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Cyclosporin-induced endothelial dysfunction and hypertension: are nitric oxide system abnormality and oxidative stress involved?

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Abstract Hypertension is a major side effect of cyclosporin (CsA). While the mechanism(s) responsible are unclear, CsA-induced endothelial dysfunction and CsA-induced hypertension have been attributed to the CsA effect on the endothelial-derived factors controlling vasomotor tone. Endothelial nitric oxide (NO) is crucial in the maintenance of a state of basal vasodilation, and recent studies have suggested an NO-mediated counterregulatory mechanism protective from CsA-induced vasoconstriction. Our study evaluates endothelial nitric oxide synthase (ecNOS) gene status (PCR analysis) and plasma levels of NO metabolites (ELISA) in kidney and heart transplant patients under chronic CsA treatment with CsA-induced hypertension. Since CsA increases superoxide production, which metabolises NO, plasma hydroperoxides from cholesterol esters and from triglycerides and peroxynitrite were also evaluated (HPLC) as an index of the presence of superoxides and of "oxidative stress". Quantification of monocyte ecNOS mRNA and NO metabolites plasma levels from patients and controls (C) demonstrated NO system upregulation in patients notwithstanding the hypertension. The mean ecNOS to β-actin ratio was 1.80 ± 0.85 in patients vs 0.40 ± 0.09 in C ($P < 0.04$). NO metabolites were 34.03 ± 14.32 μM in patients vs 11.53 ± 5.64 μM in C ($P < 0.001$). Hydroperoxides from cholesterol esters and from triglycerides were also increased in patients, 3.4 ± 1.4 vs 1.3 ± 0.6 integrated area units (i.a.u.), $P < 0.007$ and 10.6 ± 6.4 vs 1.3 ± 0.8 i.a.u., $P < 0.008$, respectively, as well as the peroxynitrite plasma level, 0.32 ± 0.11 μM/l vs undetectable in C. This study confirms a CsA-induced NO system upregulation in transplanted patients. However, the NO-mediated counterregulatory system to CsA-induced vasoconstriction, present in normals, could be canceled in patients by CsA-induced superoxide (O_2^-) and free radical production which, by increasing NO metabolism, could contribute to CsA-induced vasoconstriction and hypertension and predispose to atherosclerosis.

Key words ecNOS · Nitric oxide · Cyclosporine · Hypertension · Superoxide anions

Introduction

Increasing evidence implicates reactive oxygen species (ROS) in the pathogenesis of hypertension and its cardiovascular complications [11]. In fact, links between oxidative stress and hypertension have been recently established with the demonstration that angiotensin II increases production of ROS by vascular smooth muscle

cells [9], and by the fact that most of the actions of ROS on the development of endothelial dysfunction and hypertension have been shown to be mediated by their effects on the balance between vasoconstrictor and vasodilator pathways [9]. In particular, ROS enhance vasoconstrictor prostaglandins [13], thromboxane synthase [19] and receptors [6], inhibit prostacyclin synthase [25] and combine with nitric oxide (NO) to yield peroxynitrite, therefore, increasing NO metabolism and reducing the NO availability and thereby its efficacy as an endothelium-dependent vasorelaxing factor [11]. Furthermore, ROS, by engaging particular cell signalling processes [22], have been shown to play key roles in promoting vascular proliferation, hypertrophy and vascular remodelling [14] which are integral to the development of atherosclerosis.

The introduction of cyclosporine (CsA) is considered a milestone in immunosuppressive therapy, greatly improving morbidity and mortality of transplanted patients. However, the use of CsA for immunosuppression is complicated by serious side-effects such as hypertension, thromboembolic complications and nephrotoxicity. In particular, CsA-induced hypertension has been attributed to CsA's effect on the factors controlling vasomotor tone (for example, endothelin, thromboxane and NO) [20]. NO is crucial in the maintenance of a state of basal vasodilation and inhibition of platelet activation and its impairment by CsA has thus been advanced as a mechanism for the hypertension associated with CsA [18]. However, recent studies do not support any direct effect of CsA on NO synthesis as studies in normal human volunteers have shown that acute CsA administration produced vasoconstriction and a simultaneous increase in endothelial NO release and that CsA produced vasoconstriction in the presence of a simultaneous inhibition of NO synthase [21]. Furthermore, incubation of cultured endothelial cells with CsA showed an increased gene expression of endothelial NOS (ecNOS) [16, 21]. Given these findings it is unlikely that CsA is a direct inhibitor of the NO system and decreased NO activity is unlikely to mediate the vasoconstrictive actions of CsA. On the other hand, CsA has been shown to induce superoxide anion production which increases NO metabolism and could represent an attractive link between NO increased metabolism and reduced activity and CsA-induced vasoconstriction and hypertension.

