Cyclophilin-Mediated Pathways in the Effect of Cyclosporin A on Endothelial Cells

Role of Vascular Endothelial Growth Factor

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Abstract—The relative importance of cyclophilin (CyP) versus calcineurin (Cn)-mediated mechanisms in the effect of cyclosporin A (CsA) on endothelial cells (ECs) is largely unknown. In cultured ECs, CsA was cytotoxic/proapoptotic or cytoprotective/antiapoptotic at high or low concentrations, respectively. CsA analogs (MeVal-4-CsA and MeIle-4-CsA), which bind to CyP but do not inhibit Cn, closely reproduced the CsA effects. Based on our previous data, the role of vascular endothelial growth factor (VEGF) as a mediator of CsA-induced cytoprotection was further analyzed. The actions of CsA and CsA analogs were shifted from a protective to a cell-damaging pattern in the presence of a specific anti-VEGF monoclonal antibody (mAb). This positive interaction was further supported by a transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) by VEGF after pretreatment with either CsA or MeVal-4-CsA and an increase in the expression and synthesis of VEGF receptor 2 (VEGFR2). Of functional importance, blockade of the interaction between VEGF and VEGFR2 by a VEGFR2 mAb abolished the cytoprotective effect of CsA. In addition, preconditioning with low concentrations of CsA or CsA analogs increased both cytoprotection and VEGFR2 mRNA expression when EC were exposed to higher concentrations of CsA. In summary, our results reveal that (1) the biphasic responses to CsA in EC are related to the interaction of CsA with CyP rather than with Cn and (2) VEGF is a critical factor in the cytoprotective effect of CsA, by a mechanism that involves VEGFR2. **(*Circ Res*. 2002;91:202-209.)**

Key Words: cyclosporin A ■ cylophilin-binding analogs ■ vascular endothelial growth factor ■ vascular endothelial growth factor receptor 2 ■ cytoprotection

Toxic effects, which involve some organs with particular intensity, are the main disadvantage in the therapeutic use of cyclosporin A (CsA). Nephrotoxicity, renal vascular damage, and hypertension are the most relevant among the undesirable effects. Endothelial cell (EC) toxicity is a main feature of the CsA-induced vascular injury.[1–6]

In diverse cellular types, the mechanisms of the cytotoxic effect of CsA have not been clarified. To the contrary, the toxic mechanisms appear to be fully understood in the lymphocyte,[7,8] in which CsA binds cyclophilin (CyP), a cytosolic peptidylprolyl *cis-trans* isomerase (PPIase), and inhibits its activity.[9] The resulting CsA-CyP complex binds to and inhibits the calcium/calmodulin-dependent protein phosphatase, calcineurin (Cn).[7,8,10,11] Cn regulates the processes of dephosphorylation and nuclear import of the transcription factor; nuclear factor of activated T cells (NFAT). In the T-cell signal transduction cascade, NFAT is involved in the nuclear stimulation of the inducible expression of a number of genes, such as IL-2 or IL-4.[12] None the less, although Cn inhibition is the keystone of CsA-mediated lymphocyte effects, the issue of whether CyP or Cn is the principal mediator of CsA effects on ECs has not been yet thoroughly addressed.

A principal aspect related to the mechanisms of endothelial effects of CsA is the putative involvement of vascular endothelial growth factor (VEGF) as a main agent of its cytoprotective action. In fact, in addition to its actions aimed to promote angiogenesis, an additional property of VEGF is the induction of EC resistance against injury.[13,14] This property has been less characterized but is particularly meaningful for the present study.[13,14] VEGF exerts its effect after binding to membrane tyrosine kinase receptors (VEGFR1, VEGFR2, VEGFR3, and a complementary receptor neuropilin-1). Each of these receptors has different signal transduction properties and functions (see references 14 and 15 for review), with VEGFR2 being the more important in functional terms.[13–16]

Recent evidence has shown that circumstances as varying as hypoxia, exposure to reactive oxygen species (ROS), fibroblast growth factor-2, or rupture of interendothelial VE

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cadherin junctions can all stimulate the ECs themselves to produce VEGF in an autocrine manner.[17–19] The significance and precise biological role of this autocrine VEGF expression is still incompletely characterized, but it appears to be related to a protective mechanism.[17,20] In this regard, we have previously communicated that the blockade of the action of autocrine VEGF significantly modifies the effect of some agents on the endothelium, including CsA.[21] However, no data are available to date that identify the precise mechanisms determining the cytotoxicity or cytoprotection in CsA-treated ECs. Accordingly, the aims of the present study were, first, to ascertain whether CyP or Cn-mediated pathways are predominantly involved in CsA cytoprotective and cytotoxic actions on EC and second, to clarify, at least in part, the mechanisms linking VEGF with CsA-related cytoprotection.

