PROTECTIVE EFFECT OF L-ARGININE AGAINST NEPHROTOXICITY INDUCED BY CYCLOSPORINE IN NORMAL RATS

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Effects of L-arginine (L-arg) and aminoguanidine (AG) on the nephrotoxicity induced by cyclosporine (CsA) were investigated. After injection of CsA (15 mg kg\textsuperscript{-1} day\textsuperscript{-1} i.p. for 10 days), it induced nephrotoxicity, manifested biochemically by a significant elevation of serum urea and creatinine. In addition, a marked increase in lipid peroxides measured as malondialdehyde (MDA) as well as a significant decrease in glutathione peroxidase (GSH-Px) activity (EC.1.11.1.9) and reduced glutathione content (GSH) in kidney tissues homogenate were observed. Nephrotoxicity was further confirmed by histopathological investigation. Oral administration of L-arg (300 mg kg\textsuperscript{-1} day\textsuperscript{-1} orally) for 5 days before and 10 days concomitant with CsA injection produced a significant protection against nephrotoxicity induced by CsA. The amelioration of nephrotoxicity was evidenced by significant reductions in serum urea and creatinine concentrations. In addition, L-arg prevented the rise of MDA as well as reduction of GSH-Px activity and reduced GSH content in kidney tissue. The protective effects of L-arg against CsA-induced nephrotoxicity were further confirmed by histopathological examination. However, oral supplementation of AG (100 mg kg\textsuperscript{-1} day\textsuperscript{-1} p.o.) did not protect the kidney from the damaging effects of CsA. These results suggest that L-arg can ameliorate kidney dysfunction induced by CsA via a mechanism(s) which involves the production of nitric oxide. In addition, L-arg may therefore be a beneficial remedy for CsA nephrotoxicity and can be used to improve the therapeutic index of CsA.

\textbf{Keywords:} aminoguanidine, L-arginine, cyclosporine, oxidative stress, nephrotoxicity.

INTRODUCTION

Cyclosporine (CsA) is the immunosuppressive of choice in human organ transplantation. The clinical usage of CsA is however restricted due to both functional and structural changes in the kidney of transplant patients and experimental animals [1–3]. The renal damage consequent to CsA administration ranges from haemodynamic alteration to irreversible chronic lesion [1]. Consequently, there is great interest to increase its clinical usefulness by developing new agents in order to reduce its toxicity [3].

The mechanism of nephrotoxicity induced by CsA is thought to be due to afferent arteriolar vasoconstrictor effect and consequently decreased extremely glomerular filtration rate [4]. CsA administration produces an acute decrease in renal cortical and medullary blood flow [5]. Chronic CsA administration leads to loss of proximal tubular epithelial integrity with secondary fibrosis [6]. In addition, CsA induces structural changes due to direct vasoconstrictive effects with subsequent ischaemia. The early medullary changes such as tubulo-interstitial proliferation and macrophage infiltration correlate with the decreased urinary concentrating ability and elevated creatinine level [3]. The initial vasoconstriction induced by CsA depends on the imbalance between the various modulators of the renal vascular tone, among which are the most powerful: endothelins and nitric oxide (NO) [7]. However, lipid peroxidation and free radical generation in the tubular cells have been suggested to be responsible for the kidney damage [8, 9].

NO is one of the smallest biologically active molecules, produced from L-arginine (L-arg) by nitric oxide synthase (NOS) [10]. There are three isoforms of NOS: the endothelial type (eNOS), the neuronal type (nNOS) and the isoform expressed de novo by the exposure to pro-inflammatory cytokins, the inducible type (iNOS). Under pathological conditions iNOS catalyses an adequate quantity of iNO [11]. The L-arg/NO pathway in the vascular endothelium was discovered in the last decade. It has been shown that L-arg increases renal blood flow...
and glomerular filtration rate in normal rat through a NO-mediated mechanism [12]. In addition, Bharadway and Moore [13] showed that L-arg injection produced vasodilation in the study of perfused kidney of rat.

