the duration of the experiment. Bolus injections of acetylcholine (0.03–2 nmol, Fig. 1A) or isoprenaline (0.125–4 μmol, Fig. 1B) into the renal vasculature elicited dose-dependent decreases in the renal perfusion pressure. Infusion of L-NAME (100 μM), tetraethylammonium (3 mM), or diclophenac (7 μM) caused little increases in the renal perfusion pressure (4.1 ± 1.2, 3.6 ± 1.0, and 4.4 ± 1.4 mm Hg, respectively) but elicited significant ($P<0.05$) reductions in acetylcholine vasodilations (Fig. 1A). Comparison of the percentage reductions in the responses caused by acetylcholine (2 nmol) or isoprenaline (4 μmol) shows that the potency order of acetylcholine vasodilation inhibition was L-NAME>tetraethylammonium>diclophenac (Table 1).

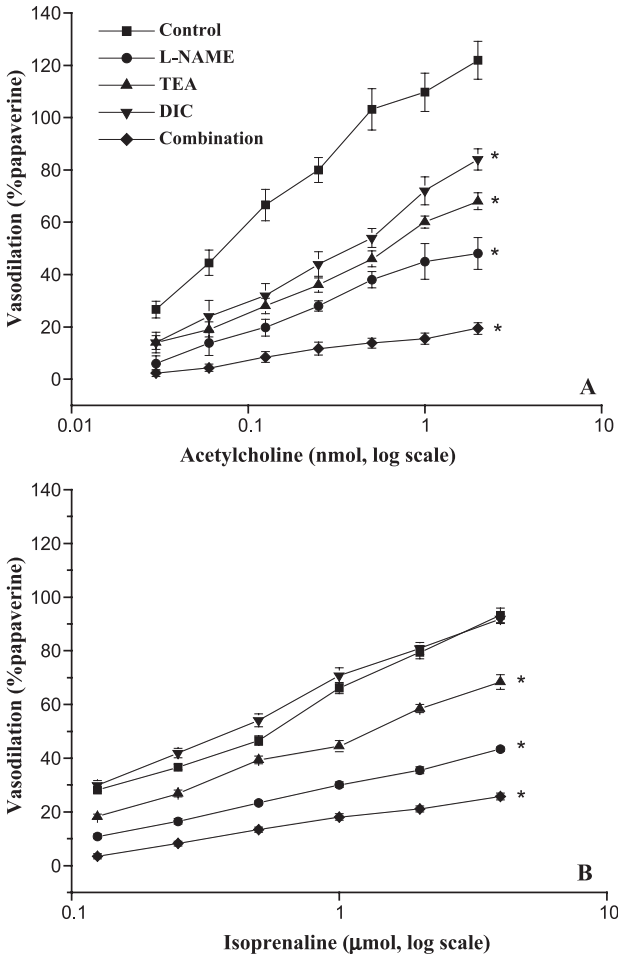

Fig. 1. Vasodilatory effects of acetylcholine (panel A) or isoprenaline (panel B) in phenylephrine (10 μM)-preconstricted isolated perfused kidneys in the absence (control) and presence of 100 μM L-NAME (nitric oxide synthase inhibitor), tetraethylammonium 3 mM (TEA, K^+ channel blocker), diclophenac 7 μM (DIC, cyclooxygenase inhibitor), or their combination. Acetylcholine or isoprenaline responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of six to eight observations. *$P<0.05$ compared with control values.

Isoprenaline vasodilations were significantly ($P<0.05$) inhibited by L-NAME or tetraethylammonium but not by diclophenac (Fig. 1B). Simultaneous infusion of a combination L-NAME, tetraethylammonium, and diclophenac abolished most of the vasodilatory effect of acetylcholine or isoprenaline (Fig. 1, Table 1). The correlation coefficients of the regression lines were highly significant ($P<0.001$) and ranged from 0.88 to 0.99.

Table 1
The percentage reductions in the vasodilatory response of acetylcholine (2 nmol) or isoprenaline (4 μmol) caused by L-NAME (100 μM), tetraethylammonium (TEA, 3 mM), diclophenac (DIC, 7 μM), or their combination

Treatment	Acetylcholine	Isoprenaline
L-NAME	57.5 ± 3.4	51.4 ± 1.2
TEA	42.1 ± 2.9	27.4 ± 1.8
DIC	28.9 ± 2.1	1.7 ± 1.3
Combination	84.1 ± 5.4	71.7 ± 2.0

Values are means ± S.E.M.