The present study extends and expands our earlier preliminary studies on kidney transplant (KT) patients [1] by increasing the number of our observations and by including heart transplant (HT) patients, and assesses the effect of chronic CsA not only on monocyte ecNOS mRNA levels and the NO system in general, but also on indicators of oxidative stress mediated by both ROS and NO-derived species as well. The studies were undertaken to test our hypothesis that to CsA-induced vasoconstriction and hypertension could contribute a pathophysiological role of CsA-induced increase in O_2^- production and its subsequent reaction with and destruction of NO.

Patients and methods

Ten patients with KTs, seven males and three females, age range 28–58 years, and ten patients with HTs, six males and four females, age range 30–48 years, with normal kidney function and with the transplant lasting for at least 1 year, chronically treated with CsA and with CsA-induced hypertension were studied and compared with ten sex- and age-matched healthy subjects (C) with no history of hypertension. Diagnosis of CsA-induced hypertension in HT patients was based on the lack of hypertension before the transplant and on the development of hypertension with permanent need for antihypertensive drugs only after CsA administration and without any improvement after the tapering of steroids associated with CsA treatments. In KT patients, diagnosis of CsA-induced hypertension was made both by the development of hypertension only after transplant, for those patients which were normotensive before the transplant, and, for those patients which were hypertensive before the transplant, by the worsening of a preexisting hypertension, also in terms of pharmacological control of blood pressure, notwithstanding the tapering of steroids associated with CsA treatment.

Blood pressure values of KT and HT patients were on average 160/105 mmHg (± 12/9 SD). All the patients were under chronic CsA treatment and CsA doses were adjusted to a trough level of 150–200 ng/ml. The antihypertensive treatment included, for all patients, dihydropiridine calcium channel blockers that in some patients were combined with angiotensin-converting enzyme inhibitors or β blockers. Although always comprising CsA, immunosuppressive protocols varied from CsA and steroids to triple therapy with CsA, steroids and azathioprine. C subjects were chosen from the staff of the Division of Nephrology of the University of Padua and were previously checked for normal values of blood pressure (on average 120/80 mmHg ± 14/9 SD) plasma electrolytes, plasma renin activity, and plasma aldosterone.

None of the subjects was taking antihypertensive or other drugs, except immunosuppressive treatment, for at least 10 days before the study. All the subjects were on a diet containing approximately 150 mmol of sodium and a definite diet with standardised dietary nitrate intake was given to the subjects for the 48 h before the study. They also abstained from food, alcohol and caffeine-containing drinks for at least 12 h before the study. No differences in the usual diet between patient and control subjects were observed using a dietary questionnaire, particularly concerning fruit and vegetable consumption. All subjects were advised to follow their usual lifestyle without intense physical activity during the day before the study. Informed consent was obtained from all the study participants, and the study protocol was approved by our institutional authorities.

Molecular biology assays

Peripheral blood monocytes (PBM) from 35 ml EDTA-anticoagulated blood, drained from the antecubital vein at 9.00 a.m. after at least 12 h of fasting, were isolated by the NycoPrep gradient method essentially as detailed by the manufacturer (NycoMed Pharma, Norway) and judged to be 85% pure and functionally alive by cytofluorometric analysis.

RNA extraction

RNA from PBM was extracted using a commercially available kit (UltraSpec RNA isolation system; Biotex, USA) with 1 ml product per ca. 5×10^6 cells. The RNA isolated had a ratio of OD 280/260 between 1.8 and 2.0.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was peformed essentially as described by the manufacturer (Gene Amp RNA PCR kit, Perkin Elmer, USA). RNA (ca. 1 μg) was reverse transcribed using the random hexamer primers, the MuLV reverse transcriptase and a Perkin Elmer 2400 thermocycler (15 min at 42 °C, 5 min at 99 °C and 5 min at 5 °C).