Aminoguanidine (AG), inhibits iNOS in a selective and competitive manner, leading to decreased generation of iNO [11]. In addition, AG is endowed with many other activities which together account for its beneficial activities. It has been found that AG inhibits diamine oxidase [14], binds to sites of non-enzymatic glycosylation and prevents further advanced glycosylation [15]. Previous study pointed to the beneficial antioxidant effects of AG [16]. In addition, AG acts as a peroxynitride scavenger [17].

The protective effects of L-arg had been previously addressed in nephrotoxicity induced by cisplatin [18]. In addition, previous reports [7, 19] showed that administration of L-arg to rats greatly ameliorated kidney dysfunction induced by CsA treatment. However, this has been recently disputed by Lassila et al. [20] who showed that treatment with L-arg did not affect renal dysfunction induced by CsA. Moreover, the beneficial effects of AG in various experimental models of cell damage induced by drugs had been reported [21, 22]. However, the effect of AG in kidney damage induced by CsA has not been addressed before. Therefore the present work was conducted to investigate the possible protective effect of L-arg and AG on nephrotoxicity induced by CsA. This aim will be achieved by studying the effect of CsA on some biochemical parameters related to renal functions. Furthermore, the possible effect of L-arg and AG on CsA-induced kidney injury will be investigated.

MATERIALS AND METHODS

Chemicals

Cyclosporin was purchased from Sandoz Pharma Ltd, (Basle, Switzerland), while AG and L-arg were obtained from Sigma (St Louis, MO, USA). Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). All the remaining chemicals were of the highest grade commercially available.

Animals

Male Swiss albino rats, weighing 200–250 g were used in all experiments. They were obtained from the Experimental Animal Care Centre of King Saud University, Riyadh, K.S.A. Animals were maintained under standard conditions of temperature 24 ± 1°C and 55 ± 5% relative humidity with regular 12 h light:12 h dark cycle and allowed free access to standard laboratory food (Purina chow) and water.

Animal treatments

The animals were divided at random into six groups of 10 animals each. The first group (control) received vehicles used for CsA (physiological saline solution, intraperitoneally (i.p.)). The second group, L-arg in drinking water (300 mg kg⁻¹ day⁻¹ p.o.). The third group, received AG in drinking water (100 mg kg⁻¹ day⁻¹ p.o.). The calculated doses of L-arg and AG were based on the average daily intake of water. The fourth group, was injected with CsA (15 mg kg⁻¹ day⁻¹ i.p.) for 10 days. In our own preliminary experiments, we used different concentrations of CsA. The selected dose of CsA induced nephrotoxicity. The fifth group received L-arg in drinking water (300 mg kg⁻¹ day⁻¹ p.o.) for 5 days, then was injected with CSA (15 mg kg⁻¹ day⁻¹ i.p.) for 10 days concomitantly with L-arg in drinking water (300 mg kg⁻¹ day⁻¹ p.o.). The last group received AG in drinking water (100 mg kg⁻¹ day⁻¹ p.o.) for 5 days, then was treated with CsA (15 mg kg⁻¹ day⁻¹ i.p.) for 10 days concomitantly with AG in drinking water (100 mg kg⁻¹ day⁻¹ p.o.). Based on preliminary data from our laboratory, the selected concentrations of L-arg and AG and the schedule of doses were chosen.

One day later, blood samples were taken by cardiac puncture, under light ether anaesthesia, into non-heparinized tubes. Serum was separated by centrifugation for 5 min at 4000 rpm and stored at −20°C until analysis. Animals were killed by cervical dislocation and the kidneys were quickly isolated, washed with saline, blotted dry on filter paper, weighed, and then 10% (w/v) homogenate of the kidney was made in ice cold saline using a Branson sonifier (250, VWR Scientific, Danbury, CT, USA).

Measurement of serum biochemical parameters

Serum creatinine and urea concentrations were determined colorimetrically as described by Bartles et al. [23], and Patton and Crouch [24], respectively, using commercially available diagnostic kits (bioMérieux-RCS Lyon-France).

