Annexin A1 protein attenuates cyclosporine-induced renal hemodynamics changes and macrophage infiltration in rats
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Received: 3 September 2011 / Revised: 30 October 2011 / Accepted: 2 November 2011 / Published online: 19 November 2011
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Abstract

Background Cyclosporine (CsA) remains an important immunosuppressant for transplantation and for treatment of autoimmune diseases. The most troublesome side effect of CsA is renal injury. Acute CsA-induced nephrotoxicity is characterized by reduced renal blood flow (RBF) and glomerular filtration rate (GFR) due to afferent arteriole vasoconstriction. Annexin A1 (ANXA1) is a potent anti-inflammatory protein with protective effect in renal ischemia/reperfusion injury. Here we study the effects of ANXA1 treatment in an experimental model of acute CsA nephrotoxicity.

Methods Salt-depleted rats were randomized to treatment with VH (vehicles 1 mL/kg body weight/day), ANXA1 (Ac2-26 peptide 1 mg/kg body weight/day intraperitoneally), CsA (20 mg/kg body weight/day subcutaneously) and CsA + ANXA1 (combination) for seven days. We compared renal function and hemodynamics, renal histopathology, renal tissue macrophage infiltration and renal ANXA1 expression between the four groups.

Results CsA significantly impaired GFR and RBF, caused tubular dilation and macrophage infiltration and increased ANXA1 renal tissue expression. Treatment with ANXA1 attenuated CSA-induced hemodynamic changes, tubular injury and macrophage infiltration.

Conclusion ANXA1 treatment attenuated renal hemodynamic injury and inflammation in an acute CsA nephrotoxicity model.

Keywords Acute renal injury · Annexin A1 · Cyclosporine nephrotoxicity · Immunosuppression · Inflammation

Introduction

Cyclosporine A (CsA) is currently widely used in the immunosuppressive protocol of solid organs and bone marrow transplantation and in the treatment of several autoimmune diseases [1]. The main side effect of CsA is nephrotoxicity, which may cause functional and structural injury [2]. Acute CsA nephrotoxicity is reversible and characterized by impairments in the glomerular filtration rate (GFR) and renal blood flow (RBF), and sublethal tubular injury [3, 4].

The pathogenesis of CsA nephrotoxicity is multifactorial with the participation of different systems, mediators and cells [1], such as the renin–angiotensin–aldosterone system [5], reactive oxygen species [6], transforming growth factor [7] and macrophages [8]. Moreover, experimental studies
have consistently demonstrated that acute CsA nephrotoxicity is a consequence of imbalance between renal vasoconstrictor and vasodilator systems, ultimately promoting afferent arteriole vasoconstriction, renal ischemia [4] and interstitial inflammation [8, 9].

The 37-kDa anti-inflammatory protein annexin A1 (ANXA1) is a glucocorticoid-regulated protein, originally termed lipocortin [10]. ANXA1 is a member of the annexin superfamily, composed of ubiquitous calcium-binding proteins which are linked to membrane-related events and cellular functions [11]. ANXA1 is highly expressed in the cytosol of neutrophils [12], monocytes [13], eosinophils [14] and epithelial cells [15]. Several studies have demonstrated an important role for ANXA1 in acute [16] and chronic [17] inflammation. In fact, ANXA1 inhibits neutrophil and monocyte adhesion and migration through inflamed postcapillary venule endothelium [12, 13], decreases neutrophil recruitment in the inflamed tissue [18] and accelerates apoptosis of polymorphonuclear cells [19]. There is much evidence demonstrating the importance and protective role of ANXA1 in cardiac, mesenteric, cerebral and renal ischemia/reperfusion (I/R) injury [20–24]. Recently, we demonstrated a structural protective role for ANXA1 in an experimental model of acute tacrolimus nephrotoxicity [25].

In the present study we used an experimental model of acute CsA nephrotoxicity, which reproduces the functional and structural changes caused by CsA in humans, to investigate the effects of pharmacological treatment with ANXA1 in CsA-induced renal injury. We described, for the first time to the best of our knowledge, that CsA augments ANXA1 expression in renal tissue and found a protective role for ANXA1 in the hemodynamic changes and inflammation caused by CsA.

