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Cyclosporin A induces prepro endothelin-1 gene transcription in human endothelial cells

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Abstract

Cyclosporin A, employed in treatment of organ allograft rejection, is associated with hypertension possibly due to endothelin-1. We studied transcriptional regulation of endothelin-1 by cyclosporin A in human endothelial cells using cell transfection experiments and reporter gene assays. Human umbilical vein endothelial cells were established expressing a fusion gene of the coding sequence of the firefly luciferase gene, placed under the control of the rat endothelin-1 promoter. Luciferase assays demonstrate 2.8-fold stimulation of the reporter gene by cyclosporin A ($P < 0.01$), and Northern blot analysis shows induction of prepro endothelin-1 mRNA. Transcription is tightly repressed in the absence of the immunosuppressant, its regulation occurs Ca^{2+}-dependent. Lack of extra- or intracellular Ca^{2+} prevents cyclosporin A-dependent endothelin-1 gene transcription and mRNA induction. These data demonstrate transcriptional regulation of endothelin-1 over a range of several orders of magnitude in human umbilical vein endothelial cells by cyclosporin A via Ca^{2+}-dependent mechanisms. They support the critical role of endothelin-1 in cyclosporin A-associated hypertension.

Keywords: Gene transfection; Reporter gene; Promoter; Luciferase

1. Introduction

Cyclosporin A has become the main immunosuppressive drug administered alone or in combination with other immunosuppressants in transplantation medicine to manage allograft rejection. Major disadvantages of this treatment concern renal functional impairment, lipid metabolism and blood pressure elevation. Among these side effects hypertension may be severe, so that ultimately, discontinuation of the drug becomes inevitable. Molecular mechanisms, underlying cyclosporin A-induced hypertension have not been clarified in detail. A variety of vasoactive peptides are speculated to contribute to this dominant side effect. Among other endothelins, potent endothelial vasoactive constrictor peptides have gained intense interest in their potential role in this clinical finding. Evidence for cyclosporin A-induced synthesis of endothelin-1 has been demonstrated in vitro and in animals (Kon et al., 1990; Bunchman and Brookshire, 1991; Haug et al., 1995a; Abassi et al., 1996; Shiraishi et al., 1997). Further data in favour for this mechanism were demonstrated with endothelin receptor antagonists that ameliorate hemodynamic response to cyclosporin A (Davis et al., 1994; Brooks and Contino, 1995; Hunley et al., 1995; Abassi et al., 1996; Bartholomeusz et al., 1996; Cavarape et al., 1998). Results from clinical studies, however, are less clear, organ transplant recipients did not demonstrate conclusive evidence in favour for endothelin-1 dependency of cyclosporin A-associated hypertension (Edwards et al., 1991; Stockenhuber et al., 1992; Grieff et al., 1993; Forslund et al., 1995; Haug et al., 1995b; Xiao et al., 1996). In addition, molecular basis of cyclosporin A-mediated endothelin-1 induction remains unclear. For one, the intracellular Ca^{2+} release upon stimulation with vasoconstrictor hormones is potentiated in the presence of cyclosporin A (Lo Russo et al., 1996; Meyer-Lehnert et al., 1997). On the other hand, while it is widely accepted that cyclosporin A stimulates basal cellular Ca^{2+} influx leading to increased intracellular Ca^{2+} pools (Pfeilschifter and Ruegg, 1987; Pfeilschifter, 1988), and inhibits the protein phosphatase calcineurin (Liu et al., 1991), neither of these mechanisms nor calcineurin are

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suspected as the underlying pathomechanism of the immunosuppressant triggering vasoconstrictor hormone release (Lo Russo et al., 1996, 1997a).