Determination of lipid peroxides, glutathione content and glutathione peroxidase enzyme activity in kidney homogenate

Glutathione contents and lipid peroxidation [malondialdehyde (MDA) production] in the kidney tissues were determined according to Eillman [25] and Ohkawa et al. [26], respectively. Glutathione peroxidase (GSH-Px) activity was measured in the kidney homogenates according to Kraus and Ganther [27].

Histopathology

Histopathological examination was performed on about 50% of randomized animals of each group. Kidney samples were taken from different areas of the kidney. The tissues were fixed for at least 48 h in 10% formalin in phosphate buffer (pH 7). The samples were then embedded in paraffin wax, cut into 5 µm sections, and stained with haematoxylin-eosin. The slides were coded and were examined by a histopathologist who was unaware of the treated groups. The nephrotoxic histological features were graded as follows:
I. Low grade tubular necrosis—small predominantly subcapsular foci of the tubular necrosis occupying and seen in up to two low power light microscopic field-marks given 1+.

II. High grade tubular necrosis—multiple and larger cortical medullary foci of tubular necrosis occupying more than two low power light microscopic field-marks given 2+.

III. Interstitial nephritis 1+.

The marking system used was according to the following:

A. No nephrotoxic effect: 0+ only
B. Mild nephrotoxic effect: 1+ only
C. Moderate nephrotoxic effect: 2+ only
D. Severe nephrotoxic effect: 3+ only

Different sections from kidney tissues were taken and examined, since the necrosis of the tubular cells is coagulative and the damage was unevenly distributed. The sections which are selected can show renal damage by light microscope examination.

Statistical analysis

Data are expressed as (means ± SEM). Statistical comparison between different groups was done using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test to judge the difference between various groups. Significance was accepted at $P < 0.05$.

RESULTS

Effects of L-arg and AG on CsA-induced changes in serum biochemical parameters

Table I demonstrates the effect of L-arg and AG on kidney damage induced by injection of CsA (15 mg kg$^{-1}$ day$^{-1}$ i.p. for 10 days). Intraperitoneal administration of CsA caused abnormal renal function in all rats. Serum urea and creatinine were significantly increased in comparison with the control group. Pre-treatment of animals with L-arg (300 mg kg$^{-1}$ day$^{-1}$ p.o.) 5 days before and concomitantly with CsA markedly reduces the rise in the level of serum urea and creatinine. However, AG pre-treatment (100 mg kg$^{-1}$ day$^{-1}$ p.o.) did not attenuate nephrotoxicity induced by CsA.

Effects of L-arg and AG on CsA-induced changes in lipid peroxides, glutathione content and glutathione peroxidase enzyme activity in kidney tissue homogenate

Table II shows the effect of L-arg and AG pre-treatment on CsA induced changes in lipid peroxides measured as MDA, glutathione content and GSHPx enzyme activity in kidney tissue homogenate. Intraperitoneal administration of CsA (15 mg kg$^{-1}$ day$^{-1}$ i.p. for 10 days) caused kidney damage which is manifested by a significant increase in MDA, a significant decrease in reduced GSH content and GSHPx enzyme activity in kidney tissue homogenate. Pre-treatment of animals with L-arg (300 mg kg$^{-1}$ day$^{-1}$ p.o.) 5 days before and concomitantly with CsA prevented the significant increase in MDA. In addition, the activity of GSHPx enzyme was greatly improved. Moreover, L-arg ameliorated the depletion of the glutathione content. However, oral supplementation of AG (100 mg kg$^{-1}$ day$^{-1}$ p.o.) did not ameliorate the depletion of reduced GSH content or decreasing GSHPx enzyme activity or prevent the rise in lipid peroxides.

