Cyclosporin A Inhibits Apoptosis of Human Endothelial Cells by Preventing Release of Cytochrome C From Mitochondria

Dirk H. Walter, MD; Judith Haendeler, PhD; Jan Galle, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Several experimental and clinical studies suggest that cyclosporin A (CSA) treatment reduces transplant atherosclerosis. Because oxidized LDL (oxLDL) is believed to play a key role in the development of atherogenesis, causing injury to the endothelium, and has been shown to induce apoptosis of endothelial cells, we investigated whether CSA inhibits oxLDL-induced apoptosis.

Methods and Results—Apoptosis was induced in human umbilical venous endothelial cells (HUVECs) by incubation of 10 μg/mL oxLDL for 18 hours. Coincubation with CSA dose dependently decreased oxLDL-induced apoptosis, with a maximal effect at 10 μmol/L. In addition, tumor necrosis factor-α– and angiotensin II–induced apoptosis was significantly prevented by CSA treatment, suggesting a general apoptosis-suppressive effect of CSA. CSA has been shown to inhibit disruption of the mitochondrial membrane function, which plays a key role in apoptosis induction. Indeed, oxLDL treatment triggered the release of cytochrome C from the mitochondria into the cytosol, indicating disturbance of the mitochondrial membrane. CSA (10 μmol/L) completely inhibited the oxLDL-induced release of cytochrome C. Moreover, tumor necrosis factor-α– and angiotensin II–induced cytochrome C release was prevented by CSA treatment.

Conclusions—OxLDL induces dysfunction of the mitochondrial membrane, leading to cytochrome C release into the cytosol, and thereby stimulates apoptosis of human endothelial cells. Apoptosis suppression by CSA correlates with the prevention of mitochondrial dysfunction and thus indicates the importance of mitochondrial destabilization in oxLDL-induced apoptosis signaling. The inhibition of apoptosis by CSA might preserve the function of the endothelium and may at least in part contribute to the antiatherogenic effects of CSA in transplant atherosclerosis. (Circulation. 1998;98:1153-1157.)

Key Words: cells ■ apoptosis ■ endothelium ■ lipoproteins ■ atherosclerosis ■ cyclosporin A

Oxidized lipoprotein is believed to play a key role as a triggering molecule that causes injury to the endothelium as an early event in atherogenesis. Further evidence indicates that the high frequency of lipoprotein abnormalities in heart transplant patients could account for the accelerated course of allograft atherosclerosis. The mechanisms by which oxidized lipoproteins induce endothelial injury, however, are not known. In vitro studies demonstrated that oxidatively modified LDL (oxLDL) has cytotoxic effects on endothelial cells. In addition, we recently demonstrated that oxLDL stimulates the cellular suicide pathway, leading to apoptosis of endothelial cells. OxLDL thereby activates the cysteine protease family recently termed “caspases,” a final common pathway of apoptosis signal transduction. Recent studies now emphasize the role of mitochondria in apoptosis signaling. Cells undergoing apoptosis show an early reduction of the mitochondrial transmembrane potential (ΔΨm), with concomitant release of the mitochondrial protein cytochrome C, which has been identified as the apoptosis-inducing factor-2 (Apaf-2). In the cytosol, cytochrome C in combination with Apaf-1 activates caspase-9, which finally leads to activation of caspase-3 and DNA fragmentation.

Several experimental and clinical studies suggest that cyclosporin A (CSA), which is commonly used to suppress allograft rejection, reduces hyperlipidemia-induced atherosclerosis and ameliorates transplant atherosclerosis. In addition, CSA has been shown to stabilize the mitochondrial transmembrane potential and thereby inhibit apoptosis induced by different stimuli.

Thus, the aim of the present study was to investigate the influence of CSA on oxLDL-induced apoptosis of human endothelial cells and to determine the involvement of mitochondrial destabilization in oxLDL-induced apoptosis. To demonstrate that the antiapoptotic effect of CSA was not
Figure 1. Inhibition of oxLDL-, TNF-α-, and Ang II–induced apoptosis by CSA. A, Apoptosis was induced in HUVECs with 10 μg/mL oxLDL and determined by morphological analysis of fluorescence-stained nuclei as representatively demonstrated in B (arrowheads indicate apoptotic cells). CSA was coincubated in the concentration indicated. *P<0.05 versus oxLDL without CSA coincubation. C, HUVECs were incubated with TNF-α (50 ng/mL) or Ang II for 18 hours in the presence or absence of CSA (10 μmol/L), and apoptosis was determined as described above. *P<0.05 versus TNF-α; **P<0.05 versus Ang II.
restricted to oxLDL-mediated apoptosis, we additionally evaluated its effects on apoptosis induced by the inflammatory cytokine tumor necrosis factor-α (TNF-α) and the proatherosclerotic factor angiotensin II (Ang II).

