Cyclosporine and Oxidized Lipoproteins Affect Vascular Reactivity
Influence of the Endothelium
Jan Galle, Ingo Schenck, Peter Schollmeyer, and Christoph Wanner

Cyclosporine and in particular oxidatively modified low density lipoproteins can both exert direct vasoconstricting effects. We hypothesized that coincubation of arteries with low density lipoproteins and cyclosporine would enhance their respective influence on vascular tone. Therefore, we investigated vascular reactivity of isolated intact rabbit renal arteries preincubated with cyclosporine in the presence of native and oxidized low density lipoproteins. After preincubation of the arteries with cyclosporine (10 μg/ml, 90 minutes), unstimulated vascular tone as well as norepinephrine-induced vasoconstrictions remained unchanged compared with controls preincubated with the cyclosporine solvent dimethyl sulfoxide. Oxidized low density lipoproteins (100 μg/ml) in the absence of cyclosporine significantly enhanced vasoconstrictions to threshold concentrations of norepinephrine (78±10 μm at 30 nM). However, after cyclosporine treatment, the oxidized low density lipoprotein–induced potentiation of contractile responses to norepinephrine was further enhanced (157±19 versus 71±11 μm). Native low density lipoproteins had no influence on vascular tone. Potentiation of norepinephrine-induced vasoconstriction by oxidized low density lipoproteins took place in either endothelium-denuded or endothelium-intact arteries, whereas the further enhancement of vascular tone after cyclosporine treatment was seen only in endothelium-intact segments. Endothelium-dependent dilations to acetylcholine were fully preserved after treatment with oxidized low density lipoproteins and cyclosporine. Indomethacin, saralasin, and the thromboxane A2 antagonist daltroban had no influence, but the Ca2+-antagonist verapamil prevented the potentiation of vasoconstrictions by cyclosporine and oxidized low density lipoproteins.

These data indicate that coincubation of isolated renal arteries with cyclosporine and oxidized low density lipoproteins potentiates norepinephrine-induced vasoconstriction by a Ca2+- and partially endothelium-dependent mechanism before the onset of severe damage of the vasculature.

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Key Words • hypertension, systemic • renal circulation • kidney • hypercholesterolemia • transplantation • endothelium • cyclosporine

Systemic hypertension is frequently observed in patients treated with cyclosporine (CyA) (for review see Reference 1). Several studies provide evidence that CyA-induced hypertension is at least in part caused by direct effects of the drug on the vasculature. In isolated human and animal arteries preincubated with CyA, enhanced constrictor responses to different agonists, 2 release of endothelial constricting factors, 3 as well as impaired release of endothelium-derived relaxing factor (EDRF) and dilator prostaglandins 4–8 have been described.

Another pathophysiological feature of patients receiving CyA treatment are raised plasma levels of low density lipoproteins (LDL). 9 Enhanced plasma levels of LDL in CyA-treated humans may be caused by the drug itself. 10 LDL exert effects on vascular tone similar to those observed under CyA treatment: in isolated arteries, LDL influence vascular reactivity by attenuation of endothelium-dependent vasodilation 11 and by increasing the sensitivity of vascular smooth muscle to contractile agonists. 12 These effects are induced particularly by oxidatively modified LDL (Ox-LDL), which accumulate in hypercholesterolemia in atherosclerotic plaques 13–14 and which have been found to bind preferentially to kidney tissue. 15 Ox-LDL also may affect kidney circulation. 16

In view of the similarities of the effects of CyA and LDL on vascular function, we hypothesized that they would potentiate one another in their influence on vascular tone. Therefore, we assessed basal and norepinephrine-stimulated tone and endothelium-dependent vasodilations in isolated rabbit renal arteries preincubated with CyA, LDL, or both.

Methods
Preparation and Oxidation of LDL
Plasma was separated from human blood, and ethylenediaminetetraacetic acid (EDTA, 0.2 mM), butylated hydroxytoluene (BHT, 20 μM), phenylmethylsulfonyl