Materials and Methods

Endothelial Cell Culture

Bovine aorta ECs were obtained, cultured, and characterized as described.[17,21,22]

Experimental Maneuvers

Confluent, growth-synchronized ECs (2 to 4 passages) without fetal calf serum (FCS) were treated with either CsA or CsA analogs (MeVal-4-CsA and MeIle-4-CsA 10 nmol/L to 50 μmol/L, 24 to 48 hours),[23] the specific Cn autoinhibitory peptide (Calbiochem),[24] the membrane nonpermeable CsA analog, 14-043,[25] and/or the corresponding vehicle. A specific blocking anti-VEGF monoclonal antibody (mAb) (1 μg/mL, Sigma, Madrid)[21,22] was used to inhibit VEGF effects. The ability of this mAb to interfere with the effects of exogenous $VEGF_{165}$ and $VEGF_{121}$ had been checked in previous studies[21,22] and in setting-up experiments. Similar incubations were done with an specific blocking anti-VEGFR2 (0.5 μg/mL) (clone 3.83, ImClone Systems Inc).[26] A nonspecific IgG antibody and a recombinant exogenous $VEGF_{165}$[21,22] were also used.

Assessment of Cell Injury by ^{51}Cr and Lactate Dehydrogenase (LDH) Release

The experiments were performed in confluent ECs, as described.[21]

Flow Cytometry

Flow cytometry was performed after propidium iodide staining, as described.[17] Cell death was analyzed using the Mccycle program (Coulter) to quantify the percentage of cells with stainability below the Go/G1 peak.

DNA Degradation

Internucleosomal DNA fragmentation of ECs was analyzed in agarose gels as described.[27]

Rhodamine 123 Fluorescence

The fluorescent intensity of rhodamine 123–labeled cells was measured, as described,[27] for evaluation of mitochondrial membrane potential.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

ECs were incubated with or without pretreatment with CsA and/or CsA analogs and stimulated with a combination of phorbol 12-myristate 13-acetate (PMA)+calcium ionophore A23187 to stimulate NFAT nuclear binding activity. Nuclear extracts were analyzed by EMSA.[11] Specificity was tested by incubating the nuclear extracts with excess (50-fold) unlabeled oligonucleotide or 1 μL anti-NFATc1 antibody (0.2 μg/mL, Santa Cruz Biotechnology, 4°C, 30 minutes before addition of the probe).

Measurement of $[Ca^{2+}]_i$

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was determined by fura2, as described.[22]

mRNA Isolation, Northern Blot, Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR), and Real-Time RT-PCR

Total RNA was isolated from ECs.[17,22] Northern blot and routine RT-PCR were performed as described,[21] using a probe and oligonucleotide primers of a common human and bovine VEGF and VEGFR2 sequences.[17,21,22] In the conditions studied, maximal expression of VEGF mRNA is evident at about 3 hours.[17,21] Quantitative PCR was also performed, to further ascertain the actual magnitude of VEGFR2 expression.[28] Supplementary information regarding this method is provided in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 28 S were used as control and the amplification products were sequenced.[17,21] To each RT-PCR reaction, 2 μCi ($\alpha^{32}P$) dCTP (>3000 Ci/mmol, Amersham) were added. The amplification cycles used for semiquantitative RT-PCR were chosen based on previous experiments.

Immunoprecipitation and VEGFR2 Western Blot

Cell lysates were obtained from ECs, and VEGFR2 immunoprecipitation and detection was performed as described by Kroll and Waltenberger.[16]

Statistical Methods

Results are expressed as mean±SEM. If not specifically mentioned, each value corresponds to 5 experiments performed in triplicate. Comparisons were done by 1-way ANOVA, Fisher and Scheffé tests, or paired and unpaired Student's *t* test when appropriate. A value of $P<0.05$ was considered significant.

Results

The exposure of ECs to CsA induced a diverse set of responses depending on the concentrations. These responses ranged from cytoprotection to injury of diverse intensity, as follows: concentrations of CsA of 1 μmol/L or higher enhanced the cytotoxic effects of FCS deprivation, whereas concentrations within the human therapeutic range (10 nmol/L) were cytoprotective, as measured by LDH release (Table 1). As a positive control, a significant decrease in LDH release was evident by incubating ECs in the presence of 20% FCS (24 hours, 81±7%; 48 hours, 70±9%; $P<0.01$ with respect to LDH release in the absence of FCS). As a confirmatory method, we measured ^{51}Cr release in samples run in parallel, which showed superimposable results in terms of cytotoxicity and cytoprotection (P=NS between percent of LDH and ^{51}Cr release, data not shown).

