Cyclosporine A Impairs Norepinephrine-Induced Vascular Contractility

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Abstract
Usage of cyclosporine A (CsA) after kidney transplantation may be associated with development of nephrotoxicity and vasculopathy, but the mechanisms by which CsA causes vascular dysfunction are still under scrutiny. We established a transplantation model and investigated the effect of CsA on vascular contractility with the aid of a pressurized myograph in comparison with control and unilaterally nephrectomized rats. Results were correlated with mRNA expression studies of α- and β-adrenoreceptors, in mesenteric resistance arteries versus the thoracic aorta. Consequences of everolimus on functional properties as well as adrenoreceptor expression were also studied. CsA significantly downregulated expression of mesenteric adrenoreceptors, whereas no effect on aortic adrenoreceptors was seen. Administration of everolimus had no influence on mRNA adrenoreceptor expression in mesenteric resistance arteries. Furthermore, contractile responses of mesenteric resistance arteries to norepinephrine were markedly reduced after treatment with CsA, while there was no difference in contraction by endothelin. Everolimus did not alter the contractility response at all. In summary, norepinephrine-induced, but not endothelin-induced, contractile responses of mesenteric resistance arteries are blunted in CsA-treated rats. This finding was accompanied by a marked downregulation of adrenoreceptors in mesenteric resistance arteries and was limited to the usage of CsA.

Introduction
Cyclosporine A (CsA) is a worldwide approved immunosuppressive drug which is commonly used to prevent allograft rejection in human organ transplantation [1]. Usage of CsA is often associated with the chronic development of nephrotoxicity, graft failure and vascular dysfunction [2, 3]. The mechanisms by which cyclosporine (CsA) causes vasculopathy after kidney transplantation are still under active scrutiny [4]. Altered sympathetic activity [5] and modified peripheral vascular function [6] are assumed mechanisms for the development of arterial

Key Words
Adrenoreceptors · Cyclosporine · Endothelin · Norepinephrine · Renal transplantation · Resistance arteries
Vascular relaxation elicited by acetylcholine [17] and increased sym-pathetic and angiotensin activities of CsA resulting in renal arterial vasoconstriction and epithelial dysfunction may also contribute to the side effects such as direct vasoconstriction [7], increased sympathetic and angiotensin activities [8, 9], endothelin release [10] as well as endothelin-mediated vasoconstric-
tion [11] and altered renal metabolism of arachidonic acid favoring a vasoconstrictor prostano
id profile [12]. Endothelial dysfunction may also contribute to the side effects of CsA resulting in renal arterial vasoconstriction and subsequent nephrotoxicity [13, 14] and hypertension [15]. Vascular relaxation elicited by acetylcholine [16], bradykinin [17], substance P [18], calcium ionophore A23187 [17], and prostaglandin E$_1$ [19] are remarkably impaired by CsA. Additionally, it was shown in uninephrecto-
imized rats that CsA significantly reduced renal blood flow, increased renal vascular resistance and that CsA-
mediated nephrotoxicity may in part be linked with the NO system [20].

Due to the interfering data on the effect of CsA on the contractile properties of resistance arteries, we estab-
lished an experimental transplantation model in rats to enlighten this question [21].

The experimental renal transplantation model without concomitant medication and clinically approved CsA trough levels and a highly standardized protocol let us exclude interference from confounding factors as seen in human organ transplantation. We investigated the vascular function of the mesenteric vascular bed with the aid of a pressurized myograph. To account for the impact of immunological effects seen after allogenic transplanta-
tion, we additionally studied unilaterally nephrecto-
mized rats treated with and without CsA, and control rats treated with and without CsA. Everolimus, as a calci-neurin-free immunosuppressive drug, was used as reference. Hypothesizing that CsA is driving its effects mainly in the endothelium of resistance arteries, we complemented these functional studies with mRNA expression studies of adrenergic $\alpha$- and $\beta$-receptors in mesenteric resistance arteries versus the thoracic aorta to account for specificity of resistance arteries.

