The effects of valsartan on renal glutathione peroxidase expression in alleviation of cyclosporine nephrotoxicity in rats

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Introduction
Transplantation medicine is a preferable treatment for patients with end-stage renal diseases. It has achieved excellent short-term outcomes, but long-term outcomes have not improved since the introduction of the cyclosporine-A (CsA) in the late 1970s.2,3 CsA, as an immuno-suppressive drug with a short-term outcome, has been widely used to avoid organ transplant rejection.2 Nephrotoxic side effect of CsA can be the Achilles' heel of this immunosuppressive drug.1,3 CsA nephrotoxicity that may eventually lead to nephropathy can be an important cause of renal dysfunction, the most important reason of graft loss.4 Although CsA-induced nephrotoxicity is a multifactorial complex,5 based on previous studies, oxidative stress...
and high reactive oxygen species (ROS) production may play an important role in its pathogenesis. Glutathione peroxidase (GPx) as an antioxidant enzyme is found in all mammalian organs. It can protect cells against oxidative damage by reducing hydrogen peroxide and organic peroxides with reduced glutathione. The level of GPx expression varies according to the types of the tissues. High amounts of GPx are detected in kidneys. Therefore, it may play an important role in renal protection against oxidative stress.

There are some present and potential strategies to diminish CsA-induced nephrotoxicity. It has been shown that antihypertensive drug, valsartan (Val), has also renoprotective effects as documented by reduced urinary albumin and protein excretion in patients with diabetes and/or chronic kidney disease. Val is an angiotensin II receptor blocker (ARB) which blocks angiotensin II type 1 (AT1) receptor and mediates the blood pressure (BP) elevating effect of oxidative stress. Therefore, it may play an important role in renal protection against oxidative stress.

Analysis of renal GPx expression
Quantitative real-time polymerase chain reaction
Total RNA was extracted from the kidney tissues using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Then, 1 μg of the total RNA was used for complementary DNA (cDNA) synthesis with Oligo (dT) primers using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA USA) according to the manufacturer’s instruction. Quantitative real-time polymerase chain reaction (PCR) analysis was performed in duplicate using SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) on the Rotor-Gene 6000 real-time PCR detection system (Corbett Research, Sydney, Australia). Results were normalized to the gene expression of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. The used specific primer-sequences were 5’-CGGTTTCCCGTG-CAATCAGT-3’ as the forward and 5’-ACACCGGGGAC-CAAATGATG-3’ as reverse primer. PCR program consisted of a preincubation step at 94°C for 7 min, followed by 40 cycles of denaturation (94°C, 20 s), annealing (64°C, 30 s), and extension (72°C, 30 s) steps with the final extension step at 72°C for 10 min. The 2−ΔΔCt formula was used to calculate the gene expression ratio of GPx to GAPDH with respect to the control group.

Biochemical parameters
Plasma concentrations of urea and creatinine (Cr) were determined by a colorimetry method using commercial reagents in an automated chemical analyzer (Roche Cobas Mira, Basel, Switzerland). Furthermore, deteriorated renal failure (DRF) index was calculated by “(Cr + urea)/2” formula as a way to better estimate the glomerular filtration rate (GFR).

Oxidative stress markers
Plasma levels of GPx were measured by a rat enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO, Wuhan, China). 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress to DNA, was also measured by ELISA.
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Malondialdehyde (MDA) level as a lipid peroxidation marker was measured by spectrophotometric method using thiobarbituric acid reactive substances (TBARS) assay according to the method of Lapenna et al.\textsuperscript{20} Protein oxidation was evaluated spectrometrically by measuring protein carbonyls according to the method described by Reznick et al.\textsuperscript{21}

**Histological assessment**

Formalin fixed kidney samples were subjected to dehydration in the ascending grades of ethanol and were kept in xylene overnight for complete dehydration. After embedding in paraffin, the tissue sections of 5 µm thick were cut and subsequently stained with hematoxylin and eosin. Thereafter, at least 10 fields of each slide were examined by a pathologist blinded to the grouping of the rats at ×200 magnification under light microscope.

**Statistical analysis**

Data are shown as mean ± standard deviation (SD). Statistical comparisons of the groups were carried out by one-way analysis of variance (ANOVA) and Bonferroni post hoc analysis. Pearson correlation coefficient was also calculated. A p value of less than 0.05 was considered statistically significant. The analyses were performed in SPSS 16.0 software.