3.2. Concentration-dependent effect of cyclosporine on vasodilatory responses to acetylcholine and isoprenaline

This experiment tested whether the inhibitory effect of cyclosporine on acetylcholine or isoprenaline vasodilations is concentration-related. As shown in Fig. 2, the consecutive infusion of increasing concentrations of cyclosporine (1, 2, and 4 μM) elicited a concentration-dependent attenuation of the vasodilatory responses to acetylcholine and isoprenaline. The percentage reductions in the vasodilatory response of acetylcholine (2 nmol) or isoprenaline (4 μmol) caused by the middle concentration (2 μM) of cyclosporine were similar (51.5 ± 1.8% vs. 56.9 ± 2.7%), suggesting comparable inhibitory effects of cyclosporine on responses of the two vasodilators.

3.3. Roles of relaxing factors in cyclosporine-induced endothelium dysfunction

Figs. 3 and 4 and Table 2 depict the effects of pretreatment with L-NAME, tetraethylammonium, or diclophenac on cyclosporine (2 μM)-evoked inhibition of acetylcholine and isoprenaline vasodilations in the rat isolated perfused kidneys preconstricted with phenylephrine. As mentioned above (experiment 1), exposure to L-NAME (Fig. 3A), tetraethylammonium (Fig. 3B), or diclophenac (Fig. 3C) significantly ($P < 0.05$) attenuated the vasodilatory responses to acetylcholine. Subsequent infusion of cyclosporine (2 μM) caused an additional attenuation of acetylcholine responses in all preparations (Fig. 3). However, as shown in Table 2, the percentage reductions in the acetylcholine (2 nmol) response caused by cyclosporine in tissues pretreated with L-NAME or tetraethylammonium were significantly ($P < 0.05$) less than the inhibition produced by the same concentration of cyclosporine when tested in the absence of either inhibitor (Section 3.2). This represented approximately 35% decline in the cyclosporine attenuation of acetylcholine vasodilations, suggesting the involvement of endothelial nitric oxide and EDHF in cyclosporine–acetylcholine interaction. In contrast, the inhibition of acetylcholine vasodilation by cyclosporine was not altered by diclophenac pretreatment (Table 2).

On the other hand, the attenuating effect of cyclosporine on the vasodilatory responses to isoprenaline was not altered in tissues pretreated with tetraethylammonium but it was virtually abolished in L-NAME-treated tissues (Fig. 4, Table 2). The effect of diclophenac pretreatment on cyclosporine–isoprenaline interaction was not tested because, as mentioned earlier (Fig. 1B), diclophenac had no effect on isoprenaline vasodilations, which rules out a possible role for vasodilator prostanoids in β-adrenoceptor-mediated vasodilations.

3.4. Effect of L-arginine on cyclosporine impairment of renal vasodilations

The effects of L-arginine supplementation on the cyclosporine-evoked attenuation of acetylcholine or isoprenaline