### Materials and Methods

**Animals/Experimental Renal Transplantation**

Animal experiments were performed following the German law of animal protection and the NIH’s principles of laboratory animal care. Male Brown Norway rats serving as donors and Lew-
is rats serving as recipients (Charles River Laboratories, Sulzfeld, Germany) (200–250 g) were kept under a conventional housing and diet (KTx+CsA). The number and treatment regimens of the different groups are listed in table 1. Briefly, naive Lewis rats with and without CsA (ctrl±CsA) (CsA, 5 mg/kg b.w./day, liquid Ne-
oral; Novartis, Basel, Switzerland, administered once daily by ga-
vage) as well as unilaterally nephrectomized Lewis rats with and without CsA (UNx±CsA) served as controls. The rats were sacri-
fied 28 days after transplantation. Detailed protocols for perfor-
man. rative renal transplantation have previously been published by our group [22]. In brief, left kidneys were explanted, flushed with cold saline and transplanted orthotopically in Lewis recipi-

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Control without CsA</th>
<th>Control with CsA</th>
<th>Control with everolimus</th>
<th>Unilateral nephrectomized without CsA</th>
<th>Unilateral nephrectomized with CsA</th>
<th>Transplanted with CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group abbreviation</td>
<td>ctrl–CsA</td>
<td>ctrl+CsA</td>
<td>ctrl+EVE</td>
<td>UNx–CsA</td>
<td>Unx+CsA</td>
<td>KTx+CsA</td>
</tr>
<tr>
<td>Number of rats, day 28</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Histopathological classification, day 28</td>
<td>Banff 1</td>
<td>Banff 1</td>
<td>Banff 1</td>
<td>Banff 1</td>
<td>Banff 1</td>
<td>Banff 1 or Banff 4 IA</td>
</tr>
<tr>
<td>Serum creatinine ± SD, day 28, mg/dl</td>
<td>0.2 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.19 ± 0.06</td>
<td>0.32 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>Immunosuppression trough levels, day 28, ng/ml</td>
<td>not detected</td>
<td>202 ± 27.7</td>
<td>4.3 ± 1.86</td>
<td>not detected</td>
<td>215 ± 29.6</td>
<td>171 ± 63.1</td>
</tr>
</tbody>
</table>

In rats treated with CsA or everolimus, trough levels were measured by liquid chromatograph–mass spectrometry once a week.

**Renal Function and Histopathological Diagnoses**

In the 28-day experimental protocol laboratory values and immunosuppression trough levels were studied on days 0, 7, 14, 21, and 28. The histopathological diagnoses according to the Banff 97 classification [23] of the kidney specimens were made by experienced pathologists.
RNA Isolation, Reverse Transcription and Real-Time PCR in Rat Specimens

After homogenization of frozen tissue sections in peqGOLD TriFast (Peqlab, Erlangen, Germany) and additional sonication, total RNA was extracted according to the manufacturer’s instructions with additional DNase digestion to remove all traces of genomic DNA. Total RNA was reverse transcribed into cDNA according to standard protocols. In brief, cDNA probes were synthesized in 20 μl reaction volume with 1 μg total RNA, 0.5 μg oligo (dT) primer (Sigma-Aldrich, Munich, Germany), 40 units of RNasin (Promega, Mannheim, Germany), 0.5 mM dNTP (Amersham, Freiburg, Germany), 4 μl 5X transcription buffer and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany) for 1 h at 37 °C. In parallel, no-RT and no-template controls were performed. Real-time PCR was performed on an ABI PRISM 7900 detection system (Applied Biosystems, Darmstadt, Germany) using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). GAPDH was used as reference gene. All water controls were negative for target and housekeeper. The sequences of the used primers are listed in table 2.

Preparation of Mesenteric Resistance Arteries

The intestines were exposed by a median incision of the abdomen and one segment of jejunum together with the mesenteric bed was quickly excised and placed in a dissection dish containing cold physiological salt solution. A 2- to 3-mm-long segment of third order branch of the superior mesenteric artery was carefully cleared from surrounding adipose tissue. After dissection the artery was transferred to the chamber of a pressure myograph (Danish Myo Technology®) for cannulation. Suffusion and peristaltic flow were started and 2 rats, 1 each, were used for each determination. Points along the vessel with use of a calibrated video system (Danish Myo Technology®) were marked and the vessel was mounted on two pipettes, secured with suture and bubbled with 95% air and 5% CO₂ to achieve a pH of 7.4. The axial length of the vessel was adjusted by moving one cannula until the vessel walls were parallel without stretch. Vessels were equilibrated under a constant intraluminal pressure (45 mm Hg) for 1 h.