Materials and methods

Animals

Male Munich–Wistar rats weighing 200–300 g (São José do Rio Preto Medical School animal facility) were housed in a temperature- and light-controlled environment. They received a low salt diet [26] and tap water ad libitum. Experiments were approved by the São José do Rio Preto Medical School Ethical Committee on Animal Experimentation (Protocol 1,082/2008).

Experimental design and pharmacological treatment

After seven days on a low salt diet, rats were randomly allocated to one of the four following groups (n = 12/group): cyclosporine A (CsA, 20 mg/kg body weight/day subcutaneously, Fujisawa, Japan) dissolved in ethanol and olive oil [3], annexin A1 mimetic peptide (ANXA1, 1 mg/kg body weight/day intraperitoneally with Ac2-26 peptide, Ac-AMVSEFLKQAWFIEENEEQYVQTVK, Invitrogen, USA) diluted in phosphate-buffered saline (PBS) [20], CsA + ANXA1, or identical volume of the respective vehicles (VH) for seven days. Diet consumption was based on pair feeding with CsA rats and weight was verified daily. After seven days of treatment, rats were allocated for GFR (n = 6/group) assessment or RBF (n = 6/group) measurement. Renal tissue was collected for histological evaluation at the end of each experiment.

Renal function studies

Animals were housed into metabolic cages for 24 h (Nalgene Co., Rochester, NY, USA) after 7 days of treatment for urinary volume collection (assessment of sodium, potassium, creatinine and osmolality). Afterwards, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg body weight) and placed on a thermostatically controlled heated table (Braile Biomédica, São José do Rio Preto, Brazil). Glomerular filtration rate was measured by inulin clearance, as described previously [26]. Results were mean of the three clearance periods and glomerular filtration rate was calculated by the standard formula.

Renal blood flow

After anesthesia, rats underwent renal hemodynamic studies as previously described [25]. RBF measurements were taken (T 106, Transonic Systems Inc., NY, USA) during 40 min. Mean arterial blood pressure measurements (Stoelting, Wood Dale, IL, USA) were taken concurrently, and the renal vascular resistance was calculated by the usual formula. At the end of the experiment, blood samples (5 mL) were drawn to determine sodium, potassium, creatinine and CsA blood levels.

Blood and urine analysis

Inulin was determined by the chemical anthrone method, and urinary and serum creatinine were determined by the Jaffé colorimetric method (ByoSystems BTS 310 spectrophotometer, Barcelona, Spain). Urinary and serum sodium and potassium concentrations were determined by an electrolyte analyzer (9,180 Electrolyte Analyzer, AVL Scientific Co., Rosweel, GA, USA). Urinary osmolality was determined by freezing point (Osmette A, Precision Systems, Natick, MA, USA). The fractional excretion of sodium (FeNa) and potassium (FeK) were calculated by the usual formulas.
Radioimmunoassay

CsA blood levels were measured by a radioimmunoassay kit (Diasorin, Stillwater, MN, USA).

Cytokine ELISA

Serum transforming growth factor (TGF-\( \beta \)) levels were measured using an ELISA kit (OpEIA; BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions.

Histopathological analyses

Renal fragments were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 4°C, dehydrated and embedded in paraffin. Sections (2–4 \( \mu \)m) were stained with hematoxylin–eosin, analyzed in five fields (41,360 \( \mu \)m\(^2\) each) from the renal cortex using a high-power objective (\( \times 40 \)) on a Zeiss-Axioskop 2 light microscope (Carl Zeiss, Jena, Germany), and the area of proximal tubules was measured using Axiovision software.

Immunohistochemistry

Renal sections (4 \( \mu \)m) underwent immunohistochemical staining [25]. They were incubated overnight at 4°C with 1:1,000 monoclonal mouse anti-rat-ED-1 (Serotec, Oxford, UK) or 1:2,000 polyclonal rabbit anti-ANXA1 (Zymed Laboratories, Cambridge, UK). Control sections were obtained by replacing the primary antibodies with equivalent concentrations of non-immune mouse or rabbit serum (Sigma–Aldrich). Sections were then incubated with a biotinylated secondary antibody, avidin-conjugated horseradish peroxidase, and enhanced with diaminobenzidine chromogenic substrate (Dako, Carpinteria, CA, USA).