**Results**

**Body weights and plasma biochemical parameters**

The basic parameters of the study groups after the treatment period are depicted in Table 1. The rats were weight matched before the intervention (p>0.05), but after the intervention, group B (CsA) had significantly lower mean body weight (BW) than the other groups (p<0.05). Furthermore, the rats receiving CsA (group B) were detected to have significantly (p<0.05) higher plasma urea, Cr and DRF-index levels than the other study groups.

**Renal expression and plasma levels of GPx**

The results of real-time PCR revealed that the renal expression of GPx was significantly (p<0.05) lower in CsA group than in other study groups (Fig. 1A). It is remarkable that the changes in plasma levels of GPx were also conformed to the results of expression alteration (Fig. 1B).

**Table 1. Biochemical parameters in the study groups**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (Control)</th>
<th>Group B (CsA)</th>
<th>Group C (Val+CsA)</th>
<th>Group D (Val)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW\textsubscript{a.i} (g)</td>
<td>209.80 ± 13.78</td>
<td>208.24 ± 11.13</td>
<td>207.77 ± 7.00</td>
<td>210.12 ± 12.39</td>
</tr>
<tr>
<td>BW\textsubscript{b.i} (g)</td>
<td>231.00 ± 16.04</td>
<td>192.47 ± 18.68</td>
<td>225.61 ± 24.55</td>
<td>242.22 ± 21.13</td>
</tr>
<tr>
<td>Cr (mg/dL)</td>
<td>1.05 ± 0.21</td>
<td>1.99 ± 0.81\textsuperscript{a}</td>
<td>1.17 ± 0.71\textsuperscript{b}</td>
<td>0.85 ± 0.77\textsuperscript{c}</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>60.14 ± 14.48</td>
<td>107.87 ± 47.66\textsuperscript{e}</td>
<td>68.86 ± 24.97\textsuperscript{d}</td>
<td>57.66 ± 23.70\textsuperscript{c}</td>
</tr>
<tr>
<td>DRF index</td>
<td>30.59 ± 7.13</td>
<td>54.93 ± 19.22\textsuperscript{f}</td>
<td>35.01 ± 11.33\textsuperscript{g}</td>
<td>29.25 ± 6.81\textsuperscript{h}</td>
</tr>
</tbody>
</table>

Abbreviations: a.i., after intervention; b.i., before intervention; BW, body weight; Cr, creatinine; CsA, Cyclosporine-A; DRF, deteriorated renal failure; Val, valsartan.

\textsuperscript{a} p<0.001 vs. control; \textsuperscript{b} p=0.021 vs. CsA; \textsuperscript{c} p<0.001 vs. CsA; \textsuperscript{d} p=0.010 vs. control; \textsuperscript{e} p=0.041 vs. CsA; \textsuperscript{f} p=0.001 vs. CsA; \textsuperscript{g} p=0.001 vs. control; \textsuperscript{h} p=0.040 vs. CsA; \textsuperscript{i} p=0.001 vs. CsA; \textsuperscript{j} p=0.001 vs. control; \textsuperscript{k} p=0.035 vs. CsA; \textsuperscript{l} p=0.001 vs. CsA.

Data are shown as Mean ± SD.

**Plasma levels of oxidative stress markers**

Plasma levels of 8-OHdG, MDA, and PCG as oxidative stress markers were measured and compared among the study groups. As presented in Fig. 2, the rats receiving CsA were detected to have significantly (p<0.05) higher plasma 8-OHdG, MDA, and PCG levels than the other study groups.

![Fig. 1. Renal expression and plasma levels of glutathione peroxidase (GPx) affected by cyclosporine (CsA) and/or valsartan (Val) in the study groups. \(A\) Real-time PCR analyses revealed that renal expression of GPx were significantly downregulated in CsA group (0.45 ± 0.12; fold change) compared to control (1.05 ± 0.35; fold change), CsA+Val (0.93 ± 0.43; fold change), and Val groups (1.43 ± 0.58; fold change); \(B\) Plasma levels of GPx in CsA group (98.57 ± 34.11; mIU/mL) were significantly lower than those in control (212.14 ± 56.75; mIU/mL), CsA+Val (192.00 ± 42.14; mIU/mL), and Val groups (265.55 ± 82.43; mIU/mL). Data are presented as mean ± SD. *p<0.05 vs. control; #p<0.05 vs. CsA.\]
Correlations of GPx, oxidative stress markers, and DRF index

As shown in Table 2, GPx was negatively correlated with oxidative stress markers and DRF index as well. The plasma level of MDA was positively correlated with 8-OHdG, as well as PCG levels. Plasma PCG and 8-OHdG levels were also positively correlated. Each of these oxidative stress markers was positively correlated with DRF index.

