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Cyclosporine A Impairs Norepinephrine-Induced Vascular Contractility

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Key Words

Adrenoreceptors · Cyclosporine · Endothelin · Norepinephrine · Renal transplantation · Resistance arteries

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.

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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

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Table 1. Experimental renal transplantation model in rats in a 28-day protocol

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

hypertension in organ transplantation recipients treated with CsA. Thereby, CsA causes several pathophysiological changes that have been regarded crucial for the side effects such as direct vasoconstriction [7], increased sympathetic and angiotensin activities [8, 9], endothelin release [10] as well as endothelin-mediated vasoconstriction [11] and altered renal metabolism of arachidonic acid favoring a vasoconstrictor prostanoid 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 E₁ [19] are remarkably impaired by CsA. Additionally, it was shown in uninephrectomized 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 established 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 transplantation, we additionally studied unilaterally nephrectomized rats treated with and without CsA, and control rats treated with and without CsA. Everolimus, as a calcineurin-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 α - and β -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 Lewis 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 \pm CsA) (CsA, 5 mg/kg b.w./day, liquid Neoral; Novartis, Basel, Switzerland, administered once daily by gavage) as well as unilaterally nephrectomized Lewis rats with and without CsA (UNx \pm CsA) served as controls. The rats were sacrificed 28 days after transplantation. Detailed protocols for performance of 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 recipients by end-to-end anastomosis of the vessels and, subsequently, the ureter of the donor and recipient. Cold and warm ischemia times were \sim 35 and 30 min, respectively. Nephrectomy of the right kidney was performed at the end of surgery. After euthanasia, mesenteric arteries and aortae thoracicae were isolated as described below. Naive Lewis rats treated with everolimus suspension according to the manufacturer's instructions (ctrl+EVE) (1.5 mg/kg b.w./day, administered once daily by gavage) served as calcineurin inhibitor-free control group.

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.

Table 2. Primers of the in vivo experiment

Gene	Forward primer	Reverse primer
GAPDH	5'-gtcgtggatctgacgtgcc-3'	5'-gatgcctgctccaccacctt-3'
rADra1 α	5'-gaatgtcctgcgaatccagt-3'	5'-gggatacgcaccatgtctct-3'
rADra1 β	5'-cctgaggatcactccaaga-3'	5'-cgactacaatgcccaaggtt-3'
rADra1 δ	5'-ccgaggtagaagcagtgcc-3'	5'-gtcagtctctcggagggtgc-3'
rADrab1	5'-acgctcaccaaccttctcat-3'	5'-cgtctaccgaagtcagagc-3'
rADrab2	5'-caggcctatgctatcgcttc-3'	5'-tgcctcggattgtctatc-3'
rADrab3	5'-cgcaccttgggtctcattat-3'	5'-gaaggcagagttggcatagc-3'

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 μ l reaction volume with 1 μ g total RNA, 0.5 μ g oligo (dT) primer (Sigma-Aldrich, Munich, Germany), 40 units of RNasin (Promega, Mannheim, Germany), 0.5 mM dNTP (Amersham, Freiburg, Germany), 4 μ l 5 \times 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 a 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 perfusion was performed using oxygenated 37°C Krebs solution (95% O₂, 5% CO₂) containing (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, disodium EDTA 0.026, and glucose 11.0; pH 7.4. 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). En-

dothelial 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: (D_r - D_c)/D_r · 100, with D_r as resting diameter and D_c as contracted diameter.

Data Analysis

Data are presented as mean \pm 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.