Once the concentration-response curve of EC cytoprotection or injury with CsA was defined, experiments were performed to ascertain the relative role of CyP and Cn as mediators of the CsA effects on ECs. CsA analogs (MeVal-4-CsA and MeIle-4-CsA) that bind to CyP but do not inhibit Cn were used for this purpose. As a quality control, this specific property was confirmed by EMSA of NFAT mobility to the nucleus. The EMSA experiments showed that 1 μmol/L CsA completely blocked the induction of NFAT DNA-binding activity from ECs stimulated with PMA plus ionophore; to the contrary, the CsA analogs failed to block it (Figure 1). The specificity of these findings was demonstrated by observing that the binding of NFAT was completely

TABLE 1. Effect of Treatment With CsA and CsA Analogs on LDH Release by EC (%)

	% LDH Release (24 hours)
Basal (vehicle, no FCS)	100±13
CsA 10 nmol/L	80±10*
CsA 1 μmol/L	103±8
CsA 10 μmol/L	145±13*
CsA 50 μmol/L	350±23†‡
MeVal-4-CsA 10 nmol/L	80±6*
MeVaL-4-CsA 1 μmol/L	106±10
MeVal-4-CsA 10 μmol/L	115±7*
MeVal-4-CsA 50 μmol/L	135±7*
MeIle-4-CsA 10 nmol/L	81±9*
MeIle-4-CsA 1 μmol/L	124±15
MeIle-4-CsA 10 μmol/L	134±10*
MeIle-4-CsA 50 μmol/L	160±10*
Cn autoinhibitory peptide 10 μmol/L	102±10
14-043 10 nmol/L	106±14
14-043 1 μmol/L	109±10
14-043 10 μmol/L	102±10

Data are expressed as LDH released at 24 hours in the absence of FCS with respect to the basal. Each value is the mean±SDM of a minimum of 5 experiments performed in triplicate.

*$P<0.05$, †$P<0.01$ with respect to the basal, respectively; ‡$P<0.005$ with respect to the same concentration of MeVal-4-CsA or MeIle-4-CsA.

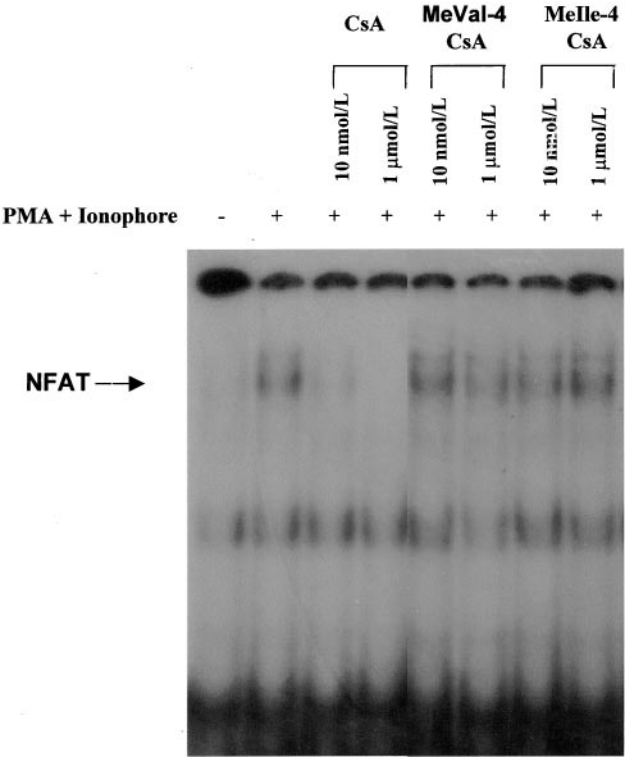

Figure 1. Kinetics and characterization of the NFAT DNA-binding complexes. Nuclear extracts from ECs stimulated with a combination of 35 nmol/L PMA+1 μmol/L ionophore (20 minutes), pretreated or not with CsA or CsA analogs (10 nmol/L and 1 μmol/L) for 2 hours were analyzed by EMSA. Arrow indicates the mobility of the specific PMA+ionophore NFAT complexes. CsA, but not CsA analogs, inhibits NFAT migration to the nucleus.

displaced from the specific bands with an excess of cold oligonucleotide, and that the addition of specific anti-NFATc1 antibody totally supershifted the NFAT complex generated with nuclear extracts from ECs stimulated with PMA plus ionophore (data not shown).

The functional properties of the analogs on ECs were analyzed by means of LDH release. The CsA analogs reproduced both the cytoprotective and cytotoxic effects observed with CsA on ECs (Table 1); however, the toxic effects of the analogs were weaker than those of CsA (Table 1), eg, $P<0.01$ between 50 μmol/L CsA versus 50 μmol/L MeVal-4-CsA or 50 μmol/L MeIle-4-CsA. To the contrary, no effect on LDH release by EC was observed on the addition of non–CsA-related Cn autoinhibitory peptide (Table 1). To further analyze the specificity of the action of the analogs, we found that the nonpermeable analog, 14-043, was devoid of any measurable effects on LDH release in the conditions of the experiments (P=NS with respect to untreated ECs; Table 1). Results of LDH release at 48 hours showed similar effects to those identified at 24 hours (data not shown).