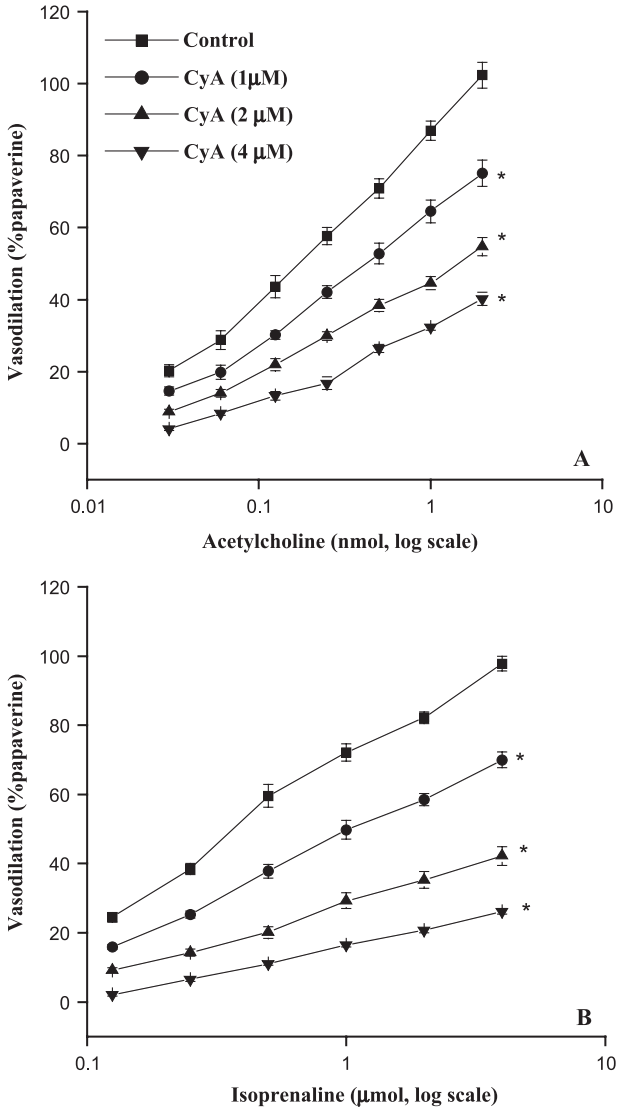

Fig. 2. Concentration-dependent effects of cyclosporine (CyA, 1–4 μM) on vasodilatory responses elicited by acetylcholine (panel A) or isoprenaline (panel B) in phenylephrine (10 μM)-preconstricted isolated perfused kidneys. Acetylcholine or isoprenaline responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of seven observations. *$P < 0.05$ compared with control values.