The purpose of the present work was to investigate regulatory signaling mechanisms of prepro endothelin-1 gene after cyclosporin A stimulation and to characterize its pathways. Using a tightly regulated transcription system composed of the firefly luciferase gene placed under the control of the rat endothelin-1 promoter, which were inserted into the genome of human endothelial cells where they were transiently expressed, we demonstrate efficient rat endothelin-1 promoter-dependent transcriptional activity in the presence of cyclosporin A. Gene transcription is repressed in the absence of the immunosuppressive drug, and lack of extra- and intracellular Ca^{2+} prevents cyclosporin A-dependent endothelin-1 gene transcription as well as prepro endothelin-1 mRNA induction. This demonstration of specific transcription activity being regulated over a range of several orders of magnitude in higher eukaryotic cells by cyclosporin A is the first direct evidence of transcriptional regulation of endothelin-1 by the immunosuppressive drug. The clinical impact, however, clearly depends on future availability of endothelin receptor antagonists or endothelin converting enzyme inhibitors that may prevent cyclosporin A-dependent hypertension.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells were obtained by primary culture of cells harvested from umbilical cords from caucasian women using the technique previously described (Marsen et al., 1995a). They were grown in fibronectin (1 $\mu g/cm^2$) coated culture flasks in Endothelial cell growth medium-1 (PromoCell, Heidelberg, FRG), supplemented with 2% fetal calf serum, 0.4% endothelial cell growth supplement, 0.1 ng/ml epidermal growth factor, 1 ng/ml basic recombinant human fibroblast growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycine sulfate, 0.05 μg/ml amphotericin B, and 10 μg/ml ciprofloxacine at 37°C, 5% CO_2, in a humidified cell culture incubator (Forma Scientific, Frankfurt/Main, FRG). On average, two umbilical cords yielded the amount of cells sufficient for one flask. Usually, cells were grown for 4–5 days before reaching 70%–80% confluency to be subcultured. Experiments were performed on confluent, contact inhibited cells, that had been kept in serum-free medium for 24 h to induce G_0/G_1 growth-arrested phase.

2.2. Non-radioactive Northern blot analysis

Total cellular mRNA was extracted from endothelial cells according to a modification of the DIG system (Boehringer-Mannheim, FRG). Briefly, endothelial cells were solubilized in lysis buffer consisting of 1.3 M guanidinium thiocyanate, 0.7 M Tris–HCl pH 8.0, 1.3 M LiCl, 65 mM EDTA, 0.25% dithiotreothol, and 0.13% laurylsarcosine. Poly-A mRNA was subsequently incubated with biotin-labeled oligo$(dT)_{20}$ probe and magnetic streptavidin particles at 37°C for 5 min. Streptavidin-mRNA complex was resuspended in buffer (10 mM Tris-buffer, 0.2 M LiCl, 1 mM EDTA, pH 7.5), repetitively washed and heat-dissociated (65°C), before being magnetically separated. RNA was then size-fractionated in a 1.2% agarose gel electrophoresis, before being transferred and immobilized on nylon membrane (Boehringer-Mannheim). Hybridization was performed as RNA–RNA hybrids because of superior strength and specificity. Membranes were hybridized simultaneously with the 1.2 kb fragment of digoxigenin-UTP labeled endothelin-1 RNA and with digoxigenin-UTP labeled β-actin RNA (Boehringer-Mannheim) for verification of equal amounts of RNA. Digoxigenin-UTP labeling had been performed by in vitro transcription of the 1.2 kb human endothelin-1 cDNA insert (Bloch et al., 1989) using SP6 RNA polymerase. RNA–RNA hybrids were visualized by chemiluminescence after binding of anti-DIG alkaline phosphatase conjugates with CDP-Star™ and exposed to Lumi-Film X-ray film (Boehringer-Mannheim). The amount of mRNA expression was quantified by densitometry using the National Institutes of Health Image® software on an Apple Macintosh® computer equipped with a Microtech® scanner, and corrected for β-actin expression.

2.3. Transfection procedures

2.3.1. Plasmids

The parent plasmid of pGL3-pr-ET-1, pΔlux-pr-ET-1, was kindly supplied by Martin Paul, FU Berlin, Germany. As described in detail elsewhere (Paul et al., 1995), it is composed of a 1495-bp fragment (positions −1329 to +166) of the rat endothelin-1 promoter, containing 100% of the promoter region, which is coupled to the coding sequence of the firefly (*Photinus pyralis*) luciferase gene, together with the polyadenylation signal of the SV40 large tumor (T) antigen gene and inserted into a modified pUC18 vector.

For means of luciferase stability, the insert was excised as a *hin*dIII–*kpn*I fragment (positions −1329 to +123) and subcloned into the pGL3 reporter gene vector (Clontech®), to yield a more stable luciferase product with augmented half-life, before this construct was employed for cell transfection experiments.