Additional, functionally meaningful confirmation of the validity of the cytoprotective actions was obtained in experiments examining the effect of pretreating the ECs with low concentrations of CsA and CsA analogs (10 nmol/L), before adding higher, cytotoxic concentrations of CsA. As can be observed in Figure 2A, pretreatment with 10 nmol/L CsA (24 hours) induced a significant increase in EC resistance against further exposure to toxic concentrations of the drugs, as assessed by LDH release. This result was further confirmed in experiments using flow cytometry (Figures 2B through 2E). Similar results were obtained by pretreating the ECs with MeVal-4-CsA (10 nmol/L) (data not shown).

Based on our previous observations, the role of VEGF as a putative mediator of cytoprotection was considered. In this regard, the protective effect of CsA and CsA analogs was blocked in the presence of an anti-VEGF mAb, which also increased the toxicity of CsA at different concentrations (Table 2). The specificity of this effect of the anti-VEGF mAb was ascertained by the following: (1) the absence of any cytotoxic or cytoprotective effect of the anti-VEGF mAb by itself (Table 2); and (2) the absence of blocking effects of a nonspecific IgG on the protective actions of CsA and/or CsA analogs (P=NS with respect to the experiments done without antibody, data not shown). VEGF has been recognized as a molecule with protective, antiapoptotic effects in ECs.[13,14] The ability of CsA and its analogs to induce apoptosis-related changes was evaluated by 3 methods, namely, flow cytometry (Figure 3A through 3D), DNA laddering (Figure 3E), and changes in mitochondrial membrane potential.[23,27] The latter are depicted in the online Figure (found in the online data supplement available at http://www.circresaha.org), which shows that treatment with CsA at toxic concentrations (10 μmol/L) induces the extrusion of rhodamine 123 from the mitochondria, therefore indicating mitochondrial depolarization. Furthermore, treatment with anti-VEGF antibody in-

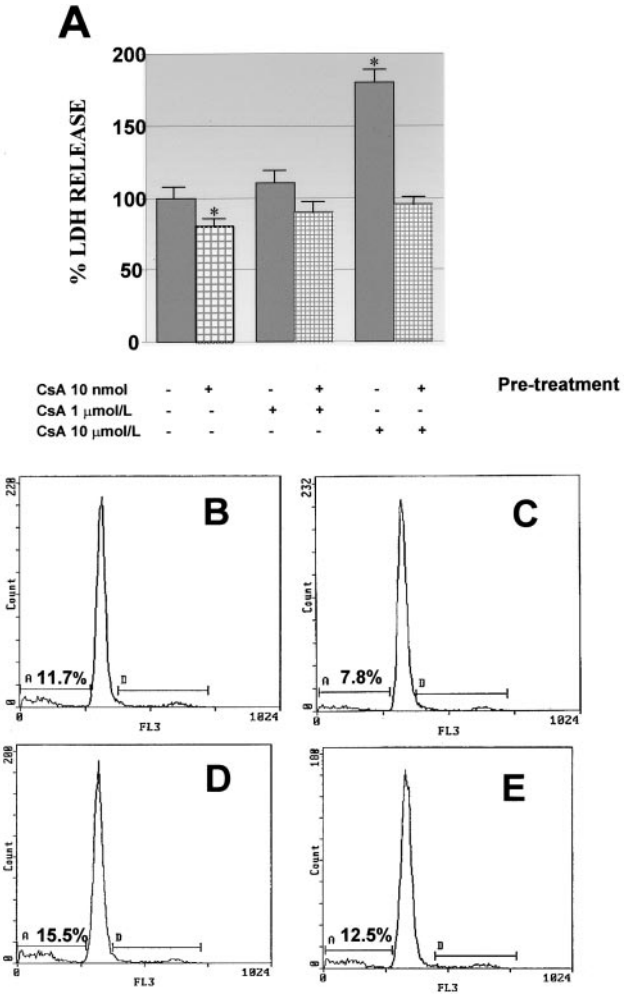

Figure 2. EC preconditioning by CsA. EC exposure to a protective concentration of CsA induces adaptation and protection of ECs against further exposure to cytotoxic concentrations of CsA. A, 24 hours after exposure to 10 nmol/L CsA and/or vehicle, ECs were re-exposed to high cytotoxic concentrations of CsA for an additional 24 hours and cell injury was assessed. Data are expressed as LDH released with respect to the basal, in the absence of FCS. B through E, Representative flow cytometry traces (propidium iodide staining) of the effect of different treatments at 24 hours, as follows: B, vehicle 24 hours plus vehicle 24 hours; C, 10 nmol/L CsA 24 hours plus vehicle 24 hours; D, vehicle 24 hours plus 10 μmol/L CsA 24 hours; E, 10 nmol/L CsA 24 hours plus 10 μmol/L CsA 24 hours. Dead cells are represented on the left of the flow cytometry trace, and their percent with respect to the total number of cells is depicted on the figure. Each trace is representative of a minimum of 5 similar experiments done in triplicate. *$P<0.01$ with respect to the respective basal.

duced the same phenomenon in ECs exposed to low (10 nmol/L) concentrations of CsA (online Figure).