PCR

PCR amplification of the cDNA was done essentially as described in the kit. The oligomer primers used were those described by Calò et al. [2], 5'3': ACATGTTTGTCTGCGGCG and GCARGGAAAAGCTCTGGGT. The reaction mixture contained 20 μl cDNA, 0.15 mM sense and antisense primer, 2.5 U *Taq* DNA polymerase (Perkin Elmer) and 0.056 mM anti-*Taq* polymerase (Clontech, USA) in a final volume of 100 μl. The conditions of amplification were set to denature at 94 °C for 1 min, to anneal at 58 °C for 1 min and to extend at 72 °C for 1 min, for a total of 35 cycles of amplification as previously reported [1, 2]. β-actin was used as a control and amplified using commercially available primers (Clontech). PCR products were separated both by electrophoresis on 4% agarose gel (NuSieve 3:1 agarose, FMC, USA) and stained with ethidium bromide and by electrophoresis on polyacrylamide gel and stained by silver staining. Molecular weight marker IX (ΦX174 Hae III; Boehringer Mannheim, Germany) was included on the gels [1, 2]. A control PCR with RNA without a reversed transcription was also performed to exclude contamination with genomic DNA.

Nucleotide sequence determination

It was performed at Primm s. r. l., San Raffaele Biomedical Science Park, Milan, Italy, to identify the amplified products using the Prism *Taq* polymerase dye terminator fluorescent sequencing kit (Perkin Elmer) and analysed using an ABI 373 automated sequencer and ABI Prism analysis software.

Quantitative evaluation of ecNOS gene products

ecNOS and β-actin gene expressions in KT, HT and C were quantified using a PCR–based semiquantitative analysis as previously reported [1, 2]. The bands corresponding to ecNOS and β-actin PCR products were visualized, photographed and densitometrically quantified by pixel density using NIH image software. The ratio between ecNOS and β-actin PCR products was used as index of ecNOS gene expression.

Plasma NO metabolites assay

The plasma level of NO metabolites was evaluated by ELISA as NO_2^-/NO_3^- after conversion of NO_3^- to NO_2^- utilising nitrate reductase and the addition of the Griess reagent included in the kit purchased from Caiman Chemicals, USA.

Plasma hydroperoxides and peroxynitrite evaluation

The plasma level of hydroperoxides from cholesterol esters and from triglycerides was determined according to Cawood et al. [4] with minor modifications. Briefly, lipids were extracted by vortexing a 1-ml of sample with 8 ml chloroform/methanol (2:1 v/v) for 2 min. After centrifugation, 5 ml of the chloroform layer were washed with 2 ml water in order to separate the two phases. Three millilitres of the chloroform phase were dried under nitrogen and the dry extract was dissolved in 50 μl exane. Twenty microlitres of the exane phase were analysed by normal-phase HPLC using a HI-bar LiChrosorb Si 60 column (Merck, Germany) with a mobile phase of exane/propan-2-ol (997:7 v/v). Isocratic HPLC analysis was performed on a Perkin Elmer apparatus compraising a Sigma 15 integrator, a series 10 pump and an LC-75 spectrophotometer with the detector set at 234 nm. The flow rate was set at 2 ml/min. The data were expressed as integrated area units (i.a.u.).

The plasma level of peroxynitrite was evaluated by HPLC as 3-nitrotyrosine following the procedure described by Kaur and Halliwell [12] with minor modifications. Briefly, 2 ml serum were analysed for nitrotyrosine after filtration through Centricon 10 filters (Amicon, USA) at 3000 g/min for 1 h at 4 °C. HPLC separation of 3-nitro-L-tyrosine was achieved using a 3-μ C-18 column (150 × 4.6 mm; Supelco, USA), with a guard column C-18 (20 × 3.2 mm; Supelco). The eluant was 0.5 mol/l KH_2PO_4-H_3PO_4 (pH 3.0), with 10% methanol (v/v) at a flow rate of 0.9 ml/min and injecting a volume of 50 μl. 3-Nitrotyrosine was detected by UV detector set at 274 and 220 nm. Using this procedure the detection limit was 0.1 μmol/l. The identification of the 3-Nitrotyrosine peak was confirmed by tests of addition of 3-nitrotyrosine standard (Sigma, USA) and by the ratio of absorbance at 220 and 274 nm in comparison with samples of 3-nitrotyrosine standard. The CV was 6.3 and 3.3% at the concentrations of 2.0 and 10.0 μmol/l respectively ($n = 15$). The mean recovery of 3-nitrotyrosine in the tests of addition was 97% ($n = 5$). In all serum samples from healthy controls ($n = 20$), 3-nitrotyrosine was under the detection limit.