2.3.2. Cell transfection experiments

Integration of genomic DNA into human umbilical vein endothelial cells was performed using liposomal transfection techniques. Briefly, in 1.5 ml serum-free cell culture medium, 1.5 μg of highly purified plasmid DNA of the rat endothelin-1 reporter gene vector (pGL3-pr-ET-1) was sus-

pended with 0.5 μg pCMVβ-galaktosidase reporter gene vector (Clontech, Heidelberg, FRG) for measurement of transfection efficiency, and 15 μl Lipofectin® (Gibco-BRL, Eggenstein, FRG) transfection medium. The reaction mixture was added to cell monolayers at 80% confluency (220.000 cells average) and incubated in a humidified 5% CO_2 atmosphere at 37°C for 3–5 h. After washing off transfection medium, incubation was continued for 24 h with the addition of 2 ml cell culture medium, containing 2% fetal calf serum with growth factors and antibiotic, before medium was exchanged for serum-free cell culture medium for an additional 24-h period. The reaction was terminated by cell lysis in 500 μl Reporter gene assay® lysis buffer (Boehringer-Mannheim). After centrifugation, cytoplasmic cell suspension was utilized for luciferase assay.

2.4. Luciferase assay

Luciferase assays utilize the ability of the *P. pyralis* luciferase gene to produce an enzyme that ATP-dependently oxidizes luciferin under light emission. Identical volumes of cytoplasmic endothelial cell extracts were analyzed in a processor controlled luminescence analyzer (Lumat LB 9507, EG and G Berthold, Wildbad, FRG), a photomultiplier tube that detects single photon emission at 20 ns intervals. 20 μl cell lysate are automatically injected with 100 μl substrate (200 mM luciferin in 25 mM glycylglycine, pH 7.8) and light emission is measured at 562 nm over 10 s time at 25°C, using the integral mode of measurement. Data are given as relative light units (RLU) which were corrected for background light emission, normalized to galactosidase activity, and compared to control conditions.

2.5. Galactosidase assay

pCMVβ-galactosidase reporter gene (Clontech), simultaneously integrated together with pGL3-pr-ET-1 during cell transfection procedures, produces an ubiquitous cellular enzyme β-galactosidase, that glycosilates lactose to galactose. Enzyme activity served as marker for integration of genomic DNA into human umbilical vein endothelial cells. Twenty microliter cell lysate were incubated with 70 μl substrate (Galacton Plus®) under gentle agitation for 45 min at pH 7.8. Using a processor-controlled luminescence analyzer, galactose was quantified at 475 nm over a 5 s period at 25°C after automatic injection of 100 μl initiation reagent which increases pH > 12 and triggers light emission. Data are given as RLU which were corrected for background light emission.

2.6. Fluorometric determination of cytosolic-free $[Ca^{2+}]_i$ concentrations

Human umbilical vein endothelial cells were grown on 13 × 20 mm glass coverslips to confluency according to the conditions outlined above. Quiescent cells were loaded with the fluorescent dye fura-2 acetoxymethyl ester (1 μM) (Molecular Probes, Eugene, OR) in serum-free medium for 40 min at 37°C and then resuspended in serum-free medium for 20 min to equilibrate cells and wash off excess dye. Prior to use, coverslips containing confluent cell monolayers were kept on ice in Krebs-Henseleit-*N*-2hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (1 × HEPES, KCl 400 mg/l, $MgSO_4$ 0.8 mM, NaCl 130 mM, glucose 5.5 mM, bovine serum albumin 1 g/l) without or with 1.25 mM $CaCl_2$. Ca^{2+}-fura-2 fluorescence of endothelial cells was measured by a modification of the method described earlier (Marsen et al., 1996), using a photocounting device AMKO LT I (Amko, Uetersen, FRG), equipped with AMKO MuLTI-scan II Gem/3 software, that was coupled to a Nikon inverse microscope (Nikon, Alsdorf, FRG). Coverslips were mounted onto a mounting device and placed under the microscope. On top of the coverslip, a bathing chamber was placed which was sealed with a rubber seal, and human umbilical vein endothelial cells were bathed with 2 ml buffer before they were stimulated according to the experimental procedure as specified in the figure legends. Using a 40-fold magnification, an area of 15–20 fura-2-labeled cells was excited at 340 and 380 nm, while emitted fluorescent light was measured at 510 nm. $[Ca^{2+}]_i$ was calculated according to the equation: $[Ca^{2+}]_i = K_d \times \{(R - R_{min})/(R_{max} - R)\} \times \{S_f/S_b\}$, where K_d is the dissociation constant of fura-2 at 37°C and pH 7 (224 nmol/l). R represents the ratio of fluorescence levels excitated at 340 and 380 nm, R_{min} and R_{max} are ratios at nominally zero Ca^{2+} (10 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)) and under saturation conditions (25 mM ionomycin), respectively. S represents the fluorescence signal at 380 nm, S_f and S_b are levels at nominally zero Ca^{2+} and under Ca^{2+} saturation corresponding to the above-mentioned conditions. All fluorescence data shown are typical for five experiment totals.