Kidney pathology

Pathological examination of the kidneys of control, L-arg and AG groups showed non-significant changes (Fig. 1). However, animals treated with CsA showed vacuolation and focal necrosis of tubular lining and interstitial cell infiltration (Fig. 2). Pre-treatment of rats with L-arg for 5 days and concomitantly with CsA for 10 days showed very mild focal tubular necrosis in comparison to CsA treated animals (Fig. 3). While the group receiving oral AG for 5 days and concomitantly...
The nephrotoxic potential of CsA, however, limits its clinical use. Consequently, there is much interest in developing new methods to abrogate renal damage by using a combination of various agents with CSA [3].

DISCUSSION

The present investigation revealed that administration of CsA (15 mg kg\(^{-1}\) day\(^{-1}\) i.p. for 10 day) resulted in an overt nephrotoxicity as evidenced in the serum by the marked elevations of urea and creatinine concentrations. In addition, nephrotoxicity was also reflected in the kidney as the depletion of reduced glutathione content which is associated with a marked reduction in GSH-Px activity and significant elevation of lipid peroxide. The nephrotoxic potential of CsA, however, limits its clinical use [7]. Consequently, there is much interest in developing new methods to abrogate renal damage by using a combination of various agents with CSA [3].

In the present study, treatments of rats with L-arg (300 mg kg\(^{-1}\) day\(^{-1}\), p.o.) did not induce any changes in the measured biochemical parameters. However, providing L-arg with drinking water (300 mg kg\(^{-1}\) day\(^{-1}\) p.o.) 5 consecutive days before and continuing during the course of CsA administration renders rats less susceptible to kidney damage induced by treatment with CsA. This protection was evidenced in the serum as the elevated level in both urea and creatinine were markedly lowered below those elicited by the nephrotoxicant. In addition, L-arg greatly ameliorated reduced levels in GSH-Px activity, reduced GSH content in kidney tissue and prevented the rise in lipid peroxides in kidney tissues. These findings may indicate a possible protective effect of L-arg against nephrotoxicity induced by CsA treatment.

This finding is in harmony with the previous reports demonstrating that exogenous supplementation of L-arg is effective in reducing renal damage induced

**Table I**

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Control</th>
<th>L-arg</th>
<th>AG</th>
<th>CsA</th>
<th>CsA + L-arg</th>
<th>CsA + AG</th>
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</thead>
<tbody>
<tr>
<td>Urea (mg%)</td>
<td>68 ± 4.8</td>
<td>70 ± 5.7</td>
<td>71 ± 6.8</td>
<td>102 ± 6.4(^\ast)</td>
<td>72.7 ± 6.3(^#)</td>
<td>97.7 ± 6.4(^\ast)</td>
</tr>
<tr>
<td>Creatinine (mg%)</td>
<td>0.7 ± 0.05</td>
<td>0.7 ± 0.1</td>
<td>0.68 ± 0.2</td>
<td>1.13 ± 0.1(^\ast)</td>
<td>0.69 ± 0.04(^#)</td>
<td>1.02 ± 0.04(^\ast)</td>
</tr>
</tbody>
</table>

All data represents mean ± SEM ($n = 10$); L-arg and AG were given orally for 5 days before and 10 days after CsA administration (300 mg kg\(^{-1}\) day\(^{-1}\) and 100 mg kg\(^{-1}\) day\(^{-1}\), respectively). Blood samples were obtained 10 days after CsA (15 mg kg\(^{-1}\) day\(^{-1}\) i.p.). * Significant difference from control group; # significant difference from CsA group. \(* P < 0.05\).

**Table II**

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Control</th>
<th>L-arg</th>
<th>AG</th>
<th>CsA</th>
<th>CsA + L-arg</th>
<th>CsA + AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol g(^{-1}) wet tissue)</td>
<td>55 ± 1.3</td>
<td>54.3 ± 1.4</td>
<td>55 ± 0.2</td>
<td>75 ± 2.0(^\ast)</td>
<td>54 ± 1.9(^#)</td>
<td>74 ± 1.17(^\ast)</td>
</tr>
<tr>
<td>GSH (µmol g(^{-1}) wet tissue)</td>
<td>5.6 ± 0.12</td>
<td>5.1 ± 0.28</td>
<td>5 ± 0.15</td>
<td>3 ± 0.05(^\ast)</td>
<td>5 ± 0.17(^#)</td>
<td>3 ± 0.24(^\ast)</td>
</tr>
<tr>
<td>GSH-Px (µmol min(^{-1}) g(^{-1}) tissue)</td>
<td>3.4 ± 0.01</td>
<td>3.3 ± 0.02</td>
<td>3.4 ± 0.02</td>
<td>2.4 ± 0.02(^\ast)</td>
<td>3.4 ± 0.02(^#)</td>
<td>3.3 ± 0.02(^\ast)</td>
</tr>
</tbody>
</table>