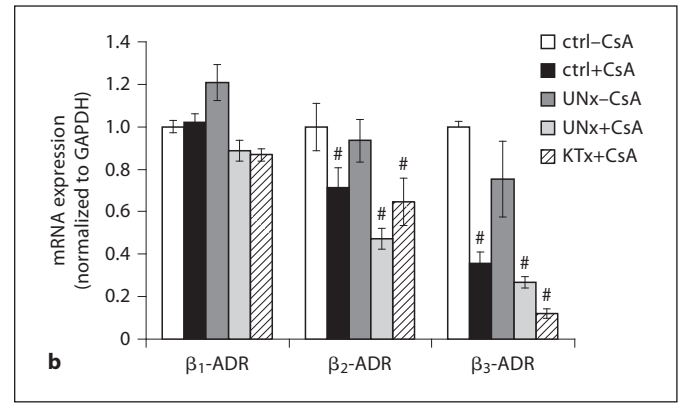
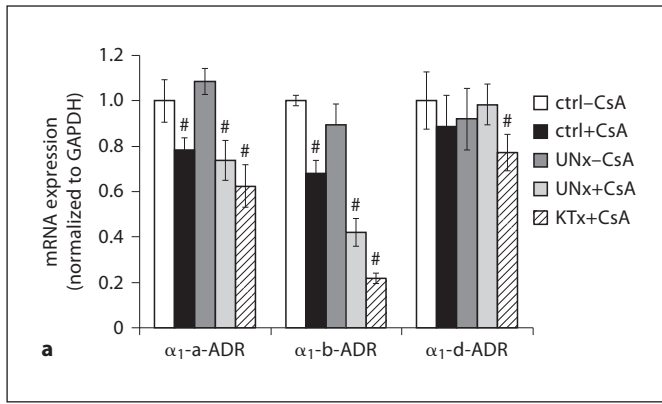


Fig. 1. mRNA expression of mesenteric α_1 - and β -ADRs. **a** mRNA expression of mesenteric α_1 (subtypes a, b, d) receptors after 28 days of CsA treatment in the different experimental groups: control rats with/without CsA (ctrl \pm CsA), unilaterally nephrectomized rats with/without CsA (UNx \pm CsA) and transplanted rats with CsA (KTx+CsA). # $p < 0.05$ was regarded as statistically significant.

b mRNA expression of β_1 -, β_2 -, and β_3 -mesenteric adrenoceptors in the different experimental groups: control rats with/without CsA (ctrl \pm CsA), unilaterally nephrectomized rats with/without CsA (UNx \pm CsA) and transplanted rats with CsA (KTx+CsA). # $p < 0.05$ was regarded as statistically significant.

mRNA Expression of Mesenteric α_1 - and β -Adrenergic Receptors (ADRs)

Administration of CsA did not significantly influence mRNA expression of mesenteric α_1 - (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 α_1 -ADRs. The α_1 -subtype a ADR was significantly downregulated in the ctrl+CsA group ($p = 0.05$) as well as in the unilaterally nephrectomized group (UNx+CsA) ($p = 0.05$) and the transplanted group (KTx+CsA) ($p = 0.003$) (fig. 1a). In accordance with α_1 -subtype a, α_1 -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 α_1 -ADRs was only significantly downregulated in transplanted rats ($p = 0.01$) (fig. 1a).

mRNA expression of β_1 -ADR was not significantly downregulated after 28 days of CsA administration. There was only a trend for downregulation in the unilaterally nephrectomized as well as the transplanted rats (fig. 1b). mRNA expression of β_2 -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 β_2 -ADR, β_3 -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).

mRNA expression of α_1 - (subtypes a, b, d) and β -ADRs (subtypes 1, 2, 3) was neither significantly influenced after 6 (data not shown) nor 28 days in thoracic aorta (fig. 2a). Additionally, administration of everolimus had no influence on mRNA expression of α_1 - (subtypes a, b, d) and β -ADRs (subtypes 1, 2, 3) in mesenteric resistance arteries (fig. 2b) as well as the thoracic aorta in control rats in the 28-day protocol (data not shown).

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 α_1 -a-, b-, d- and β -adrenoceptors. We were able to demon-