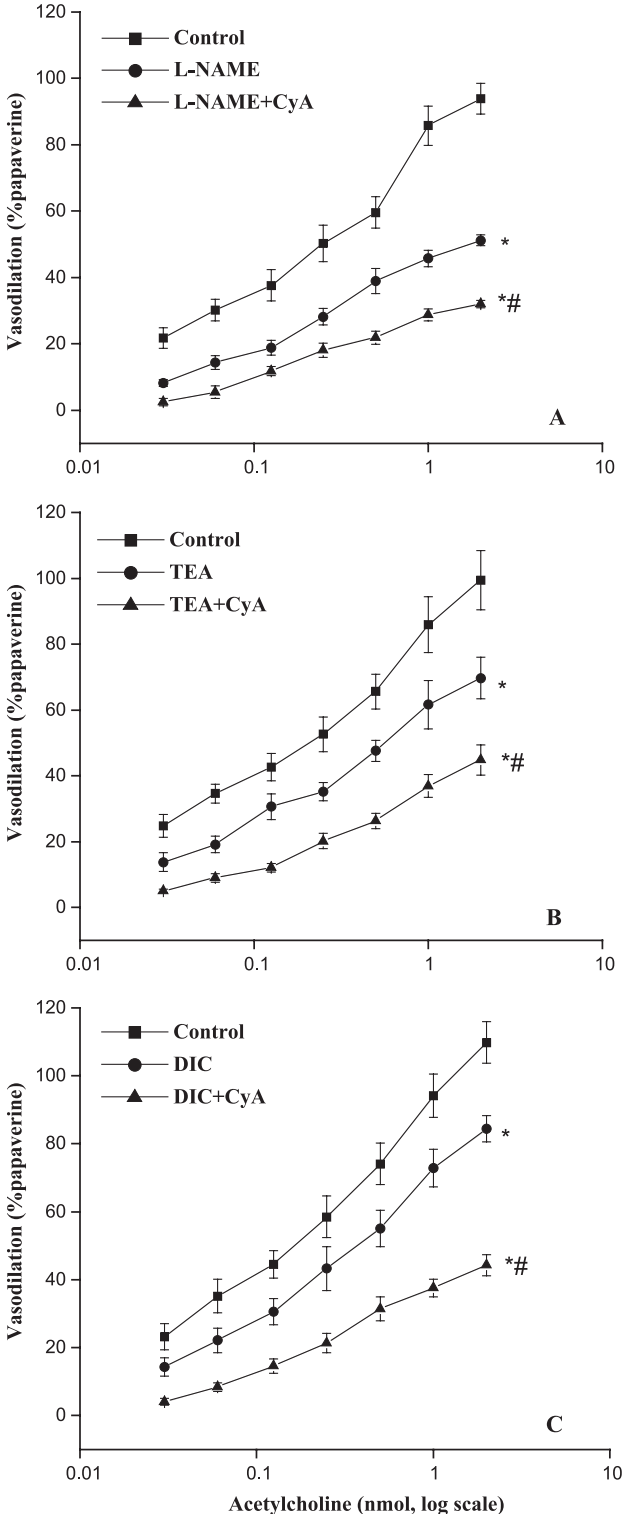

Fig. 3. Effect of cyclosporine (CyA, 2 μM) on vasodilatory effects of acetylcholine in phenylephrine (10 μM)-preconstricted isolated perfused kidneys pretreated with 100 μM L-NAME (nitric oxide synthase inhibitor, panel A), tetraethylammonium 3 mM (TEA, K^+ channel blocker, panel B), or diclophenac 7 μM (DIC, cyclooxygenase inhibitor, panel C). Acetylcholine responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of six observations. $^{*,\#}P<0.05$ compared with control and inhibitor (L-NAME, TEA, or DIC) values, respectively.

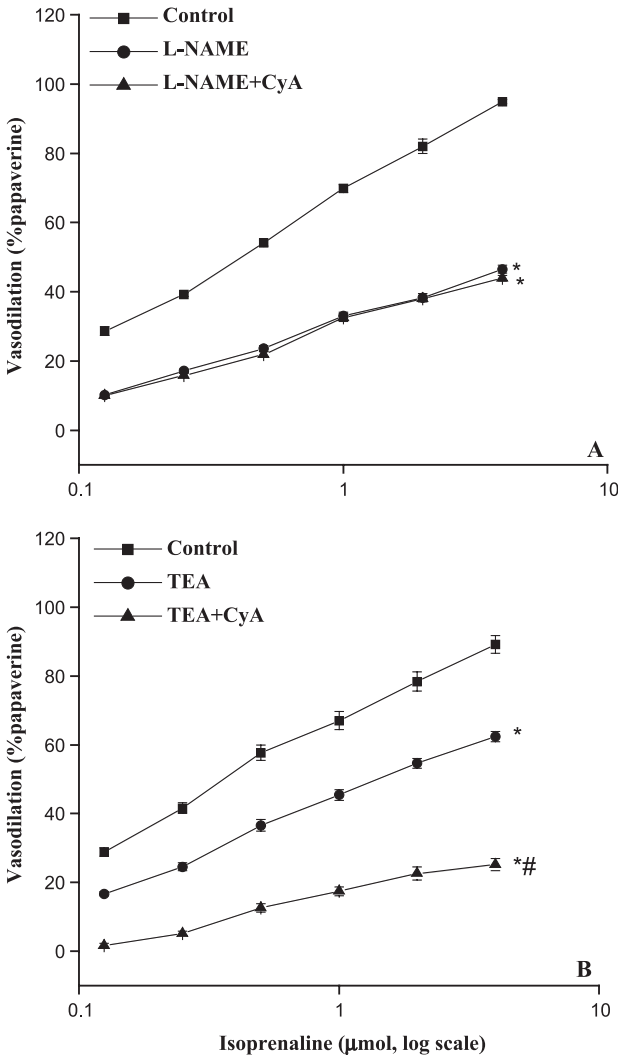

Fig. 4. Effect of cyclosporine (CyA, 2 μM) on vasodilatory effects of isoprenaline in phenylephrine (10 μM)-preconstricted isolated perfused kidneys pretreated with 100 μM L-NAME (nitric oxide synthase inhibitor, panel A) or tetraethylammonium 3 mM (TEA, K^+ channel blocker, panel B). Isoprenaline responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of seven observations. $^{*,\#}P<0.05$ compared with control and inhibitor (L-NAME or TEA) values, respectively.

vasodilations are illustrated in Fig. 5. Continuous infusion of L-arginine (100 μM) completely abolished the cyclosporine-evoked reductions in isoprenaline vasodilations (Fig. 5B).

Table 2
The percentage reductions in the vasodilatory response of acetylcholine (2 nmol) or isoprenaline (4 μmol) caused by cyclosporine (CyA, 2 μM) in the absence and presence of L-NAME (100 μM), tetraethylammonium (TEA, 3 mM), or diclophenac (DIC, 7 μM)

Treatment	Acetylcholine	Isoprenaline
CyA	51.5 ± 1.8	56.9 ± 2.7
CyA (L-NAME)	37.5 ± 0.7[a]	5.3 ± 2.0[a]
CyA (TEA)	35.9 ± 3.1[a]	59.4 ± 3.1
CyA (DIC)	47.3 ± 2.9	–

Values are means ± S.E.M.
[a] $P<0.05$ compared with CyA values in the absence of the inhibitors.