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fluoride (PMSF, 1 mM) (Sigma, Munich, Germany), and chloramphenicol (10 mg/dl, Boehringer-Mannheim, Mannheim, Germany) were added to avoid autodigestion, proteolytic digestion, and bacterial growth.17 LDL were isolated by sequential ultracentrifugation18 and oxidized as described recently in detail.19 Briefly, LDL (8–12 mg protein per milliliter, protein concentration determined by the method of Bradford20) were isolated after isolation and desalted by gel filtration and kept in the dark at 4°C for no longer than 3 weeks. LDL prepared by this method are referred to as native LDL (N-LDL). For oxidative modification, antioxidant-free LDL (0.3 mg protein per milliliter) were incubated with CuSO4 (5 μM) for 20–24 hours at 23°C. The degree of oxidation was quantified by three different methods: 1) the absorption increase at 234 nm wavelength, indicating conjugated diene formation of fatty acids;21 2) the increase in relative mobility on agarose gel electrophoresis, which demonstrated fractionation of apolipoprotein B-100.12 The oxidative process was stopped by adding BHT (20 mM) and EDTA (0.2 mM) when the formation of conjugated diene as index for lipid peroxidation was about 80% of its maximum. Homogeneity of LDL was tested by agarose gel electrophoresis (lipidophor electrophoresis kits, IMMUNO, Heidelberg, Germany). The relative mobility of Ox-LDL at agarose gel electrophoresis as an index for lipoprotein oxidation was then 1.3 compared with N-LDL. Finally, Ox-LDL were desalted, concentrated, and stored as N-LDL. In total, eight different LDL preparations were used in this study.

Drugs
Cyclosporine (Sandoz, Basel, Switzerland) was dissolved in dimethyl sulfoxide (DMSO; SERVA, Heidelberg, Germany) and further diluted in Tyrode’s solution of the following composition (mM): Na+ 144, K+ 4, Ca2+ 1.6, Mg2+ 1.0, Cl− 140, HCO3− 11.9, H2PO4− 0.4, calcium disodium EDTA 0.025, glucose 11; Po 120 mm Hg, pH 7.4. The final concentrations of CyA and DMSO in Tyrode’s solution were 10 μg/ml and 0.1%, respectively. Indomethacin, acetylcholine, and verapamil were purchased from Sigma. Indomethacin was dissolved in ethanol (0.1 M NaHCO3, 1:3, vol/vol). Daltroban (Boehringer-Mannheim) was dissolved in 0.1 M NaOH and, as indomethacin, norepinephrine (NE, Hoechst, Frankfurt, Germany), saralasin (Sigma), and the other drugs, were further diluted with Tyrode’s solution.

Vessel Preparation and Diameter Determination
Full details of this experimental set-up have been published earlier.23 Briefly, intact segments of the renal artery (0.8 cm in length) were obtained from rabbits of either sex (2.5–3.5 kg). In some of the experiments, the endothelium was removed mechanically by gently rubbing the segments over a rough steel cannula. Absence of endothelium was proven by the lack of a dilator response to acetylcholine.23 The segments were cannulated at both ends with steel cannulas and placed in an organ bath (37°C) containing oxygenated Tyrode’s solution (37°C, pH 7.4). Perfusion routes for bath perfusion and intraluminal perfusion were separate, and drugs could be administered to either route independently. Outer vascular diameters were recorded continuously by a photoelectric device. The transmural pressure was adjusted hydrostatically to 50 mm Hg by elevating the outflow tubing (isobaric conditions). After an initial period (60 minutes) of equilibration in the organ bath, the intraluminal perfusion was started with Tyrode’s solution (1,735±26 μm, n=88).

The influence of CyA on norepinephrine-induced vasoconstriction was determined after 90 minutes of preincubation of the arteries with 10 μg/ml CyA. This concentration of CyA exceeds plasma levels of CyA-treated patients by a factor of 5–100, but is comparable to those used in other in vitro studies. The duration of the cyclosporine incubation was chosen to limit the length of the experiments to a point where the endothelial function in terms of the endothelium-dependent vasodilatations was still fully intact. Single experiments with shorter incubation periods (30 or 60 minutes, n=6) revealed no influence of CyA on contractile responses. In each experiment, another segment of the same animal preincubated with an equivalent amount of DMSO (final concentration, 0.1%), the solvent for CyA, served as simultaneous control. DMSO at this concentration had no influence on basal or agonist-stimulated vascular tone. N- or Ox-LDL (100 μg/ml) were added to the intraluminal perfusion lining before and after incubation of the segments with CyA. Contractile responses were elicited by adding cumulative doses of norepinephrine (0.003–1 μM) either to the intraluminal perfusion lining or to the organ bath. Vasodilations were elicited by adding cumulative doses of acetylcholine (0.01–1 μM) to the intraluminal lining of norepinephrine-preconstricted arteries.