Table 1 Renal functional parameters, cyclosporine blood levels and plasma TGF-\( \beta \) levels in vehicle (VH), annexin A1 (ANXA1), cyclosporine A (CsA) and combination (CsA + ANXA1) groups after 7 days of treatment

<table>
<thead>
<tr>
<th></th>
<th>VH</th>
<th>ANXA1</th>
<th>CsA</th>
<th>CsA + ANXA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mmHg)</td>
<td>117 ± 4</td>
<td>119 ± 2</td>
<td>107 ± 1</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>GFR (mL/min/100 g)</td>
<td>0.92 ± 0.08</td>
<td>0.96 ± 0.07</td>
<td>0.32 ± 0.05***</td>
<td>0.39 ± 0.05***</td>
</tr>
<tr>
<td>SCR (mg/dL)</td>
<td>0.68 ± 0.06</td>
<td>0.61 ± 0.04</td>
<td>1.07 ± 0.08**</td>
<td>1.07 ± 0.05**</td>
</tr>
<tr>
<td>UV (( \mu )L/min)</td>
<td>10.8 ± 1.4</td>
<td>13.4 ± 2.1</td>
<td>10.5 ± 1.9</td>
<td>11.1 ± 0.9</td>
</tr>
<tr>
<td>UOs mU(( \mu )mol/Kg)</td>
<td>298 ± 23</td>
<td>224 ± 24</td>
<td>328 ± 40</td>
<td>299 ± 33</td>
</tr>
<tr>
<td>FeNa (%)</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>FeK (%)</td>
<td>2.21 ± 0.22</td>
<td>2.74 ± 0.60</td>
<td>2.45 ± 0.47</td>
<td>2.46 ± 0.35</td>
</tr>
<tr>
<td>CsA (ng/mL)</td>
<td>–</td>
<td>–</td>
<td>2.480 ± 129</td>
<td>2.509 ± 176</td>
</tr>
<tr>
<td>TGF-( \beta ) (pg/mL)</td>
<td>–</td>
<td>–</td>
<td>749 ± 223</td>
<td>436 ± 189</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n = 6 rats/group); MABP mean arterial blood pressure; GFR glomerular filtration rate; SCR serum creatinine; UV urinary volume; UOs mU(\( \mu \)mol/Kg) urinary osmolality; Fe fractional excretion; Na sodium; K potassium; CsA blood trough level and serum TGF-\( \beta \). ***p < 0.001 versus VH and ANXA1

Fig. 1 Protective effects of annexin A1 (ANXA1) in acute cyclosporine (CsA) nephrotoxicity. Rats on low-salt diet were treated with VH (vehicles, 1 mL/kg body weight/day), ANXA1 (1 mg/kg body weight/day intraperitoneally), CsA (20 mg/kg body weight/day subcutaneously) and CsA + ANXA1 for seven days. a Pharmacological treatment with ANXA1 attenuated CsA-induced renal blood flow (RBF) decrease and b renal vascular resistance (RVR) increase. Data are mean ± SEM (n = 6 rats/group). *p < 0.05 versus VH, ANXA1 and CsA + ANXA1.
a high-power objective (×40), counting cells in 25 fields (41,360 μm² each). Densitometric analysis for ANXA1 immunostaining was performed on an arbitrary scale ranging from 0 to 255 by Axiovision software on a Zeiss-Axioskop two-light microscope.

Statistical analysis

Results are mean ± SEM. Statistical evaluations of CsA and TGF-β levels were performed using the two-tailed, unpaired Student’s t test. Comparisons between all groups were performed using one-way ANOVA followed by the Bonferroni post-hoc test. In all cases, p < 0.05 was taken as significant.

Results

Body weight and blood pressure

Body weight measurements demonstrated a significant (p < 0.05) weight loss in the CsA (−7.0 ± 2.6 g) and CsA + ANXA1 (−7.3 ± 2.1) groups compared to progressive body weight gain in VH (4.7 ± 1.1) and ANXA1 (5.7 ± 2.1) animals. Mean arterial blood pressure was similar in all groups (Table 1).

Renal function analysis

CsA-treated animals had a significant decrease in GFR and increase in SCr compared to VH and ANXA1 (p < 0.001). ANXA1 did not protect against CsA-induced GFR reduction and SCr increase (Table 1).