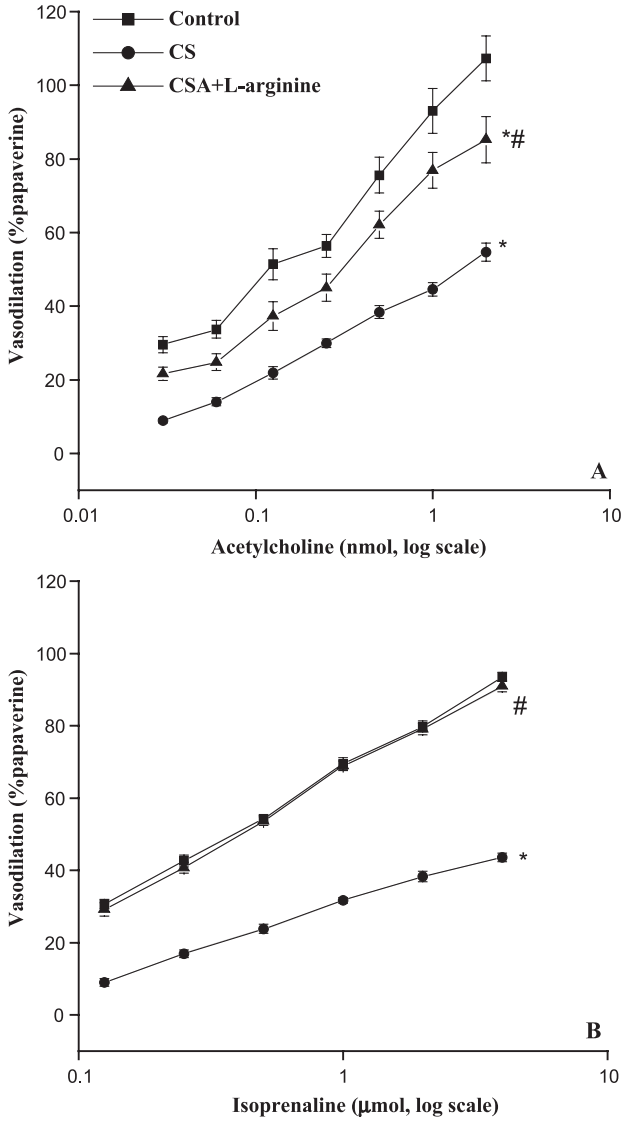

Fig. 5. Effect of L-arginine supplementation (100 μM) on cyclosporine (CyA, 2 μM)-induced attenuation of acetylcholine (panel A) or isoprenaline (panel B) vasodilations in phenylephrine (10 μM)-preconstricted isolated perfused kidneys. Acetylcholine or isoprenaline responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of seven to eight observations. $*,^{\#}P<0.05$ compared with control and CyA values, respectively.

Vasodilatory responses to isoprenaline in tissues receiving cyclosporine + L-arginine were similar to those obtained in control tissues (Fig. 5B). Acetylcholine vasodilations in cyclosporine-treated tissues were partially increased after L-arginine infusion but remained significantly less compared with acetylcholine responses in control tissues (Fig. 5A).

3.5. Interaction between nitric oxide and EDHF

Whether a mutual facilitation exists between nitric oxide and EDHF on endothelial function was investigated in this experiment. The effects of L-NAME or tetraethylammonium followed by the combination of the two inhibitors on acetylcholine renal vasodilations are shown in Fig. 6. The initial exposure to L-NAME (Fig. 6A) or tetraethylammonium (Fig. 6B) caused significant decreases in acetylcholine responses; the percentage reduction in the response caused by acetylcholine 2 nmol amounted to 44.8 ± 0.6% and 35.4 ± 1.4%, respectively. The sequential exposure of L-NAME- or tetraethylammonium-treated tissues to tetraethylammonium (Fig. 6A) and L-NAME (Fig. 6B) caused further attenuation of acetylcholine vasodilations. The additional attenuation caused L-NAME (46.2 ± 1.9%) or tetraethylammonium (33.0 ± 0.4%) in tissues pretreated with