3. Results

3.1. Cyclosporin A stimulates prepro endothelin-1 gene transcription via promoter activation

Previously, we have demonstrated induction of prepro endothelin-1 mRNA by cyclosporin A (Marsen et al., 1996). Now, we analyzed the effect of cyclosporin A on endothelin-1 promoter activity in human umbilical vein endothelial cells and compared it to Thrombin, a known agonist capable to induce the prepro endothelin-1 gene (Yanagisawa et al., 1988; Kohno et al., 1990; Marsen et al., 1995a,b, 1996) (Fig. 1). Human umbilical vein endothelial cells, transfected with the rat endothelin-1 promoter construct were treated with 5 μM cyclosporin A for

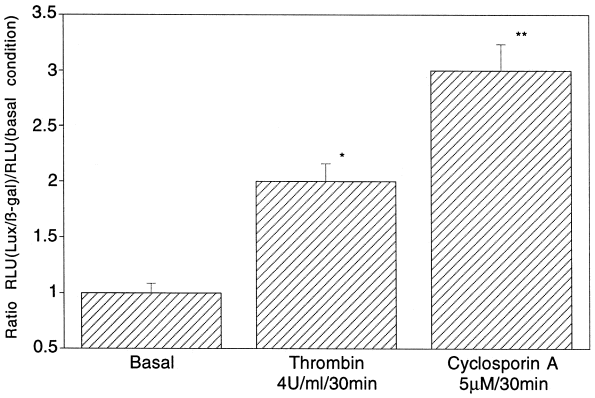

Fig. 1. Luciferase assay in human umbilical vein endothelial cells, transiently transfected with the fusion gene construct of the rat endothelin-1 promoter coupled to a luciferase reporter gene (pGL3-pr-ET-1), that had been exposed to cyclosporin A (5 μM/30 min) and Thrombin (4 U/ml per 30 min) as indicated. After addition of luciferin substrate, emitted light is measured at 562 nm, corrected for background and normalized for galactosidase activity. Luciferase activity showed significantly stronger induction for cyclosporin A than for Thrombin. Data represent five transfections each and are given as mean ± S.E.M. Analysis of unpaired data determined significant differences at a 95% ($^{*}P < 0.05$) and 99% confidence interval ($^{**}P < 0.01$) for stimulated vs. unstimulated conditions.

30 min to induce endothelin-1 expression. This short incubation period results primarily from necessities of transfection assays. Due to its lipophilic behavior and extensive distribution with its majority residing as non-quantifiable amount outside the extracellular compartment concentrations of the drug were chosen as 5 × peak plasma concentrations measured in vivo. These concentrations are well within a range of experimentally employed concentrations to investigate acute effects of the immunosuppressant (Benigni et al., 1992). At the end of the incubation period, cells were lysed and analyzed for induction of the rat endothelin-1 promoter by luciferase assay. For comparison, unstimulated control cells and Thrombin-treated (4 U/ml per 30 min) human umbilical vein endothelial cells were employed to verify transcriptional regulation of the prepro endothelin-1 gene. Cyclosporin A demonstrated induction of luciferase activity at 30 min by 280% compared to unstimulated control conditions. Absolute induction of luciferase activity revealed stronger induction of the endothelin-1 promoter for cyclosporin A than for Thrombin ($P < 0.01$) (Fig. 1). Induction is mediated via receptor-independent mechanisms, pretreatment of human umbilical vein endothelial cells with the selective endothelin receptor antagonist BQ 123 (500 nM/30 min) or with the non-selective compound PD 142843 (1 μM/30 min), revealed independence of agonist-mediated endothelin-1 promoter activity of autocrine regulation as previously postulated (Saito et al., 1995) (Fig. 2). Activation of a presently unknown target protein at receptor level, resulting in stimulation of phospholipase C and potentiation of inositol trisphosphate with subsequent Ca^{2+} release was ruled out with the synthetic phospholipase C inhibitor ET18-OCH_3 (10 μM/20 min), revealing independence of endothelin-1 promoter activity from this intracellular second messenger (Fig. 3).