Twenty-four-hour urinary output was similar in all groups. Fractional excretion of sodium (FeNa) and potassium (FeK), and urinary osmolality (UOsm) were similar and very low in all groups, reflecting the low salt diet (Table 1).

CsA blood levels

CsA levels were similar in the CsA and CsA + ANXA1 groups (Table 1).

Fig. 2 Annexin A1 partially protected against cyclosporine (CsA)-induced histological changes in an acute CsA nephrotoxicity model. a, b Morphological analysis of renal tissue from vehicle (VH) control and annexin A1 (ANXA1) groups showing typical glomeruli (GR) and proximal tubules (PT). c Micrograph showing the renal cortex of a salt-depleted rat given CsA, 20 mg/kg body weight/day during one week, showing tubular dilatation and macrophage infiltration (arrows). d ANXA1 gave partial protection against tubular injury. e Morphometric analysis of renal proximal tubular dilatation area. Data are mean ± SEM (n = 6 rats/group). ***p < 0.001, **p < 0.01 versus VH and ANXA1; *p < 0.05 versus CsA. Hematoxylin–eosin stain. Bars = 20 μm.
TGF-β levels

TGF-β levels were undetectable in the VH and ANXA1 groups and elevated in CsA animals. There was a trend to lower TGF-β in the CsA + ANXA1 group, but it was not statistically significant (Table 1).

Annexin A1 attenuated CsA-induced hemodynamic changes

Treatment with CsA caused a significant decrease in the renal blood flow and a significant increase in the renal vascular resistance (RVR) compared to VH and ANXA1 (p < 0.05). The administration of ANXA1 significantly attenuated CsA-induced RBF reduction and RVR increase (Fig. 1).

Annexin A1 attenuated CsA-induced histopathological changes

Analysis of renal tissue from VH and ANXA1 animals showed normal renal structures, without injury (Fig. 2a, b). In contrast, CsA-treated animals showed acute tubule-interstitial damage characterized by tubular dilatation and infiltration of macrophages (Fig. 2c). ANXA1 administration conferred partial protection against CsA-induced proximal tubule injury (Fig. 2d). These results were confirmed by morphometric analysis of cortical proximal tubules (Fig. 2e).

CsA-induced macrophage influx

A large number of infiltrated macrophages were detected in the cortical interstitial sections of CsA-treated rats at seven days of treatment (Fig. 3a). ANXA1 treatment significantly reduced CsA-induced renal macrophage infiltration (Fig. 3b, c).

Cyclosporine increases annexin A1 expression

ANXA1 expression was detected in Bowman’s capsule epithelium and in the macula densa in VH and ANXA1 animals (Fig. 4a, b), as well as in the medullary and papillary collecting ducts (data not shown). CsA- and CsA + ANXA1-treated rats showed a significant augmentation in ANXA1 expression in Bowman’s capsule epithelium and in the macula densa (Fig. 4c, d). Figure 4e reports the densitometric analysis of endogenous ANXA1 expression, demonstrating an exacerbated ANXA1 response associated with acute CsA nephrotoxicity.

Discussion

In this study we observed a partial protective effect of ANXA1 against the acute hemodynamic changes and tubular injury caused by CsA, paralleled by a partial suppression of CsA-induced macrophage infiltration and attenuation of CsA-induced tubular injury. We also
observed that CsA treatment augmented renal expression of ANXA1, similar to that demonstrated in renal ischemia/reperfusion models. Afferent arteriole vasoconstriction is considered the main mechanism responsible for the pathogenesis of CsA-induced acute renal injury [1]. This event is mediated by the imbalance of vasoactive factors, i.e. an increase of vasoconstrictors (such as angiotensin II and endothelin) and a decrease in vasodilators (such as prostaglandins and nitric oxide) causing vasoconstriction [1, 4, 27]. ANXA1 is an important endogenous anti-inflammatory mediator, which is activated in response to cellular or tissue injury [28, 29]. In recent years several studies have shown a protective role for ANXA1 and its peptide Ac2-26 in cardiac, mesenteric, cerebral and renal ischemia/reperfusion (I/R) injury [20–23, 30].