Based on the aforementioned results, the hypothesis was raised that CsA might have induced increased expression of VEGF mRNA. We believe this is not the case because an increase in VEGF mRNA expression was not detected by either RT-PCR or Northern blot (Figure 4A and 4B) in ECs exposed to diverse concentrations of CsA or CsA analogs. A small, but consistent degree of expression of VEGF mRNA was evident in ECs in baseline conditions, as previously shown.[17,21]

TABLE 2. VEGF as a Mediator of Endothelial Cytoprotection: Critical Role of VEGFR2

	% LDH Release
Basal (vehicle, no FCS)	100±13
Anti-VEGF mAb	95±11
CsA 10 nmol/L	80±10*
CsA 10 nmol/L+anti-VEGF mAb	101±7
CsA 1 μmol/L	103±8
CsA 1 μmol/L+anti-VEGF mAb	110±9
CsA 10 μmol/L	145±13*
CsA 10 μmol/L+anti-VEGF mAb	356±23†
CsA 50 μmol/L	350±23†
CsA 50 μmol/L+anti-VEGF mAb	423±31†
MeVal-4-CsA 10 nmol/L	80±6*
MeVal-4-CsA 10 nmol/L+anti-VEGF mAb	105±6
Melle-4-CsA 10 nmol/L	81±9*
Melle-4-CsA 10 nmol/L+anti-VEGF mAb	110±7
Anti-VEGFR2 mAb	104±10
CsA 10 nmol/L+ anti-VEGFR2 mAb	103±4
CsA 1 μmol/L+anti-VEGFR2 mAb	133±9*
MeVal-4-CsA 10 nmol/L+anti-VEGFR2 mAb	108±6
$VEGF_{165}$ 0.5 nmol/L	87±6*
$VEGF_{165}$ 0.5 nmol/L+ CsA 10 nmol/L	78±9*
$VEGF_{165}$ 0.5 nmol/L+ MeVal-4-CsA 10 nmol/L	75±6*
$VEGF_{165}$ 0.5 nmol/L+ Melle-4-CsA 10 nmol/L	76±7*

Percent LDH release by ECs at 24 hours of incubation in the absence of FCS. Anti-VEGF mAb (1 μg/mL), recombinant exogenous $VEGF_{165}$, and anti-VEGFR2 mAb (clone 3.83, 0.5 μg/mL) were present from 60 minutes before the addition of CsA or CsA analogs and during the entire incubation time. Data are expressed as LDH released with respect to the basal (no FCS); each value is the mean±SDM of a minimum of 5 experiments done in triplicate.

*$P<0.05$ with respect to the basal; †$P<0.001$ with respect to the basal.

In view of these results, which ruled out increased VEGF expression by CsA as the mechanism underlying the protective effect by CsA and CsA analogs, the possible existence of positive interactions between CsA or CsA analogs and VEGF intracellular signaling was assessed. The corresponding results are depicted in Table 3. First, we have found a transient increase in VEGF-induced $[Ca^{2+}]_i$ in ECs preincubated for 24 hours with 10 nmol/L CsA or 10 nmol/L MeVal-4-CsA, compared with the transient $[Ca^{2+}]_i$ in ECs preincubated only with vehicle ($P<0.001$). To the contrary, no differences were found in the ATP-induced transient $[Ca^{2+}]_i$ in ECs preincubated for 24 hours with vehicle, 10 nmol/L CsA, or 10 nmol/L CsA analog against control cells (Table 3; P=NS between the 3 conditions).

Moreover, in LDH release experiments assessing the protective effect versus damage caused by the absence of FCS, we found that EC protection by exogenous recombinant $VEGF_{165}$, added at 0.5 nmol/L, was potentiated after pretreatment with 10 nmol/L of CsA or MeVal-4-CsA (Table 2). The concentration of VEGF used corresponds to the reported affinity constant of VEGF for VEGFR2.[13,14] As a further illustration of the effects of exogenous $VEGF_{165}$, it attenuated