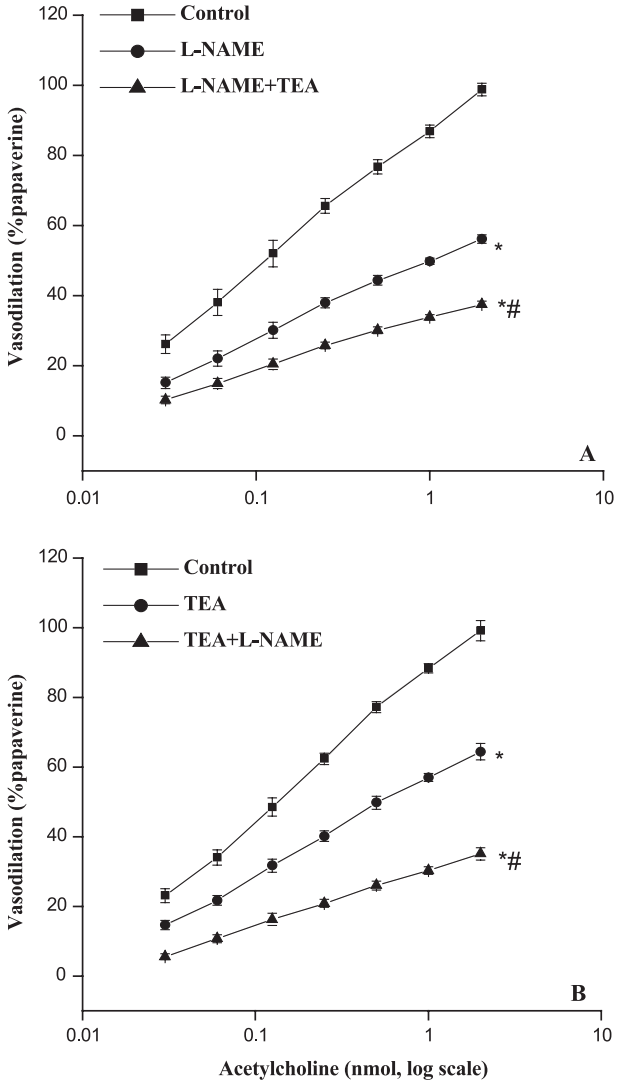

Fig. 6. Individual and combined effects of 100 μM L-NAME (nitric oxide synthase inhibitor) and tetraethylammonium 3 mM (TEA, K^+ channel blocker) on vasodilatory responses to acetylcholine in phenylephrine (10 μM)-preconstricted isolated perfused kidneys. Acetylcholine responses are expressed as percentages of papaverine (50 nmol)-induced vasodilations. Values are mean ± S.E.M. of five observations. $*P<0.05$ compared with control values; $^{\#}P<0.05$ compared with L-NAME (panel A) or TEA (panel B) values.

tetraethylammonium and L-NAME, respectively, was similar to the effect of either inhibitor when tested alone.

4. Discussion

The impairment of vascular endothelium function plays a central role in the cyclosporine-induced nephrotoxicity, microvascular thromboses, and hypertension (Shulman et al., 1981; Gerkens, 1989; Gossmann et al., 2001). The primary goal of the present study was to evaluate the roles of major relaxing factors of the endothelium in the cyclosporine–endothelium interaction in the rat isolated perfused kidney. More specifically, the current study presented a comparative investigation of the differential contributions of endothelial nitric oxide, EDHF, and vasorelaxant prostaglandins, to the cyclosporine impairment of the acetylcholine receptor- and β-adrenoceptor-mediated vasodilations in the renal vasculature. Studies were carried out in the rat isolated perfused kidney pre-constricted with phenylephrine to determine the acute effects of cyclosporine on the dose–vasodilatory response curves of acetylcholine or isoprenaline in the absence and presence of L-NAME, tetraethylammonium, or diclophenac (inhibitors of nitric oxide synthase, K^+ channels, and cyclooxygenase, respectively). The main findings of the study are: (i) whereas all three relaxing factors contributed to acetylcholine vasodilations, responses to isoprenaline were mediated via nitric oxide and EDHF but not prostaglandins; (ii) cyclosporine elicited a concentration-dependent attenuation of acetylcholine or isoprenaline vasodilations; (iii) the identity of the relaxing factor(s) involved in cyclosporine-evoked attenuation of acetylcholine (nitric oxide and EDHF) and isoprenaline (nitric oxide) vasodilations depended on the nature of he vasodilatory stimulus; and (iv) L-arginine supplementation fully abolished the attenuating effect of cyclosporine on isoprenaline vasodilations in contrast to a partial counteraction of cyclosporine impairment of acetylcholine vasodilations. Collectively, these findings demonstrate that cyclosporine attenuates cholinergically and β-adrenergically mediated renal vasodilations, which may contribute to the drug-related vascular toxicity at least at the kidney level. Further, the contributions of the relaxing factors of the endothelium to the vasodilatory responses to acetylcholine or isoprenaline as well as to the attenuation of these responses by cyclosporine are not uniform.