A possible explanation for the attenuation in CsA-induced hemodynamic and tubular injury in ANXA1-treated animals is given by the observed reduction in the number of macrophages infiltrating the renal tissue [26]. This early macrophage trafficking was consistently demonstrated in previous studies of CsA nephrotoxicity [8, 9]. Previous results from our group have shown that clodronate-induced macrophage depletion alleviates hemodynamic changes in a model of CsA nephrotoxicity similar to that used in the present study [26]. Activated macrophages produce several substances with vasoactive properties and are likely part of the mechanisms causing functional changes in CsA nephrotoxicity [26]. It is noteworthy that the protective effects of ANXA1 on CsA-induced RBF impairment were not accompanied by a simultaneous action on CsA-induced GFR fall. A possible explanation is that the mechanisms promoting structural and hemodynamic damage might be dissociated from those causing functional changes in CsA nephrotoxicity [9, 31]. In the cardiac I/R injury model, the protective effect of ANXA1 and its mimetic peptide is mediated by the formylated peptide receptor (FPR) and is related to the reduction in Fig. 4 Analysis of annexin A1 expression in renal tissue by immunohistochemistry staining. a, b Vehicle (VH) and annexin A1 (ANXA1) groups demonstrated modest staining with anti-ANXA1 in Bowman’s capsule epithelium and macula densa (arrowheads). c, d CsA-treated animals (CsA alone or with ANXA1) showed intense immunostaining of endogenous ANXA1 (arrowheads). GR glomeruli. e Mean optical densitometry (MOD) analysis in renal tissue immunostained for ANXA1. Values are mean ± SEM (n = 6 rats/group). ***p < 0.001 versus VH and ANXA1. Hematoxylin counterstain. Bars = 20 μm.
leukocyte transmigration, and IL-1β levels in the heart [22]. Interestingly, CsA and ANXA1 are both ligands of the formyl peptide receptor [32], which may interfere with ANXA1 effects mediated by this same receptor and consequently jeopardize a possible protective effect [33].

Only one week of CsA administration was sufficient to increase plasma levels of TGF-β. A possible source for this early over-production of TGF-β is the infiltrative macrophages. In fact, these inflammatory cells are a well-known source of pro-fibrotic cytokines including TGF-β, which has an important role in cellular proliferation [34] and extracellular matrix deposition [35]. Even low doses of CsA increased renal tissue expression of TGF-β [9] and this cytokine accelerated matrix protein production in chronic CsA nephrotoxicity [36]. ANXA1 has also been shown to regulate TGF-β expression, by enhancing Smad signaling [37].

A novel result in the present study is the significant CsA-induced increase in the renal expression of endogenous ANXA1. Similarly, we have shown that salt-depleted tacrolimus treatment augments renal ANXA1 expression [25]. ANXA1 expression has previously been shown to increase in response to glucocorticoids [38], inflammation [28], heat or chemical cellular damage [39] and pro-inflammatory cytokines [40]. Epithelial cells express a high amount of ANXA1 and most of the properties of this anti-inflammatory molecule, such as its anti-proliferative action, inhibition of cell activation, and inhibition of both phospholipase A2 activity and prostanoid generation [15] are important components of the cell defense against I/R injury [41]. In fact, ANXA1 expression is very different in the normal and in the post-ischemic rat kidney. In a normal kidney ANXA1 is mainly found in Bowman’s capsule epithelium, macula densa and medullary and papillary collecting ducts, with light staining at the thick ascending limb. After ischemia/reperfusion, ANXA1 renal tissue expression increases significantly [42], as observed in the present study. These data suggest that endogenous ANXA1 increase might be an attempt to protect cell function and structure against ischemia reperfusion injury [43]. In fact, recent data from our group demonstrated that mimetic peptide Ac2-26 treatment protected against ischemia/reperfusion-induced renal structural and functional injury regulating neutrophil and monocyte transmigration to kidney tissue [24].

In conclusion, the present data provides in-vivo evidence that ANXA1 mimetic peptide significantly attenuated CsA-induced renal hemodynamic and tubular injury and kidney macrophage infiltration in a rodent model of acute CsA nephrotoxicity.

Acknowledgments Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Grant 2008/01.048-9 to L.P.A.); Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (Grant 306,074/2007-9 to S.M.O. and 307,371/2006-9 to E.A.B.).

Conflict of interest The authors have no competing financial interests to disclose in relation to this manuscript.

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