Experimental Protocol

Experiments were performed under no-flow conditions. Vascular reactivity was tested with norepinephrine (NE) (10⁻⁵ M). Endothelial function was pretested with acetylcholine (10⁻⁴ M). Contraction to NE was achieved with cumulative doses from 10⁻⁸ to 10⁻⁴ M. Vascular reactivity was additionally assessed in response to cumulative doses of endothelin (10⁻⁹ to 10⁻⁶ M). The changes in internal diameter as well as media thickness of vessels in response to each increase in intravascular pressure was measured at three points along the vessel with use of a calibrated video system (Danish Myo Technology®). Contraction to NE and endothelin was calculated as follows: (Dₑ – Dᵣ)/Dᵣ * 100, with Dₑ as resting diameter and Dᵣ as contracted diameter.

Data Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using SigmaStat 3.0 software. Groups were compared using t test, one-way ANOVA, two-way ANOVA, or ANOVA for repeated measurements as appropriate. Post-hoc testing was performed using Bonferroni (two-way ANOVA) or Newman-Keuls (one-way and repeated measures) test. p < 0.05 was significant.

Results

Characterization of the Different Groups

At day 28 there was almost no difference in serum creatinine and histopathological diagnoses between the examined groups: in accordance with control and unilaterally nephrectomized rats, 10 out of 12 KTx+CsA rats showed normal histopathological classification (Banff 1), the other 2 rats showed only moderate signs of rejection (Banff 4 IA) (table 1). There were no differences in resulting renal function or measured CsA trough levels (table 1). Everolimus-treated rats matched the CsA-treated rats in matters of resulting serum creatinine and Banff classification. The abbreviations, numbers, treatment regimens, histopathological diagnosis, and data corresponding to the renal function of the different groups of rats are summarized in table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-gtcgtgtgatctgatcgctgc-3’</td>
<td>5’-gaattctgctctccacca-3’</td>
</tr>
<tr>
<td>rADra1α</td>
<td>5’-ctgaggtcactcactcagca-3’</td>
<td>5’-cgctacatctccacccaggtt-3’</td>
</tr>
<tr>
<td>rADra1β</td>
<td>5’-ccgtaagatcactctcagcc-3’</td>
<td>5’-gctcagttctggaggttc-3’</td>
</tr>
<tr>
<td>rADra2α</td>
<td>5’-agctccacacctccctcag-3’</td>
<td>5’-cgtcaggttccacgac-3’</td>
</tr>
<tr>
<td>rADraβ</td>
<td>5’-caggctatgtcactgcctc-3’</td>
<td>5’-tgctcaggttgcctactc-3’</td>
</tr>
<tr>
<td>rADrb</td>
<td>5’-cggctctgggtccattatt-3’</td>
<td>5’-aaggcagagtgcacgac-3’</td>
</tr>
</tbody>
</table>

Table 2. Primers of the in vivo experiment

CsA Impairs Vascular Contractility

mRNA Expression of Mesenteric α1- and β-Adrenergic Receptors (ADRs)

Administration of CsA did not significantly influence mRNA expression of mesenteric α1- (subtypes a, b, d) and β-ADRs (subtypes 1, 2, 3) in a day 6 protocol (data not shown).

In the day 28 protocol, administration of CsA significantly downregulated expression of mesenteric α1-ADRs. The α1-subtype a ADR was significantly downregulated in the ctrl+CsA group (p = 0.05) and the transplanted group (KTx+CsA) (p = 0.003) (fig. 1a). In accordance with α1-subtype a, α1-subtype b was also significantly downregulated in the identical groups (ctrl+CsA p = 0.04, UNx+CsA p = 0.01, KTx+CsA p = 0.003). mRNA expression of subtype d of mesenteric α1-ADRs was only significantly downregulated in transplanted rats (p = 0.01) (fig. 1a).

mRNA expression of β1-ADR was not significantly downregulated after 28 days of CsA administration. There was only a trend for downregulation in the unilaterally nephrectomized group as well as the transplanted rats (fig. 1b). mRNA expression of β2-ADR was significantly downregulated in all three CsA-treated groups (ctrl+CsA p = 0.05, UNx+CsA p = 0.0004, KTx+CsA p = 0.03) (fig. 1b). In accordance with β2-ADR, β3-ADR was also significantly downregulated after administration of CsA in these groups (ctrl+CsA p = 0.03, UNx+CsA p = 0.003, KTx+CsA p = 0.006) (fig. 1b).