In agreement with earlier reports (Vargas et al., 1994; Stephan et al., 1995), pharmacological studies conducted in the present study showed that renal vasodilatory effect of acetylcholine is largely endothelium dependent and involves increased production of endothelial relaxing factors such as nitric oxide, EDHF, and prostaglandins. Similarly, the vasodilatory effect of isoprenaline depends on the endothelium because the use of L-NAME or tetraethylammonium significantly attenuated isoprenaline vasodilations and their combination abolished 70% of the isoprenaline response. In contrast, vasorelaxant arachidonic acid metabolites are unlikely to be involved in isoprenaline vasodilations because the latter were not altered after cyclooxygenase inhibition by diclophenac. It cannot be argued that the concentration of diclophenac (7 μM) employed in the present study might have not been enough to inhibit cyclooxygenase activity because the same concentration of diclophenac significantly attenuated acetylcholine vasodilations. Further, effective cyclooxygenase inhibition has been achieved by even lower concentrations of the drug in reported studies (Kaw and Hecker, 1999; Kalliovalkama et al., 1999). Surprisingly, Rubanyi and Vanhoutte (1985) reported that cyclooxygenase inhibition accenuates isoprenaline vasodilations in canine coronary arteries probably due to the inhibition of vasoconstrictor prostanoids. Notably, controversial reports are available in the literature with regards to the endothelium-dependence of β-adrenoceptor vasodilations. Isoprenaline relaxations are dramatically abolished by endothelium removal in some studies (Rubanyi and Vanhoutte, 1985; Van der Zypp et al., 2000) but not in others (De Mey and Vanhoutte, 1982). Nitric oxide synthase inhibition also produces variable effects on β-adrenergic relaxations (Moncada et al., 1991; Van der Zypp et al., 2000). These discrepancies may relate to differences in factors such as tissues, animal species, and age (De Mey and Vanhoutte, 1982; Rubanyi and Vanhoutte, 1985; Moncada et al., 1991; Van der Zypp et al., 2000).

An important objective of the present study was to characterize the roles of relaxing factors of the endothelium in the cyclosporine-evoked endothelium dysfunction. The interaction of cyclosporine with renal vasodilations was evaluated in the absence and presence of selective inhibitors of relaxing factors of the endothelium (L-NAME, tetraethylammonium, or diclophenac). We reasoned that the ability of one or more of these inhibitors to attenuate the cyclosporine–endothelium interaction might highlight the involvement of the respective endothelial factor(s) in cyclosporine effects. Under these circumstances, the inhibitor drug would act to eliminate sites at which cyclosporine possibly acts to inhibit endothelial function. The results showed that cyclosporine elicited a concentration-dependent and equipotent attenuation of the vasodilatory responses to acetylcholine and isoprenaline in the renal vasculature. The present study provided the first experimental evidence that the nature of the endothelial relaxing factors involved in the interaction of cyclosporine with the two vasorelaxants was not identical. Comparison of the percentage reductions in the vasodilatory responses caused by the highest dose (2 nmol) of acetylcholine showed that the inhibition of nitric oxide synthase or blockade of K^+ channels with L-NAME and tetraethylammonium, respectively, produced significant and equivalent attenuation (35%) of the inhibitory effect of cyclosporine on acetylcholine vasodilations in renal tissues. These findings may suggest important roles for endothelial nitric oxide and EDHF in cyclosporine-induced impairment of acetylcholine renal vasodilations. In contrast, the lack of an effect of cyclooxygenase inhibition by diclophenac on cyclosporine