Histological findings

Histological changes including hyperemia, inflammatory cell infiltration, and vacuolization were exhibited in the CsA treated rats. Val ameliorated these histological changes (Fig. 3).

Discussion

CsA as a calcineurin inhibitor prevents calcineurin, a protein-phosphatase involved in the activation of T-cells, and reduces the activity of the immune system. As an immunosuppressant drug, CsA is widely used to prevent organ transplantation rejection, but its nephrotoxic side effect may lead to dramatic problems post-transplantation. There is much evidence that suggests oxidative stress may have an important role in CsA-induced toxicity. In the present study, we demonstrated that the plasma levels of 8-OHdG, a direct indicator of oxidative DNA damage; MDA, a marker determining the degree of lipid peroxidation; and also protein oxidation marker, PCG, were significantly higher in CsA group compared with the other study groups. Plasma levels of GPx were also decreased by CsA treatment. This result can confirm the oxidative stress-inducing effect of CsA. GPx acts in the scavenging and inactivating of ROS, thereby protecting the body against oxidative stress. Reduced renal GPx level in the present study might be a reason of oxidative stress elevation by CsA. Another mechanisms can also be assumed. Yoon et al. in their study showed that CsA could downregulate the antioxidant enzymes, manganese superoxide dismutase (MnSOD) and hemeoxygenase-1.

Based on previous studies, the adverse effects of CsA can be alleviated by some agents such as SKF 106203 (a leukotriene receptor antagonist) and paricalcitol (an active and nonhypercalcemic analog of vitamin D). Moreover, recently hydrogen sulphide was introduced as a new ther-
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CsA could deteriorate renal function possibly through renal GPx downregulation and oxidative stress enhancement. Val treatment might ameliorate CsA-induced nephrotoxicity via renal GPx upregulation that could eventually lead to oxidative stress decrease.

Fig. 4. Mechanism of valsartan (Val) effect in alleviation of cyclosporine (CsA) nephrotoxicity. CsA could deteriorate renal function possibly through renal GPx downregulation and oxidative stress enhancement. Val treatment might ameliorate CsA-induced nephrotoxicity via renal GPx upregulation that could eventually lead to oxidative stress decrease.

apogetic agent that can reverse the vasoconstriction changes associated with CsA treatment during reperfusion. Due to the renoprotective effect of Val, we initially hypothesized that it might also lead to the diminution of the nephrotoxicity of CsA. It has been shown that Val treatment does not interfere with immunosuppressive therapy. Val can therapeutically be administered to patients with kidney diseases, as hypertension is a prominent cause and also outcome of kidney diseases. Effects of different ARBs on BP are close to each other, but some studies have shown that Val is the most specific, most effective, and the safest drug of all. These factors may make Val to be distinct among other agents in alleviating the CsA nephrotoxicity. The exact mechanism of Val renal protection has not yet been clear. Some previous studies have demonstrated that the renoprotective effect of Val is independent from BP changes. In our study, the dose of Val was 30 mg/kg/day; based on previous studies, this dose of Val does not change BP in the rats. Due to the elimination of BP lowering role, the other potential mechanisms for the renal protection of Val against CsA nephrotoxicity could be possible. Jiao et al. and Wu et al. in their study showed that Val could decrease TGF-β1 and MCP-1 expression and oxidative stress in cultured in high-glucose and oxidative stress conditions. Jiao et al. in their study showed that Val could decrease TGF-β1 and MCP-1 expression and oxidative stress in cultured in high-glucose and oxidative stress conditions. These mechanisms might be related with the renal protection of Val.

Conclusion

In the present study, it was demonstrated that CsA could deteriorate the renal function possibly through the downregulation of renal GPx and enhancement of oxidative stress enhancement. Val treatment might ameliorate CsA-induced nephrotoxicity via renal GPx upregulation that could eventually lead to oxidative stress decrease (Fig. 4). Further studies are needed to better understand the mechanisms involved in CsA nephrotoxicity and Val renal protection as well.

Ethical approval

The Animal protocol in the present study was based on the Guide for Research Highlights

What is current knowledge?

√ CGPx and oxidative stress may have important roles in CsA nephrotoxicity and Val renal protection as well.

What is new here?

√ Val could alleviate the CsA-induced nephrotoxicity via the upregulation of renal GPx and reduction of oxidative stress.
Care and Use of Laboratory Animals [U.S. Department of Health, Education, and Welfare (DHEW), Publication number 78-23, National Institutes of Health (NIH), revised 1978] and local guidelines for compassionate use of animals in research.

Competing interests
Authors declare no conflict of interests.

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References