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium (Cell Systems/Clonetics) supplemented with hydrocortisone (1 μg/mL), bovine brain extract (12 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), epidermal growth factor (10 ng/mL), and 10% fetal calf serum until the third passage. Human LDL was isolated by sequential ultracentrifugation and oxidized as described previously. Antioxidant-free LDL (0.3 mg protein/mL) was incubated with CuSO₄ (5 μmol/L) for 24 hours at 23°C. The degree of oxidation was assessed by 2 different methods, the increase in mobility on agarose gel (1.4 versus native LDL) and the formation of thiobarbituric acid–reactive substances (3.4 ± 0.8 μmol/L).

**Detection of Cell Death**

For morphological staining of nuclei, cells were centrifuged (10 minutes, 700g), fixed in 4% formaldehyde, and stained with 4′,6-diamidino-phenylindole (0.2 μg/mL in 10 mmol/L Tris/HCl, pH 7, 10 mmol/L EDTA, 100 mmol/L NaCl) for 20 minutes; 500 cells were counted by 2 independent, blinded investigators. Lactate dehydrogenase was determined as described previously.

**Western Blot**

After incubation of HUVECs (1 × 10⁷ cells) for 18 hours, cells were scraped off the plates and pelleted by centrifugation at 800g for 10 minutes. Then, cells were resuspended in 1.2 mL of cold lysis buffer (20 mmol/L HEPES, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L PMSF) and incubated for 3 minutes on ice. Cells were homogenized with 10 strokes and centrifuged at 750g for 15 minutes at 4°C. The supernatant (cytosolic fraction) was removed, and the mitochondrial pellet was resolved in 50 μL of lysis buffer. Proteins were loaded onto 15% SDS-polyacrylamide gels. Antibody against cytochrome C (Pharmigen) was added in a final dilution of 1:500 in TBS-5% milk powder-1% FCS-0.5% Tween 20. Blots were reprobed with actin.

**Statistical Analysis**

Data are expressed as mean ± SEM from ≥ 3 independent experiments. Statistical analysis was performed with ANOVA followed by modified least significant difference test (SPSS software).
Results

Effect of CSA on Apoptosis of HUVECs

Exposure of HUVECs to oxLDL has been shown to induce apoptosis in a time- and concentration-dependent manner. To evaluate the effect of CSA, apoptosis was stimulated with 10 µg/mL oxLDL for 18 hours and determined by morphological analysis of fluorescence-stained nuclei (Figure 1A and 1B). Coincubation with CSA dose dependently reduced oxLDL-stimulated apoptosis, with complete inhibition obtained at 10 µmol/L CSA (Figure 1A). We excluded necrotic cell death by measuring the release of the cytosolic enzyme lactate dehydrogenase, which was not significantly changed in oxLDL-treated HUVECs (104±5% compared with control cells). Furthermore, CSA (10 µmol/L) did not induce necrotic cell death when added alone or in combination with oxLDL (101±2% compared with control). Control experiments demonstrated that the solvent ethanol did not affect basal or oxLDL-induced apoptosis (data not shown).

To test whether CSA also inhibits apoptosis induction by other proinflammatory or proatherosclerotic factors, apoptosis of HUVECs was stimulated by TNF-α or Ang II as previously described, and the effects of CSA were investigated. Incubation of 50 ng/mL TNF-α for 18 hours triggered apoptosis of HUVECs as demonstrated by morphological analysis of fluorescence-stained nuclei (Figure 1C). The addition of 10 µmol/L CSA completely abolished TNF-α–induced apoptosis (Figure 1C). In addition, Ang II–stimulated apoptosis was also suppressed by CSA coincubation (Figure 1C), suggesting a general apoptosis-suppressive effect of CSA.

Effect of CSA on Mitochondrial Transmembrane Permeability Transition

CSA has been shown to inhibit the disruption of mitochondrial membrane function, a common manifestation of apoptosis induced by several proapoptotic stimuli. To test whether inhibition of mitochondrial disruption may account for the antiapoptotic effect of CSA, we first tested the effect of oxLDL on mitochondrial permeability. Disruption of mitochondrial membrane function results in the specific release of the mitochondrial enzyme cytochrome C into the cytosol, and cytochrome C was detected by Western blotting. As shown in Figure 2A, incubation of HUVECs with oxLDL (10 µg/mL) for 18 hours induced the release of cytochrome C into the cytosolic fraction 2-fold compared with the cytochrome C amount determined in the cytosolic fraction of control cells. Importantly, coincubation of oxLDL with CSA completely suppressed the release of cytochrome C, even below basal levels (Figure 2A). The increase of cytochrome C in the cytosolic fraction correlated with a reduction of ~20% of the mitochondrial protein after oxLDL stimulation. Moreover, coincubation with CSA significantly reversed the decrease of cytochrome C in the mitochondrial fraction. Equal loading was confirmed by reprobing the blots with antibodies against actin (Figure 2A). Moreover, CSA completely prevented TNF-α– and Ang II–induced cytochrome C release (Figure 2B). CSA thereby suppressed cytochrome C levels in the cytosolic fraction below basal levels (Figure 2B).