impairment of acetylcholine vasodilations argues against a possible role for prostaglandins in cyclosporine–acetylcholine interaction. On the other hand, the attenuation of isoprenaline vasodilations by cyclosporine seems to be mediated solely via the inhibition of endothelial nitric oxide activity because cyclosporine effects were completely abolished in tissues pretreated with L-NAME but remained intact in tissues exposed to tetraethylammonium. These findings together with the observations that L-arginine supplementation of cyclosporine-treated preparations completely normalized isoprenaline vasodilations and caused a partial restoration of acetylcholine vasodilations establish pharmacological evidence that the cyclosporine-induced inhibition of isoprenaline vasodilations is mediated exclusively via inhibition of nitric oxide activity whereas the inhibition of acetylcholine responses by cyclosporine involves both nitric oxide and EDHF components.

Given that the present study was undertaken in the isolated perfused kidney as a whole, whether the altered renal endothelial function caused by cyclosporine or selective inhibitors of endothelial relaxing factors involved interaction with similar or different sites (e.g. afferent or efferent arterioles) of the renal vasculature cannot be ascertained from the present findings. Nonetheless, evidence is available that the adverse renovascular effects of cyclosporine are confined to the small renal arteries and afferent arterioles (Myers et al., 1988). Further, nitric oxide has been shown to exert a greater vasodilatory effect in the afferent than in the efferent glomerular arterioles (Ito et al., 1993). Similarly, a selective modulatory role for EDHF on afferent but not efferent arteriolar tone has been documented (Wang and Loutzenhiser, 2002). These findings together may infer a possible role for vascular endothelium of the preglomerular vessels as a shared target for cyclosporine and selective inhibitors of endothelial relaxing factors.

Notably, tetraethylammonium was employed in the present study to verify the involvement of K^+ channels in the cyclosporine–endothelium interaction. Tetraethylammonium suffers two limitations. First, it has been shown to alter nitric oxide-mediated responses (Mieyal et al., 1998) and, therefore, may affect data interpretation and conclusions. Nevertheless, the present study presented evidence that argues against the presence of a mutual facilitation between endothelial nitric oxide and EDHF (Lindauer et al., 2003). This is because the attenuation of renal acetylcholine vasodilations caused by pharmacologic inhibition of each of these two factors by L-NAME and tetraethylammonium, respectively, was not altered when tested in the presence of the inhibitor of the other factor, suggesting that the two endothelial factors (nitric oxide and EDHF) act independently. The second limitation that pertain to the use of tetraethylammonium is its nonselectivity towards various types of K^+ channel, which are believed to contribute differently to vasodilator responses in the rat perfused kidney (Mieyal et al., 1998; Wang et al., 2003). It is important, therefore, to evaluate the effect of selective K^+ channel inhibitors such as glibenclamide (inhibitor of ATP-sensitive K^+ channels), charybdotoxin (inhibitor of Ca^{2+}-activated K^+ channels) and aminopyridine (inhibitor of voltage-gated K^+ channels) on the renal effects of cyclosporine. This issue will be addressed in future studies.

In summary, the present study evaluated the roles of relaxing factors of the endothelium (nitric oxide, EDHF, and prostaglandins) in the cyclosporine-induced attenuation of renovascular vasodilations evoked by activation of muscarinic (acetylcholine) receptors and β-adrenoceptors (isoprenaline) in the renal vasculature. The results demonstrated that cyclosporine attenuated the vasodilatory responses elicited by acetylcholine or isoprenaline in a concentration-related manner. The attenuation of isoprenaline vasodilations by cyclosporine is exclusively mediated via the inhibition of endothelial nitric oxide activity because (i) the attenuation of isoprenaline vasodilations by cyclosporine was abolished in the presence of the nitric oxide synthase inhibitor L-NAME; and (ii) L-arginine supplementation fully reversed the attenuating effect of cyclosporine on isoprenaline vasodilations. On the other hand, the inhibition of endothelial nitric oxide and EDHF activities similarly contribute to cyclosporine–acetylcholine interaction. These findings may add insights into the understanding of circulatory mechanisms involved in the adverse vascular effects known to associate cyclosporine therapy (Shulman et al., 1981; Gerkens, 1989; Gossmann et al., 2001).

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