Taken together, these data are the first direct demonstration — employing gene transfection assays — of transcriptional regulation of the prepro endothelin-1 gene by cyclosporin A.

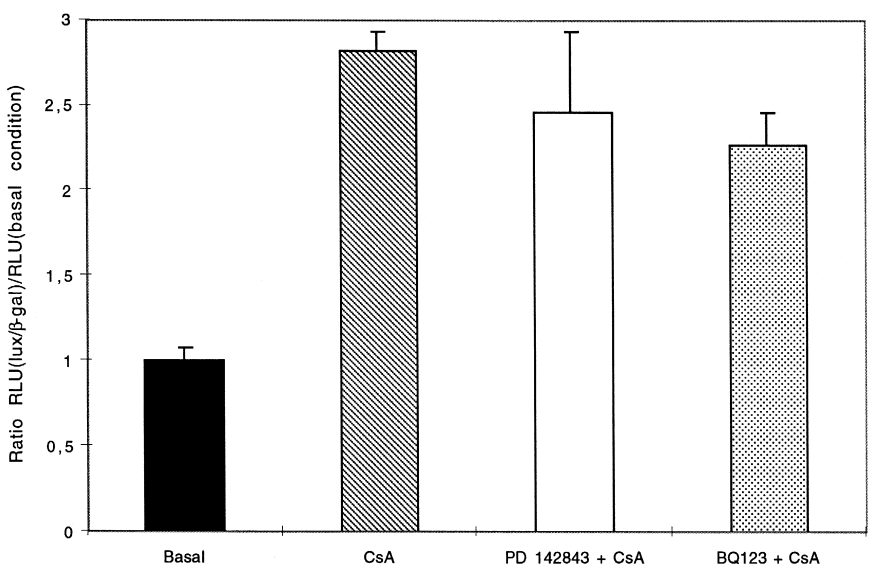

Fig. 2. Luciferase assay in human umbilical vein endothelial cells, transiently transfected with the fusion gene construct of the rat endothelin-1 promoter coupled to a luciferase reporter gene (pGL3-pr-ET-1) that were incubated with the selective endothelin receptor antagonist BQ 123 (500 nM/30 min) or with the non-selective compound PD 142843 (1 μM/30 min) prior to cyclosporin A (5 μM/30 min). After addition of luciferin substrate, emitted light is measured at 562 nm, corrected for background and normalized for galactosidase activity. Luciferase activity showed independence of endothelin-1 promoter activity from autocrine regulation by cyclosporin A. Data represent five transfections each and are given as mean ± S.E.M.