Discussion

The results of the present study demonstrate that CSA inhibits oxLDL-induced apoptosis of human endothelial cells. The mechanism underlying the protective effect of CSA appears to involve stabilization of mitochondria, thereby preventing the release of the mitochondrial protein cytochrome C into the cytosol. Cytosolic cytochrome C has been shown to be necessary to activate the apoptosome complex leading to caspase activation and apoptosis induction. The findings of the present study may give mechanistic insights into CSA action and may support the results of several experimental studies that demonstrate an antiatherosclerotic effect of CSA in transplant atherosclerosis as well as hyperlipidemia-induced atherosclerosis.

In cardiac heart transplants, accelerated coronary atherosclerosis has become the principal cause of late death and allograft dysfunction. Accelerated atherosclerosis is assumed to be mediated by alloimmunity; however, additional “antigen-independent” mechanisms may also account for the development of transplant atherosclerosis. Thus, the high frequency of lipoprotein abnormalities in patients with heart transplantation and the prevention of allograft failure by the use of HMG-CoA reductase inhibitors suggest an important role of lipoproteins in transplant atherosclerosis. Moreover, oxLDLs is believed to play a key role in causing endothelial cell injury, thereby contributing to atherosclerotic lesion development. Stimulation of the endogenous suicide cell death pathway by oxLDL in endothelial cells may be one cause of endothelial injury. We now demonstrate that apoptosis induction by oxLDL is prevented by the immunosuppressive agent CSA in a dose-dependent manner. Moreover, the apoptosis-suppressive effect of CSA is not restricted to oxLDL-induced apoptosis but also extends to apoptosis induced by TNF-α or the proatherosclerotic factor Ang II, suggesting a general antiapoptotic effect of CSA on endothelial cell apoptosis.

Apoptosis refers to the morphological alterations exhibited by actively dying cells. Activation of the caspases cascade, the final common effector proteases mediating apoptosis signaling, has been demonstrated to play a central role in endothelial cell apoptosis. Recent evidence now suggests that dysfunction of the mitochondria with a release of cytochrome C precedes activation of caspases and DNA fragmentation. Indeed, injection of cytochrome C into the cytosol of different cell lines induces activation of caspase-3 and subsequent apoptosis. The findings of the present study demonstrate that oxLDL stimulation induces the release of mitochondrial cytochrome C into the cytosol, which is essential for activation of caspase-3, the central executioner of apoptosis. Thus, oxLDL-induced cytochrome C release may lead to activation of caspase-3, which we previously demonstrated is stimulated by oxLDL. Importantly, the inhibitory effect of CSA on oxLDL-induced apoptosis appears to be mediated by inhibition of the mitochondrial dysfunction, as demonstrated by the prevention of cytochrome C release. Indeed, this concept is in accordance with...
previous studies that demonstrate that CSA potently inhibits the reduction of the mitochondrial transmembrane potential and thereby suppresses apoptosis of other cell types.11

CSA is known to prevent atherosclerosis.8,9,16 The results of the present study demonstrating an inhibition of endothelial cell apoptosis by CSA might provide a clue to explain the antatherosclerotic effects of CSA. The concept that CSA treatment suppresses transplant atherosclerosis is supported by the finding that the use of therapeutic levels of cyclosporine in an experimental model of transplant atherosclerosis clearly has a substantial inhibitory effect on the development of transplant atherosclerosis.9 Because a large portion of CSA is bound to LDL in plasma, effective lipid lowering may therefore lead to more free CSA in the blood. The results of the present study demonstrating an inhibition of endothelial cell apoptosis-inducing factor. However, contradictory effects of CSA have also been demonstrated in some experimental models that revealed an acceleration of atherosclerosis.22,23 This controversy might be due to different dosages used and the end point examined.

In summary, the present investigation demonstrates that oxLDL-induced dysfunction of the mitochondrial membrane potential, leading to cytochrome C release into the cytosol, and thereby stimulated apoptosis of human endothelial cells. Apoptosis induction by oxLDL as well as by TNF-α and Ang II was completely inhibited by CSA. Apoptosis suppression by CSA correlated with the prevention of mitochondrial dysfunction and thus indicates the importance of mitochondrial destabilization in oxLDL-induced apoptosis signaling. The inhibition of apoptosis by CSA might preserve the function of the endothelium and may at least in part contribute to the antitherogenic effects of CSA in transplant atherosclerosis.

Acknowledgments
We would like to thank Christine Goebel for expert technical assistance. This work was supported from the Deutsche Forschungsgemeinschaft (DFG) (Ga 431/2–1, Di 60012–2, and 5FB553). Dr Dimmeler has a fellowship from the DFG (Di 600/2–1).

References
Cyclosporin A Inhibits Apoptosis of Human Endothelial Cells by Preventing Release of Cytochrome C From Mitochondria
Dirk H. Walter, Judith Haendeler, Jan Galle, Andreas M. Zeiher and Stefanie Dimmeler

_Circulation._ 1998;98:1153-1157
doi: 10.1161/01.CIR.98.12.1153

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/98/12/1153

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/