The effects of saralasin (1 μM), verapamil (1 μM), and the thromboxane A2 antagonist daltroban (1 μM) were determined by comparing norepinephrine-induced vasoconstriction in the presence of CyA and Ox-LDL before and after 20 minutes of preincubation with the respective antagonist. Some of the experiments were performed in the presence of indomethacin (10 μM) added to the intraluminal perfusion lining. The effectiveness of the indomethacin treatment in terms of suppression of prostacyclin formation has been proven in earlier studies by measurement of the stable prostacyclin metabolite, 6-ketoprostaglandin F1α.23 Indomethacin did not significantly alter vascular reactivity before or after treatment with either CyA or lipoproteins.

Statistics
All data are presented as mean±SEM. Dilator responses are expressed in a percentage of the initial steady-state constriction induced by norepinephrine. Differences were tested with Student’s t test for unpaired data. The significant shift in the plots in Figure 4 was determined using the one-way analysis of variance for repeated measurements, followed by a point by point comparison using the unpaired t test. Differences were considered significant at an error probability of p<0.05.

Results
Effects of Native and Oxidized LDL on Unstimulated and Norepinephrine-Stimulated Renal Arteries
Intraluminal perfusion of unstimulated renal arteries with N-LDL and Ox-LDL (100 μg/ml) caused no
FIGURE 1. Representative diameter (D) recordings in two endothelium-intact rabbit renal artery segments from one animal perfused with oxidized low density lipoproteins (Ox-LDL, 100 μg/ml), preincubated with either cyclosporine (CyA, 90 minutes 10 μg/ml, right panel) or the CyA solvent dimethyl sulfoxide (DMSO, 90 minutes 0.1%, left panel). Left panel: In the segment preincubated with DMSO, Ox-LDL elicited no vasoconstriction in the absence of norepinephrine (NE). In contrast, vasoconstriction elicited by threshold concentration of NE (30 nM) was potentiated by Ox-LDL. Right panel: In the segment preincubated with CyA, Ox-LDL elicited no vasoconstriction in the absence of NE. However, the potentiation of NE-induced vasoconstriction by Ox-LDL was further enhanced.

Influence of Cyclosporine on Vasomotor Effects of Native LDL and Oxidized LDL

In the absence of norepinephrine, N-LDL and Ox-LDL elicited no vasomotor effects in renal artery segments preincubated for 90 minutes with CyA. However, in the presence of norepinephrine contractile responses to Ox-LDL were further enhanced when the segments were preincubated with CyA (Figures 1 and 3, middle pair of columns, n=14 in each series).

FIGURE 3. Bar graph shows contractile responses elicited by threshold concentration of norepinephrine (NE, 30 nM) in endothelium (E)-intact and endothelium-denuded rabbit renal arteries preincubated with either cyclosporine (CyA, 10 μg/ml) or its solvent dimethyl sulfoxide (DMSO, 0.1%) in the absence or presence of oxidized low density lipoproteins (Ox-LDL, 100 μg/ml). Left pair of columns: Contractile responses to NE did not differ between the endothelium-intact CyA-treated segments and the control segments in the absence of Ox-LDL. n=14 in each series. Middle pair of columns: In endothelium-intact arteries, the potentiation of NE-induced contractile responses by Ox-LDL was significantly further enhanced in the CyA-treated segments versus the DMSO-treated controls. n=10 in each series. *p<0.05. Right pair of columns: In endothelium-denuded segments, contractile responses to NE were significantly enhanced in the presence of Ox-LDL but did not differ between the CyA-treated segments and the control segments. n=8 in each series.

Influence of Cyclosporine on Norepinephrine-Induced Vasoconstriction

When the renal artery segments were preincubated for 90 minutes with 10 μg/ml CyA, contractile responses to threshold concentration of norepinephrine did not differ significantly between CyA-treated segments and control segments (preincubated with an equivalent amount of DMSO, the solvent for CyA) (Figures 1 and 3, left pair of columns, n=14 in each series).

FIGURE 2. Bar graph shows effects of native (N-LDL) and oxidized (Ox-LDL) low density lipoproteins (100 μg/ml for 20 minutes) on basal, unstimulated vascular tone and on contractile responses elicited by threshold concentration of norepinephrine (NE) in endothelium-intact rabbit renal arteries. N-LDL and Ox-LDL had virtually no effect on basal vascular tone, whereas NE-induced contractile responses (78±10 μm) were significantly potentiated by Ox-LDL but not by N-LDL. n=9 in each series. *p<0.05.
Norepinephrine-Induced Vasoconstriction in the Presence of Cyclosporine and Oxidized LDL

Preincubation with cyclosporine (CyA) and oxidized low density lipoproteins (Ox-LDL) significantly enhanced contractions induced by norepinephrine in rabbit renal arteries. The enhancement was independent of the endothelial lining and was partially mediated by the Ca2+ channel blocker verapamil. Preincubation with CyA had no influence on the vasomotor responses induced by Ox-LDL in the CyA-treated or control segments.