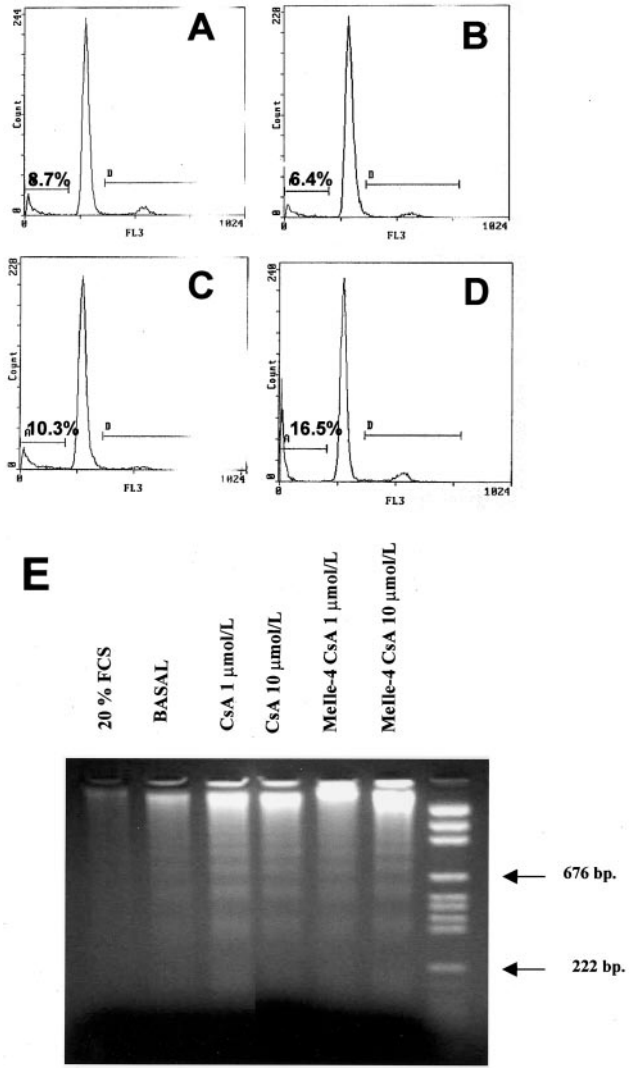

Figure 3. Effect of treatment with CsA on ECs. ECs were exposed to different concentrations of CsA and/or CsA analogs for 24 hours. A through D, Representative flow cytometry traces (propidium iodide staining). Dead cells are represented on the left of the flow cytometry trace, and their percent with respect to the total number of cells is depicted on the figure. A, B, C, and D correspond to vehicle, 10 nmol/L, 1 µmol/L, and 10 µmol/L of CsA, respectively. E, Laddering of DNA in agarose gel. Each trace is representative of a minimum of 4 similar experiments. *$P<0.01$ with respect to the corresponding basal.

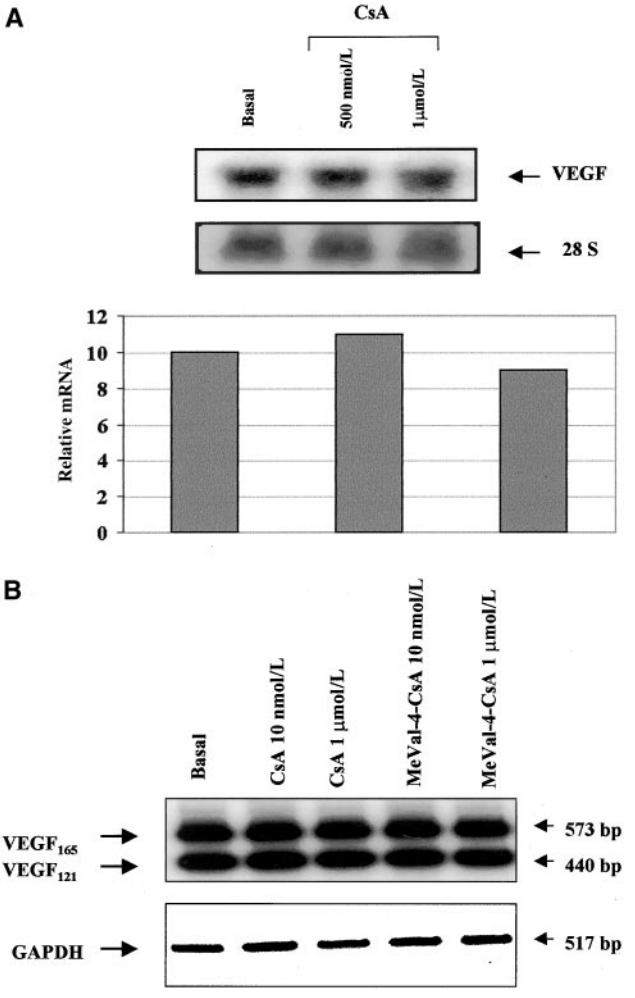

Figure 4. Northern-blot (A) and RT-PCR (B) analysis for VEGF with total RNA from ECs untreated or treated with different concentrations of CsA or CsA analogs (3 hours). No increase in VEGF mRNA expression was detected at any of the conditions studied.

the cytotoxicity of higher doses of CsA (LDH release: CsA 1 µmol/L=103±8% versus CsA 1 µmol/L+$VEGF_{165}$ 0.5 µmol/L=91±7%; $P<0.05$, n=4).

In an effort to characterize the mechanism of the potentiated VEGF response, we analyzed the role of VEGFR2, the main receptor responsible for VEGF-induced signals in ECs.[16] In this regard, the protective effect of CsA and CsA analogs was blocked in the presence of an anti-VEGFR2 mAb; furthermore, the anti-VEGFR2 mAb also increased the toxicity of CsA (Table 2).