Statistical analysis

Data expressed as mean ± SD were analysed using Student's *t*-test for unpaired data. Differences at a 5% level ($P < 0.05$) or less were considered significant. Data were evaluated on a Power Macintosh 5400/180 computer (Apple Computer, USA) using the Statview II statistical package (BrainPower, USA).

Results

Figure 1 is a representative assay for ecNOS RT-PCR amplified oligonucleotides from a HT patient, a KT patient and a C. It clearly shows PCR products which have the expected size for ecNOS and β-actin gene products from patients and C monocyte RNA. The identity of the amplified product was also confirmed by sequencing the PCR fragment and when compared to the reported human ecNOS sequence [17] it showed 100% identity (nucleotide sequence position: 6714–7015; exon 25).

The level of ecNOS gene expression evaluated by the ratio of ecNOS PCR product to β-actin PCR product was significantly increased in KT and HT patients in

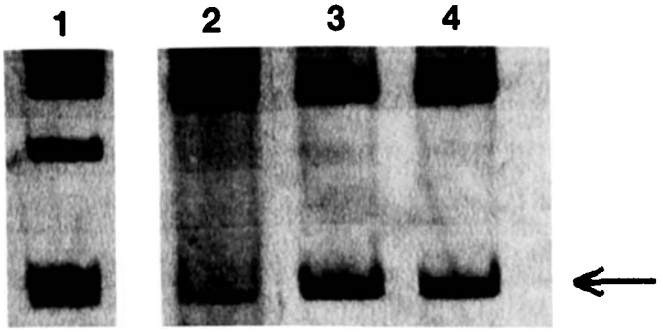

Fig. 1 Endothelial nitric oxide synthase (ecNOS) (*arrow*) and β-actin (*upper band*) PCR products of a representative heart transplant patient (*lane 3*), a representative kidney transplant patient (*lane 4*) and a representative control subject (*lane 2*). Molecular weight marker IX (ΦX174 Hae III; *lane 1*)

Table 1 Monocyte endothelial nitric oxide synthase *(ecNOS)* gene expression, nitric oxide *(NO)* metabolites, hydroperoxides and peroxynitrites plasma levels in transplanted patients and controls. (*KT* kidney transplant, *HT* heart transplant, *i.a.u.* integrated area units, *n.d.* not detectable)

Parameters	Patients: KT and HT	Controls
ecNOS/β-actin ratio (densitometric units)	1.80 ± 0.85*	0.40 ± 0.09
Plasma NO_2^-/NO_3^- (μM/l)	32.03 ± 11.62**	10.37 ± 4.86
Hydroperoxides:		
From colesterol esters (i.a.u.)	3.4 ± 1.4****	1.3 ± 0.6
From triglycerides (i.a.u.)	10.6 ± 6.4***	1.3 ± 0.8
Peroxynitrites (as nitrotyrosine; μM/l)	0.32 ± 0.11	n.d.

* $P < 0.03$
** $P < 0.004$
*** $P < 0.007$
**** $P < 0.008$

comparison to C, therefore confirming our preliminary results [2]. The mean ecNOS to β-actin ratio was 1.80 ± 0.85 in patients vs 0.40 ± 0.09 in C, $P < 0.03$ (Table 1). NO_2^-/NO_3^- plasma level was also increased in patients: 32.03 ± 11.62 vs 10.37 ± 4.86 μM, $P < 0.004$ (Table 1). Lipid hydroperoxides were increased in patients 3.4 ± 1.4 vs 1.3 ± 0.6 i.a.u., $P < 0.007$ and 10.6 ± 6.4 vs 1.3 ± 0.8 i.a.u., $P < 0.008$ from cholesterol esters and triglycerides, respectively (Table 1). Nitrotyrosine, an indicator of peroxynitrite levels, which is normally undetectable in healthy subjects [16] was also elevated in patients 0.32 ± 0.11 μM/l (Table 1).