Inhibition of Norepinephrine-Induced Vasoconstriction in the Presence of Cyclosporine and Oxidized LDL by Verapamil

To study whether the vasoconstrictions are linked to a transmembranous Ca2+ influx, we preincubated segments with the Ca2+ channel blocker verapamil. Verapamil significantly suppressed norepinephrine-induced (30 nM) vasoconstrictions in both the controls and CyA-treated segments (data not shown). Furthermore, contractile responses to NE were no longer potentiated by Ox-LDL in the CyA-treated or control segments (Table 1, n=9).

Influence of Cyclosporine and Oxidized LDL on Endothelium-Dependent Vasodilation to Acetylcholine

Several studies describe the attenuation of endothelium-dependent vasodilation after CyA treatment. However, under our experimental conditions, acetylcholine-induced, endothelium-dependent vasodilations were not influenced by CyA or Ox-LDL.
were not impaired after CyA and Ox-LDL treatment (Figure 5). Thus, impaired release of EDRF could not account for the potentiation of the contractile responses.

Discussion

The aim of this study was to investigate the effects and interactions of cyclosporine and LDL on basal, unstimulated vascular tone and on norepinephrine-induced vasoconstriction in isolated rabbit renal arteries. Basal, unstimulated vascular tone was influenced neither by LDL nor by CyA. However, norepinephrine-induced vasoconstrictions were potentiated by Ox-LDL. In the absence of CyA, Ox-LDL in concentrations corresponding to those estimated in arterial walls14 potentiated vasoconstrictions by a direct effect on vascular smooth muscle without involvement of the endothelium. After CyA treatment, the Ox-LDL-induced potentiation of contractile responses was further enhanced by an endothelial-dependent mechanism. N-LDL had no influence on vascular tone. The Ca$^{2+}$ antagonist verapamil prevented the potentiation of contractile responses by CyA and Ox-LDL, whereas saralasin, indomethacin, and the thromboxane A$_2$ antagonist daltroban were without effect. The endothelial integrity in terms of endothelium-dependent dilation to acetylcholine was well preserved under our study conditions.

In the present study, no enhancement of basal and norepinephrine-stimulated vascular tone has been observed after CyA treatment alone. In many other studies investigating the role of CyA on vascular functions, cremophor was used as a solvent.24 It has been shown that cremophor itself acts as a potent vasoconstrictor.25 In our hands, cremophor in clinically used concentrations dose-dependently elicited strong vasoconstrictions in the rabbit renal artery that exceeded those induced by CyA and Ox-LDL alone (data not shown). This indicates that cremophor may have caused in part the vasoconstrictor effects of CyA as demonstrated in other studies. An important difference of our experimental protocol to other investigations is the short exposure time of the vessels to CyA. In aortas obtained from rats under long-term CyA treatment, phenylephrine-induced vasoconstrictions were enhanced.7 In contrast, a more recent study in rats treated with CyA for 2 weeks demonstrated a rightward shift of the dose-response curve for norepinephrine.26 It appears that reasons for these discrepancies could be solvents for CyA, different exposure time, and administration routes of CyA. Investigation of short-term effects of CyA treatment may be of particular clinical interest since CyA solved in cremophor is routinely administered intravenously to patients during the initial phase of organ transplantation. Therefore, tissue perfusion may decrease shortly after administration of the drug27 and not only under long-term treatment.

Another important finding was that acetylcholine-induced, endothelium-dependent vasodilations were fully preserved under our study conditions. This indicates an intact function of the endothelium in contrast to studies with longer exposure periods where relaxations to acetylcholine (but not to nitrovasodilators) were reduced.6,7 Again, the most likely interpretation for this discrepancy is the relatively short duration of the CyA treatment in our study. However, the intact endothelial function and the unchanged contractile responses after short-term CyA treatment alone made it possible to detect the endothelium-mediated effect of cyclosporine in the presence of Ox-LDL, which has not been described before.