Contractility of Mesenteric Arteries

Contractile responses of mesenteric resistance arteries to NE were markedly reduced after treatment with CsA in control rats (ctrl+CsA p < 0.05 for maximal stimulation; fig. 3) and unilaterally nephrectomized rats (UNx+CsA p < 0.05; fig. 4). In transplanted rats treated with CsA, the response to NE was diminished compared to UNx+CsA rats (fig. 4). Contractile responses to endothelin were identical in all groups (fig. 5). In contrast, everolimus did not alter the contractility response to NE in control rats (fig. 3).

Discussion

In our experimental model the effect of CsA on vascular contractility in mesenteric resistance arteries was studied on a pressurized myograph. Additionally, these results have been completed by mRNA analyses of α1-a-, β1-, β2- and β3-adrenoreceptors. We were able to demon-
strate that NE-induced, but not endothelin-induced, contractile responses of mesenteric resistance arteries are blunted in CsA-treated rats after 28 days. Detected findings were limited on the usage of CsA and could not been demonstrated using everolimus, a calcineurin inhibitor-free immunosuppression. The findings may be caused by the significant downregulation of $\alpha_1$-b-, $\alpha_1$-d-, $\beta_2$-, and $\beta_3$-adrenergic receptors in mesenteric resistance arteries, whereas mRNA expression of any investigated $\alpha$- and $\beta$-ADR was not significantly altered in the thoracic aorta. Our data are in accordance with functional studies showing that vascular contractility is decreased in mesenteric resistance arteries in response to NE after CsA treatment [24]. In contrast to other experimental data with unphysiologically high doses of CsA, we were able to show that the usage of CsA with clinical approved trough levels [25]...
not only altered vascular contractility but also maximum contraction in response to NE. These effects were demonstrated after 28 days of CsA treatment and are in accordance with data on the endothelial and vascular smooth muscle function after 3 weeks of CsA treatment [26]. We have to state that the observed differences were seen predominantly with supraphysiologic doses. Nevertheless, this illustrates a general hyporesponsiveness to NE. The influence of CsA only on resistance arteries underlines the physiological relevance of the detected results due to a well-known fact that these vessels are the main target of endothelial dysfunction. Besides mesenteric resistance arteries, renal resistance arteries may also be affected and this may in part contribute to CsA-induced nephrotoxicity and renal vasculopathy. However, our results are to a degree inconsistent with findings that postulated that resistance vessels obtained from transplanted kidneys were more sensitive to NE [27]. The findings of a decreased sensitivity to noradrenaline are in contrast to previous reports of an enhanced vascular sensitivity to adrenergic agonists in the presence of CsA: intramuscular administration of surplus doses of CsA (20 mg/kg/day) for 7 days enhanced vasoconstriction in response to noradrenaline [28]. Hence these results have been demonstrated not in resistance but large vessels. In accordance, Tavares et al. [29] postulated an increased catecholamine release from the sympathetic nervous end-
ADRs in bovine pulmonary artery smooth muscle cells [44]. In accordance with these studies, we could see a marked downregulation of all β2- and β3-adrenoreceptor subtypes under CsA therapy in mesenteric resistance arteries, but not in the thoracic aorta. The additional downregulation of β2- and β3-adrenoreceptors in our study in contrast to previous work [33] may be caused by the different time course of CsA treatment (28 days vs. 6 weeks), the different dosages that were used and the differences of the vessel region investigated. In future the consequences of CsA on specific α-/β-adrenoreceptor expression in comparison with specific α- or β-adrenoreceptor blockers have to be examined. Whether CsA directly reduces blood flow and increases vascular resistance or whether altered serum concentration of NO or the deleterious toxic effects of CsA due to increased production of free radicals and ROS [45] effect detected results, remains unresolved. However, as renal vessels are resistance vessels comparable with mesenteric resistance arteries, the detected results during CsA treatment may be regarded as surrogate of CsA-mediated renal vasculopathy.

However, in contrast to Takeda et al. [46, 47], who reported that CsA increased endothelin production and endothelin mRNA levels in rat mesenteric arteries, we did not observe any effects on endothelin-mediated contraction in CsA-treated rats. The effects having been found in the experimental protocol by Takeda et al. may have been caused by surplus doses of CsA (25 mg/kg/day).

In summary, we found that NE-induced, but not endothelin-induced, contractile responses of mesenteric resistance arteries are blunted in CsA-treated rats, even with clinically approved CsA trough levels. This finding was accompanied by a marked downregulation of α- and β-adrenoreceptors in mesenteric resistance arteries, but not in the thoracic aorta. Our findings were also limited to the usage of CsA, whereas everolimus neither induced functional changes nor modified adrenoreceptor expression.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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