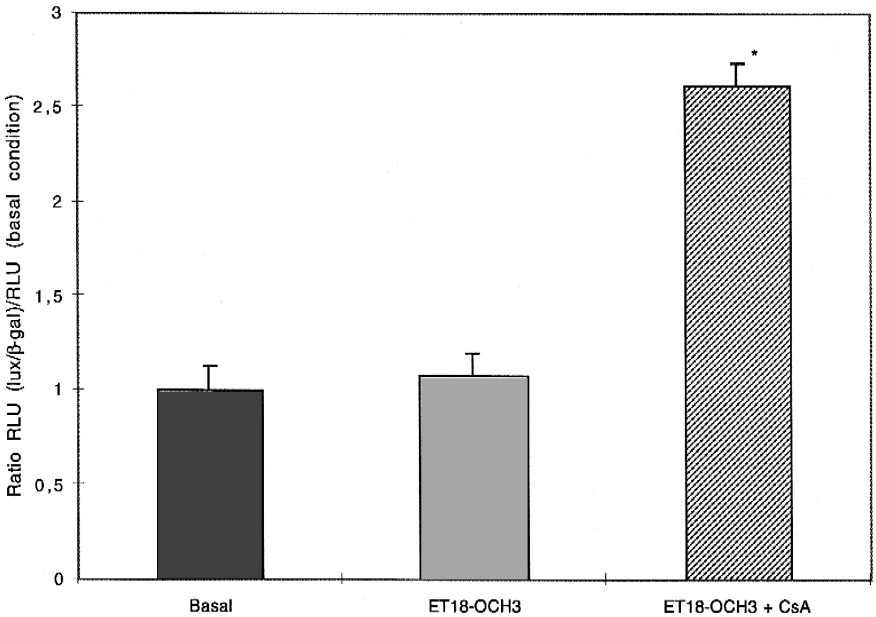

Fig. 3. Luciferase assay in human umbilical vein endothelial cells, transiently transfected with the fusion gene construct of the rat endothelin-1 promoter coupled to a luciferase reporter gene (pGL3-pr-ET-1), that were incubated with the phospholipase C inhibitor ET18-OCH_3 (10 μM/20 min) alone or prior to cyclosporin A (5 μM/30 min). After addition of luciferin substrate, emitted light is measured at 562 nm, corrected for background and normalized for galactosidase activity. Luciferase activity showed independence of endothelin-1 promoter activity from phospholipase C activation. Data represent five transfections each and are given as mean ± S.E.M. Analysis of unpaired data determined significant differences at 95% ($^*P < 0.05$) for stimulated vs. unstimulated conditions.

3.2. Cyclosporin A-stimulated prepro endothelin-1 gene expression is mediated by cytosolic-free $[Ca^{2+}]_i$

Intracellular free Ca^{2+} concentrations $[Ca^{2+}]_i$ have been demonstrated to be significantly increased after cyclosporin A exposure, and $[Ca^{2+}]_i$ is a crucial intracellular signaling mechanism in regulation of prepro endothelin-1 mRNA expression (Lo Russo et al., 1996; Marsen et al., 1996; Meyer-Lehnert et al., 1997). We verified induction of cytosolic-free $[Ca^{2+}]_i$ after cyclosporin A (5 μM) stimulation by fluorometric analysis in human umbilical vein endothelial cells monolayers. $[Ca^{2+}]_i$ increased from 79 nM resting value to peak levels of 143 nM (Fig. 4). In the absence of extracellular Ca^{2+} (Ca^{2+} free buffer), the

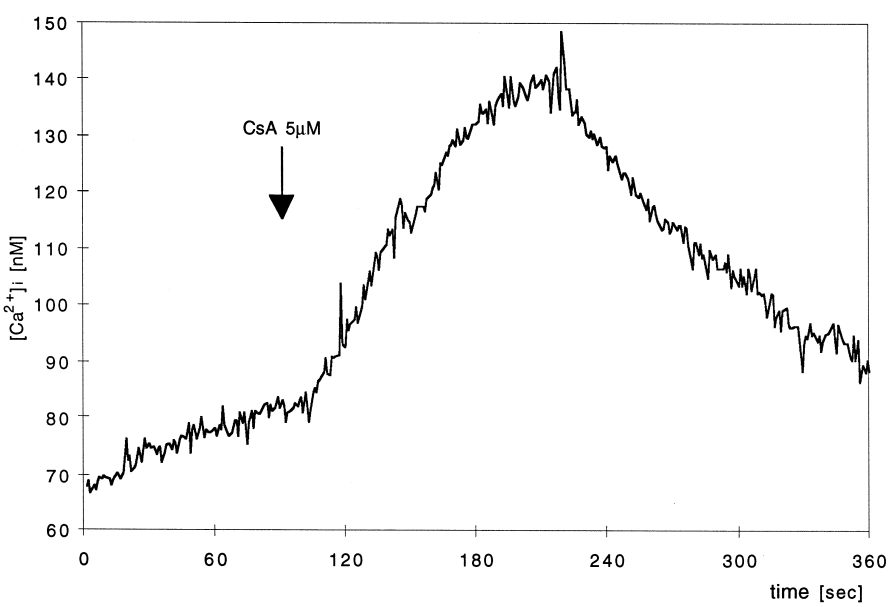

Fig. 4. Fluorometric analysis of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by treatment with 5 μM cyclosporin A (CsA) (arrow). Confluent adherent human umbilical vein endothelial cells, grown on glass coverslips were loaded with fura-2 acetoxymethylester, and $[Ca^{2+}]_i$ changes were determined as described under Section 2. Traces from consecutive coverslips were superimposed for comparison.