These findings implicate that the underlying mechanisms for the potentiation of vasoconstriction by CyA and Ox-LDL are at least in part different from effects of CyA alone, such as augmented transmural nerve stimulation.28 It has been suggested that attenuation of endothelial dilator capacities by CyA might result in enhancement of vascular tone.7 However, impairment of endothelial function in terms of a reduced release of vasodilator agonists such as EDRF or prostacyclin cannot account for the potentiation of vasoconstriction since endothelium-dependent vasodilations were not attenuated after preincubation with CyA and Ox-LDL. The inhibition of formation of cyclooxygenase products by indomethacin was also without effect.

Several studies demonstrated a role of vasoconstrictor prostaglandins and thromboxane A$_2$ in the CyA-induced nephrotoxicity of chronically treated animals.29,30 In these studies, the animals were set on a cyclosporine-containing diet for 2–12 weeks. However, a potential role of these substances in the enhancement of contractile responses in our short-term experiments appears unlikely in view of the lack of influence of both indomethacin and the thromboxane A$_2$ antagonist. A likely interpretation could be the different lengths of treatment periods.

It has been shown that CyA at high concentration (2 mg/ml) induces the endothelial release of angiotensin II.3 Consequently, angiotensin II might be a mediator of CyA-induced vasoconstrictions. However, in our segments treated with Ox-LDL and 10 µg/ml CyA, saralasin, a competitive angiotensin II antagonist, elicited no effect on contractile responses. Therefore, angiotensin

![Figure 5. Vasodilations evoked by cumulative doses of acetylcholine (0.003–0.3 µM) in endothelium-intact rabbit renal arteries after preincubation with cyclosporine (CyA, 10 µg/ml, 90 minutes) and oxidized low density lipoproteins (Ox-LDL, 100 µg/ml, 20 minutes) or their respective solvents as controls. Vasodilations are expressed as percentage of precontraction induced by norepinephrine (100 nM). Acetylcholine-induced, endothelium-dependent vasodilations did not differ between the CyA- and Ox-LDL–treated segments and the controls. For symbols without error bars, the SEM was less than symbol size. n=6 in each series.](http://hyper.ahajournals.org/figure)
II does not seem to mediate the potentiation of norepinephrine-induced vasoconstriction. The 200-fold lower CyA concentration in our experiments may be insufficient to induce an endothelial angiotensin II release.

However, it should be emphasized that the lack of effectiveness of the various antagonists only refers to the short-term CyA treatment and may be different under chronic conditions.

An important finding was the dependence of the CyA-induced potentiation of contractile responses on an intact endothelial lining. After endothelial denudation, CyA no longer elicited enhancement of vasoconstriction. This implies the release of an endothelium-derived vasoconstrctor factor induced by CyA. The enhancement of vasoconstriction by Ox-LDL alone was not diminished after endothelial denudation, but reached the same magnitude as in the endothelium-intact arteries. The latter observation is in accordance with a previous study from this laboratory, where Ox-LDL potentiated vasoconstriction elicited by different contractile agonists in endothelium-denuded human mammary and rabbit femoral arteries by a direct interaction with vascular smooth muscle. Thus, the influence of Ox-LDL alone on agonist-induced vasoconstriction was mediated by an endothelium-independent mechanism, in contrast to the endothelium-dependent further enhancement induced by CyA. In recent studies, it has been suggested that CyA induces the release of endothelin from human endothelial cells and dog renal arteries. Recently, Yang et al. have demonstrated that threshold concentrations of endothelin potentiate norepinephrine-induced contractile responses of isolated arteries. Therefore, it is tempting to speculate that CyA induces the release of endothelin, thereby leading to the further enhancement of vasoconstriction already potentiated by Ox-LDL. The inhibition of the contractile responses by verapamil is in accordance with this hypothesis since the effects of endothelin on norepinephrine-induced vascular tone were also suppressed by a Ca2+ antagonist in the study of Yang et al.

Several studies provide evidence that the alteration of renal hemodynamics in CyA-treated animals involves the sympathetic nervous system. Thus, potentiation of norepinephrine-induced vasoconstriction may be a mechanism for impaired renal hemodynamics leading to decreased glomerular filtration rates. It has been shown that patients under CyA treatment frequently exhibit lipid disorders, which favor the formation of Ox-LDL. We speculate that CyA and Ox-LDL synergistically enhance vascular tone.

In summary, these data indicate that coexistence of isolated renal arteries with CyA and Ox-LDL potentiates norepinephrine-induced vasoconstriction by a Ca2+- and partially endothelium-dependent mechanism before the onset of severe damage of the vasculature in terms of attenuation of endothelium-dependent vasodilations.

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