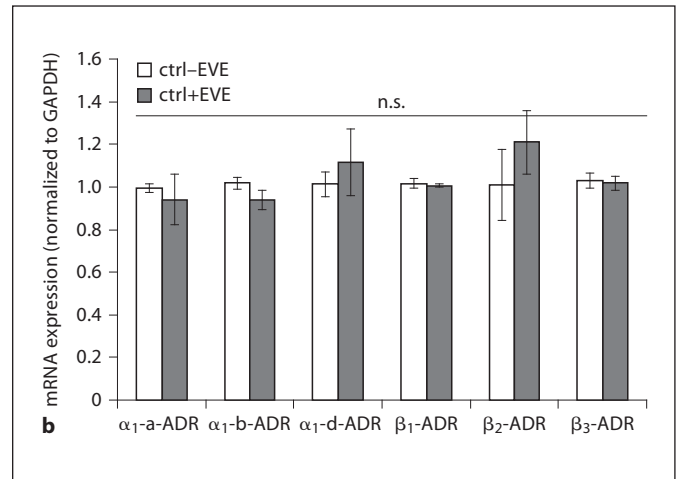
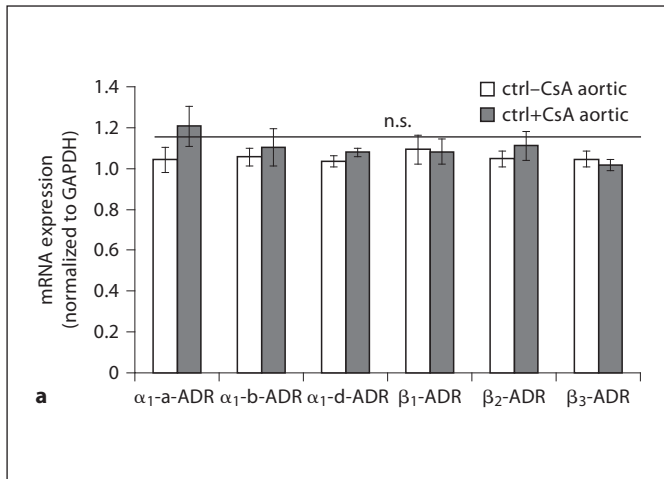


Fig. 2. mRNA expression of aortic ADRs and effect of everolimus in control rats. **a** mRNA expression of aortic α_1 - (subtypes a, b, d) and β_1 -, β_2 -, and β_3 -ADRs after 28 days of CsA treatment in control rats with/without CsA (ctrl±CsA). $p < 0.05$ was regarded as

statistically significant. **b** Effect of everolimus on mRNA expression of mesenteric α_1 - (subtypes a, b, d) and β_1 -, β_2 -, and β_3 -ADRs in control rats treated with/without everolimus (ctrl±EVE) for 28 days. # $p < 0.05$ was regarded as statistically significant.

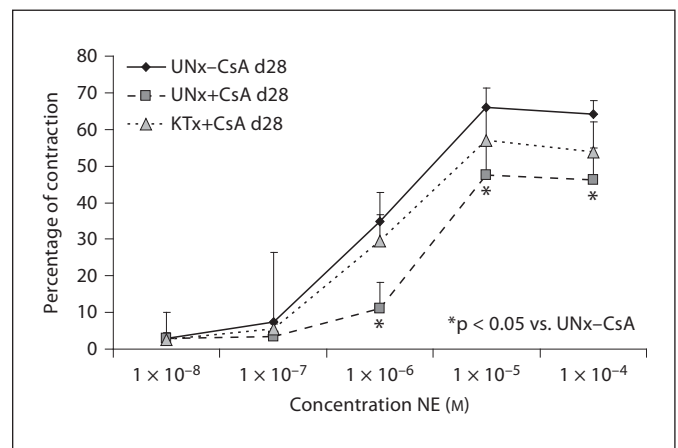
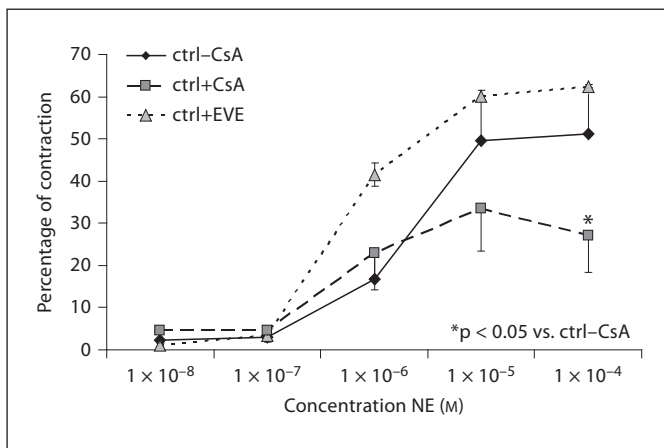


Fig. 3. Contraction of mesenteric resistance arteries under stimulation with cumulative doses of NE in control rats treated with/without CsA for 28 days (ctrl-CsA vs. ctrl+CsA) and control rats treated with everolimus (ctrl+EVE). * $p < 0.05$ at maximum stimulation.

Fig. 4. Contraction of mesenteric resistance arteries under stimulation with cumulative doses of NE in unilaterally nephrectomized rats treated with/without CsA (UNx-CsA, UNx+CsA) for 28 days and transplanted rats treated with CsA (KTx+CsA). * $p < 0.05$ was regarded as statistically significant.