Discussion

Our data clearly demonstrate that CsA-induced vasoconstriction and hypertension cannot be a consequence of decreased levels of ecNOS as both ecNOS mRNA as well as NOS enzymatic activity, as indicated by increased levels of plasma NO metabolites, NO_2^-/NO_3^-, were increased in our patients. To our knowledge, this is the first study that evaluates NO system status (production of mRNA for ecNOS and NOS activity) "ex vivo" in transplanted patients. These results mirror the results reported in cultured endothelial cells which, upon incubation with CsA, showed an increased expression of ecNOS [16, 21]. The mechanism(s) coupling CsA to ecNOS levels, however, remain incompletely defined.

One possible mechanism which could link CsA to overexpression of ecNOS mRNA levels could be the result of the effect of CsA on the activity of calcineurin, a Ca^{++}-calmodulin-dependent phosphatase which is inhibited by a CsA-cyclophillin complex [15]. CsA effects on calcineurin, a major mediator of Ca^{++}-stimulated gene expression [8], reduce its phosphatase activity which in turn may remove calcineurin's inhibitory effect on ecNOS gene expression, thereby stimulating ecNOS transcription. The demonstration that chronic CsA treatment leads to increased plasma NO metabolites indicates that CsA is not a direct inhibitor of ecNOS, but rather the effects of CsA in transplanted patients must occur downstream from the generation of NO. Therefore, other pathways must be involved to explain the CsA-induced hypertension of our patients in terms of reduced NO activity. Reports in the literature combined with our data suggest that a counterregulatory pathway exists that prevents CsA-induced hypertension in normals [21], but is apparently absent or impaired in transplanted patients. One potential mechanism is that CsA increases the turnover of NO such that the level of NO present is insufficient to maintain vasodilation. The result is vasoconstriction and hypertension.

One attractive potential pathway that could link NO metabolism involves CsA induction of superoxide production (O_2^-) [7]. The O_2^- anions produced then react with NO, thereby destroying it and go on subsequently to produce NO_2^-/NO_3^- via the formation and breakdown of peroxynitrite, an even more highly reactive free radical. This destruction of NO induced by CsA would explain the presence of vasoconstriction despite increased ecNOS mRNA as well as NOS activity as testified by increased NO_2^-/NO_3^- which suggests, if anything, that NO production is increased. This contention is further bolstered by the increased peroxynitrite level, as indicated by increased nitrotyrosine, an indicator of peroxynitrite level [12], present in transplanted patients but undetectable in controls where no trace of this compound is normally detected [12]. Moreover, the presence of oxidative stress and of diminished NO levels

are further suggested by the increased levels of lipid hydroperoxides noted in our study, given also the reports by us and others suggesting an antioxidant role for NO [3, 5, 10, 24]. Finally earlier studies make it unlikely that impaired O_2^- metabolism is responsible for the increased O_2^- levels reported upon CsA treatment as CsA has been shown to raise red blood cell levels of superoxide dismutase [23].

In conclusion, our study results strongly support a central role of NO in the control of vascular tone and specifically in CsA mediated vasoconstriction. To our knowledge this is the first study which evaluates the impact of NOS gene expression, NO system status and the role of "oxidative stress" "ex vivo" in transplanted patients chronically treated with CsA and on CsA-mediated vasoconstriction and hypertension, making therefore more reliable the extrapolation of our results to the "in vivo" clinical settings of long-term CsA treatment. Thus, these results more strongly suggest that efforts to identify interventions such as exogenous antioxidants (for example vitamin E or drugs with antioxidant activity) that reduce the level of O_2^-, or the usage of NO as an antioxidant may provide a means to ameliorate the vasocontriction, hypertension and their complications associated with long-term CsA treatment in transplanted patients, such as endothelial dysfunction and atherosclerosis.

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