To further understand the mechanisms involved, an increased expression of VEGFR2 mRNA was found after 4 hours of incubation with either CsA or CsA analogs. This effect peaked between 8 to 16 hours of stimulation and disappeared at 32 hours (data not shown and Figures 5A through 5C). Of additional interest for the aforementioned preconditioning phenomenon, pretreatment of EC with low cytoprotective concentrations (10 nmol/L) of CsA or its analog MeVal-4-CsA favored the increased expression of VEGFR2 when ECs were re-exposed to higher (1 µmol/L) concentrations of CsA (Figure 5D). In agreement with the findings on VEGFR2 expression, an increased synthesis of

TABLE 3. [Ca^{2+}] Transient in ECs When the Cells Were Stimulated With VEGF or ATP

		Peak, nmol/L	
	Baseline, nmol/L	VEGF 0.5 nmol/L	ATP 1 mmol/L
Vehicle	94±8	130±16	178±19
CsA 10 nmol/L	105±10	219±27*	195±23
Me Val-4-Csa 10 nmol/L	100±10	241±15*	174±25

ECs were preincubated during 24 hours with either vehicle, 10 nmol/L CsA, or 10 nmol/L MeVal-4-CsA. Each value corresponds to the mean±SDM of a minimum of 5 triplicate experiments.

*$P<0.05$ with respect to ECs preincubated with vehicle.

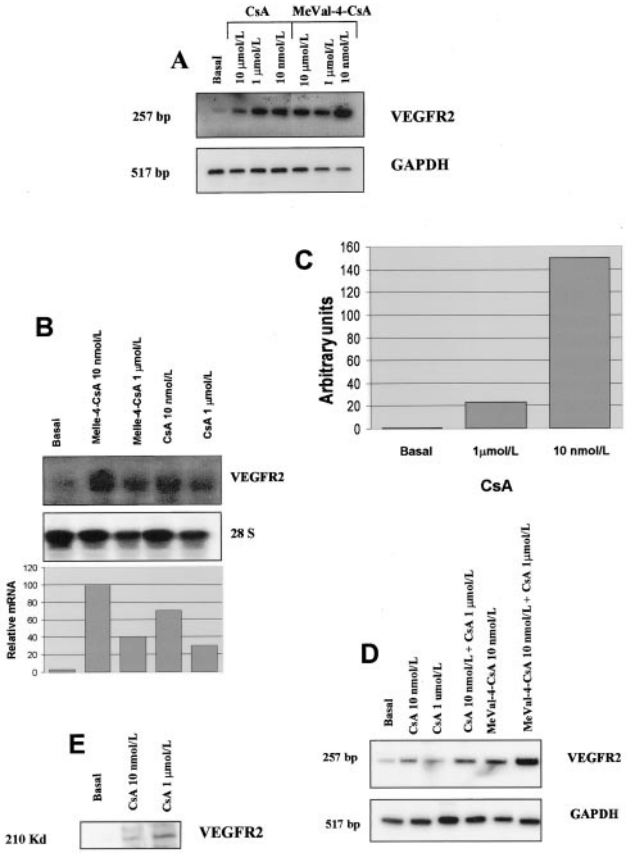

Figure 5. RT-PCR (A), Northern-blot (B), quantitative (real-time) RT-PCR (C), immunoprecipitation, and Western blot of VEGFR2. A through C, ECs untreated or treated with different concentrations of CsA and/or CsA analogs for 16 hours. D, RT-PCR of RNA from ECs re-exposed to 1 μmol/L of CsA for additional 16 hours, after 24-hour exposure to 10 nmol/L of CsA or Me-Val-4-CsA. E, Immunoprecipitation and Western blot of cell lysates from ECs untreated or treated with different concentrations of CsA for 36 hours.

VEGFR2 protein was found when ECs were incubated with different concentrations of CsA (Figure 5E).

Discussion

The principal results of our study are (1) that the effects of CsA on cytoprotection and cytotoxicity in ECs are conveyed through CyP-mediated rather than by Cn-mediated mechanisms and (2) that VEGF (via VEGFR2) has a major role in the cytoprotective effects of CsA at low concentrations.

The experiments with CsA analogs, namely, MeVal-4-CsA and MeIle-4-CsA, which bind to CyP but do not inhibit Cn, were instrumental in elucidating the relative role of CyP and Cn. To our knowledge, no data are available to date that allow discrimination of the relative contribution of CyP and Cn in terms of EC response to CsA. The finding that CsA analogs bind only to CyP to reproduce both the cytoprotective/antiapoptotic and cytotoxic/proapoptotic effects observed with CsA on ECs, strongly suggests that, in the case of ECs and within a certain range of concentrations, binding to CyP rather than Cn inactivation is the relevant phenomenon. This result was further substantiated by the absence of effects of the Cn autoinhibitory peptide and the cell nonpermeable analog, 14-043.

The mechanism by which CsA and CsA analogs promote EC toxicity and apoptosis are not clearly established, but changes in mitochondrial membrane potential have consistently been found in cells treated with CsA[23,27]; these results are in close agreement with our findings in which the cytotoxic effects were concentration-dependent and 1 μmol/L concentration of CsA appears to be the first at which toxicity develops consistently.