All data represents mean ± SEM ($n = 10$); L-arg and AG were given orally for 5 days before and 10 days after CsA administration (300 mg kg\(^{-1}\) day\(^{-1}\) and 100 mg kg\(^{-1}\) day\(^{-1}\), respectively). Blood samples were obtained 10 days after CsA (15 mg kg\(^{-1}\) day\(^{-1}\) i.p.). * Significant difference from control group; # significant difference from CsA group. \(* P < 0.05\).
by CsA, possibly through the NO pathway [7, 19]. In addition Assiss et al. [28], and De Nicola et al. [29], showed that oral supplementation of L-arg prevents nephrotoxicity induced by chronic administration of CsA due to formation of more NO which may enhance vasodilatation and consequently reduce the kidney function impairment. Oriji and Keiser [30] reported that CsA administration inhibits the endothelial NOS enzyme and this inhibition can be overcome by parenteral administration of L-arg. Therefore, the protective effect of L-arg against kidney dysfunction may be related to its reported vasodilatory effect. However, the results of the present study revealed that L-arg prevents the rise in lipid peroxides and the significant decrease in reduced GSH content and GSH-Px activity induced by CsA treatment. So it seems that the protective effect induced by L-arg against nephrotoxicity induced by CsA may involve an additional non-haemodynamic cytoprotective effect. However, it is difficult to assess which of these properties is responsible for the protective effect induced by L-arg.

Bobadilla et al. [5], studied the role of NO in renal haemodynamic abnormalities and concluded that the production of NO seems to be normal in CsA nephrotoxicity. In addition, the renal vasoconstriction associated with CsA is not mediated by NO deficiency. Moreover, Lassila et al. [20] reported that pre-treatment with L-arg did not affect renal damage induced by CsA. Recently, Zhang et al. [31], reported that L-arg supplementation in young recipients with chronic renal allograft dysfunction did not improve renal function. This discrepancy with our results may be due to the L-arg dose schedule. In the present study, L-arg was orally supplemented before and during CsA administration.

The rationale for L-arg dose schedule in this study was to maintain sufficient plasma concentration before, during and after the critical periods of CsA-induced renal damage, since the biochemical changes that occur in the kidney after CsA administration are of crucial importance in determining the extent of nephrotoxicity [32].

In the present study, treatment of rats with AG (100 mg kg−1 day−1 p.o.) did not induce any changes in the measured biochemical parameters. In addition, oral supplementation of AG in drinking water for 5 days to the rats, followed by CsA administration (15 mg kg−1 day−1 i.p.) for 10 days, did not result in any protection against kidney damage induced by CsA. This finding was further confirmed by a histopathological study. This finding supports the previous report of Wu et al. [33], who studied the effect of AG and CsA on iNOS of mouse cultured mTAL cells and demonstrates that AG inhibits iNOS in a very similar way to inhibition of iNOS by CsA. These findings suggest that CsA-induced nephrotoxicity cannot be protected by iNOS blockade or ameliorated by the substrate for NO production.

In conclusion, the results of the present study may indicate that L-arg is beneficial as a protective agent against nephrotoxicity induced by CsA possibly through implication of NO. In contrast, AG did not attenuate renal damage induced by CsA treatment. Further studies are greatly needed to elucidate the exact mechanism(s) of protection and the effect of L-arg on the pharmacological effect of CsA.

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