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 be demonstrated using everolimus, a calcineurin inhibitor-free immunosuppression. The findings may be caused by the significant downregulation of α_1 -b-, α_1 -d-, β_2 - and β_3 -adrenoreceptors in mesenteric resistance arteries,

whereas mRNA expression of any investigated α - and β -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]

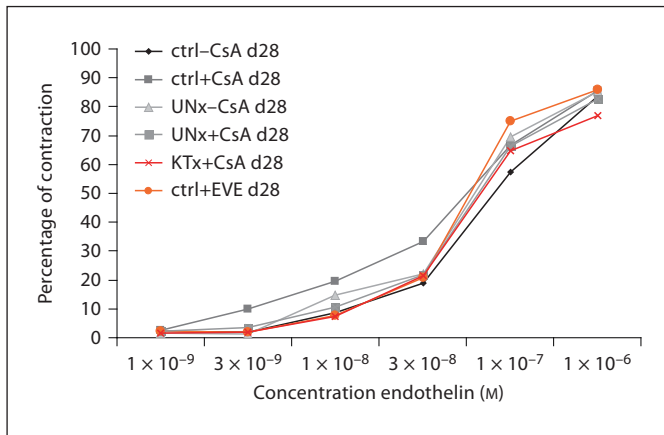


Fig. 5. Contraction of mesenteric resistance arteries under stimulation with cumulative doses of endothelin in control rats treated with/without CsA (ctrl-CsA vs. ctrl+CsA), unilaterally nephrectomized rats treated with/without CsA (UNx-CsA, UNx+CsA) and transplanted rats treated with CsA (KTx+CsA), as well as control rats with everolimus (ctrl+EVE). Experimental course for 28 days.

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 some 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-

ings of rat aorta after CsA treatment for 7 weeks with surplus doses of CsA (30 mg/kg/day). Therefore, the importance of examined vessel size as already previously discussed [30, 31] is crucial for the detected results and may explain the differences between our findings and previous reports [32–34] which focused on aortic but not mesenteric arteries. Apart from the studied vessel size, detected results depend on the used dosage of CsA, e.g. subcutaneous administration of almost nephrotoxic dosages of CsA (60 mg/kg/day) for 5 days caused enhanced vasoconstriction induced by NE [35] and also increased catecholamine release (30 mg CsA/mg·kg [29]). Thus, clinically approved CsA trough levels as used in our experimental model may cause effects totally different from those caused by almost toxic CsA doses. The reduced response to noradrenaline by mesenteric arteries may be even regarded as an escape mechanism for the CsA side effects.

Looking for the underlying pathophysiological mechanisms, we focused on mRNA expression of α - and β -ADRs. The existence of α -adrenoceptors in rat aorta [36, 37] as well as in rat mesenteric resistance arteries [38] was shown previously by several groups. An interaction of α_1 -adrenoceptors under exposure to CsA has already been described [39] and Tavares et al. [37] also detected a significant downregulation of α -adrenoceptor expression after CsA treatment. In contrast, in our study the expression level of α -adrenoceptors was only significantly downregulated in the mesenteric resistance arteries, whereas it remained unchanged in the aorta. In accordance with the functional data, detected findings were limited on the usage of CsA and could not be demonstrated using everolimus, neither in the thoracic aorta nor in mesenteric arteries. These findings underline the physiological relevance of our data since resistance vessels are believed to be crucial in peripheral control of vasculopathy and systemic blood pressure [40]. The downregulation of α_1 -adrenoceptors may to some degree be caused by modified activation of protein kinase C [37] as well as by structural damage and modified vessel architecture [41].

To distinguish if reduced response to NE-induced contractile response was only caused by modified α_1 expression, we also investigated the expression of β_1 -, β_2 - and β_3 -adrenoceptors after CsA treatment in mesenteric resistance arteries as well as in the thoracic aorta. The presence of these receptors in these vessel regions has been described previously [42, 43]. It has been shown that CsA treatment provokes a downregulation of β_1 cardiac adrenoceptors [33] as well as a downregulation of β -

ADRs in bovine pulmonary artery smooth muscle cells [44]. In accordance with these studies, we could see a marked downregulation of all β_2 - and β_3 -adrenoreceptor subtypes under CsA therapy in mesenteric resistance arteries, but not in the thoracic aorta. The additional downregulation of β_2 - and β_3 -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 contrac-

tion 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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