Our results suggest that the role of CyP is more relevant than that of a mere relay step in the pathway leading to Cn blockade. Although Cn blockade is critical for the immunosuppressive effects of CsA in lymphoid cells, it was not as clear whether Cn was equally important for the other putative effects of CsA in diverse types of cells, eg, ECs. Actually, to our knowledge, the present study is the first that addresses this issue by a direct experimental approach. Our results suggest that there are important differences between ECs and lymphocytes in terms of CsA effects. CyP of the A subtype (CyP-A) is the predominant PPIase present in the cytoplasm of mammalian cells.[29] The relative affinity of CyP-A to CsA increases in the 4-substituted derivates.[23,29] accordingly, the inhibitory constant of PPIase activity is around 7 nmol/L for CsA and 4 nmol/L for MeVal-4-CsA[23,25]; therefore, implying the possible occurrence of a slightly stronger effect of MeVal-4-CsA compared with CsA. This difference might become more relevant at the lower concentrations of the drug.

The fact that CsA and CsA analogs produce both proapoptotic and antiapoptotic effects, depending on their concentrations, can be related to the inhibition of PPIase activity. CyP could be involved in signal transduction pathways through conformational alteration of transcription factors, ion channels, or protein kinases, which could stimulate protective mechanisms at low concentrations. These mechanisms may become overwhelmed by higher amounts of the drugs. Putative candidates involved in EC protective mechanisms include heat shock proteins,[30] ROS,[3,31] nitric oxide,[32] and Bcl-2.[33]

Based on our previous observations,[17,21] we assessed the role of VEGF as a putative mediator of EC cytoprotection. Our results show a complete inhibition of the CsA and CsA analogs' protective effect on exposure to anti-VEGF mAb; therefore, these findings specifically support the relevant role of VEGF in the protection of ECs against deleterious factors, ie, FCS deprivation. In the experimental conditions used, namely, absence of FCS, no exogenous VEGF was present. Therefore, the inhibitory effect of the anti-VEGF mAb can only be attributed to blockade of endogenous, autocrine VEGF. Next, we asked whether, as observed with EC challenge by other agents, eg, ROS, cytochalasin D, or anti–VE-cadherin antibody,[17,18,21] CsA was capable of inducing increased expression of autocrine VEGF. This was not the case, and the negative results of these experiments indicated that the response of the expression of VEGF was rather different with CsA than with the aforementioned agents. Accordingly, additional experiments were performed to help elucidate the possible mechanism of the VEGF effect. First, the finding of increased transient VEGF-induced $[Ca^{2+}]_i$ in

ECs preincubated with 10 nmol/L of CsA or MeVal-4-CsA strongly suggested that a mechanism potentiating the effect of VEGF was acting under these conditions. The fact that transient ATP-induced $[Ca^{2+}]_i$ was not affected in the same conditions provides evidence for the specificity of the effect found with VEGF.

In view of the absence of increased VEGF expression, a probable explanation for the increased VEGF effect was the overexpression of VEGF receptors. To clarify this point, we analyzed the role of VEGFR2 in ECs. In this regard, the reversal of the protective effects of CsA and CsA analogs with a specific anti-VEGFR2 mAb strongly favored the importance of this receptor in the observed effects of VEGF. The finding of increased expression of VEGFR2 mRNA and the increased amount of VEGFR2 protein in ECs treated or pretreated with CsA and CsA analogs, offered an explanation for the previous results and provided sound mechanistic support for the preconditioning phenomenon induced by low (10 nmol/L) CsA and MeVal-4-CsA. Moreover, the feature that both CsA and CsA analogs induced a similar increase in VEGFR2 expression and synthesis supports the hypothesis that a CyP-mediated mechanism is involved in this particular mechanism. The fact that VEGFR2 increased with both cytoprotective and cytotoxic doses of CsA and/or CsA analogs on ECs indicates that the inductive actions on VEGFR2 expression are operative within an ample range of concentrations, in spite of the fact that the protective actions due to VEGFR2 are overwhelmed by exposure to high concentrations of these drugs. The apparent differences in intensity of VEGFR2 mRNA expression and VEGF protein could be traced either to the different sensitivity of the techniques or to real divergences in the regulation of the mRNA expression and rate of protein synthesis.

In addition to the enhanced VEGFR2 expression, the possibility that increased transient $[Ca^{2+}]_i$ in CsA- or and MeVal-4-CsA–treated ECs is indicative of facilitation of one or several intracellular signaling pathways cannot be ruled out.

In summary, our results disclose 2 principal aspects of EC response to CsA, namely the predominant role of CyP-mediated mechanisms and VEGF, by a mechanism that involves VEGFR2. In a more general view, our data add further interpretative resources to understand the defensive responses of endothelium and to design strategies to prevent endothelial injury by toxic agents.

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