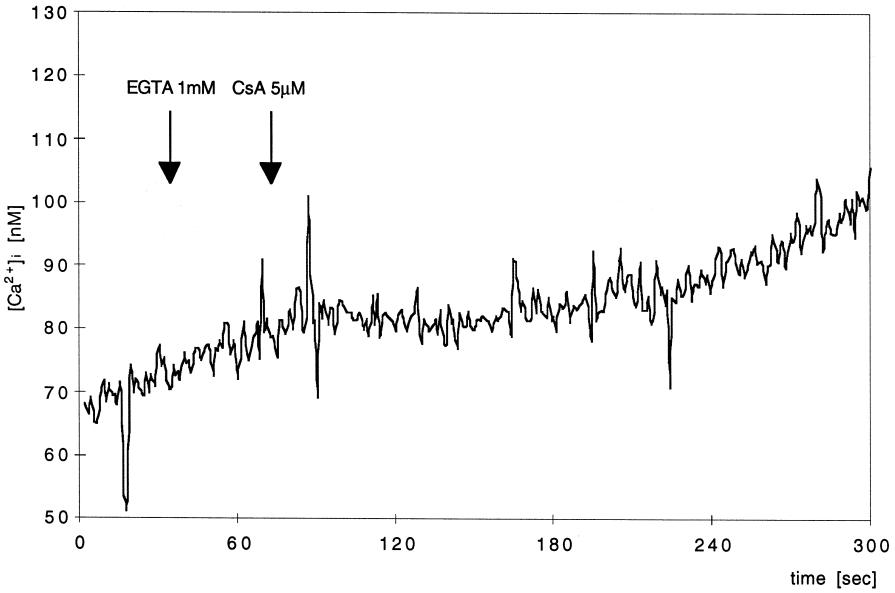

Fig. 5. Fluorometric analysis of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by treatment with 5 μM cyclosporin A (CsA) in the absence of extracellular Ca^{2+} (pretreatment with EGTA 1 mM for 30 s) (arrows). Confluent adherent human umbilical vein endothelial cells, grown on glass coverslips were loaded with fura-2 acetoxymethylester, and $[Ca^{2+}]_i$ changes were determined as described under Section 2. Traces from consecutive coverslips were superimposed for comparison.

release of cytosolic-free $[Ca^{2+}]_i$ after cyclosporin A challenge was attenuated at lower Ca^{2+} concentrations (Fig. 5).

3.3. Chelation of cytosolic-free $[Ca^{2+}]_i$ inhibits cyclosporin A-stimulated prepro endothelin-1 mRNA expression

Human umbilical vein endothelial cells were treated with the Ca^{2+} chelators EGTA and 1,2-bis(*o*-amino-5′methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethylester (MAPTAM) to reduce the extracellular and/or intracellular Ca^{2+}-content before they were challenged with cyclosporin A. Northern blot analysis were employed for quantification of prepro endothelin-1 mRNA expression (Fig. 6). EGTA (1 mM/30 s) treatment significantly reduced cyclosporin A-stimulated prepro endothelin-1 mRNA induction by $>70\%$ to below background levels, however, MAPTAM (100 mM/15 min) treatment demonstrated to be more effective in terms of prepro endothelin-1 mRNA inhibition. When both substances, EGTA and MAPTAM, were added together, to chelate extracellular as well as intracellular Ca^{2+} concentrations, cyclosporin A stimulation was unable to induce prepro endothelin-1 mRNA expression further than in MAPTAM pretreated conditions.

3.4. Cyclosporin A-stimulated prepro endothelin-1 promoter activation is regulated by intracellular Ca^{2+} concentrations

Ca^{2+}-chelating agents in transiently transfected human umbilical vein endothelial cells were also employed to determine if cyclosporin A-stimulated endothelin-1 promoter activity is a Ca^{2+}-dependent process. Human umbilical vein endothelial cells were pretreated with MAPTAM (100 mM/15 min) or EGTA (1 mM/30 s) alone to

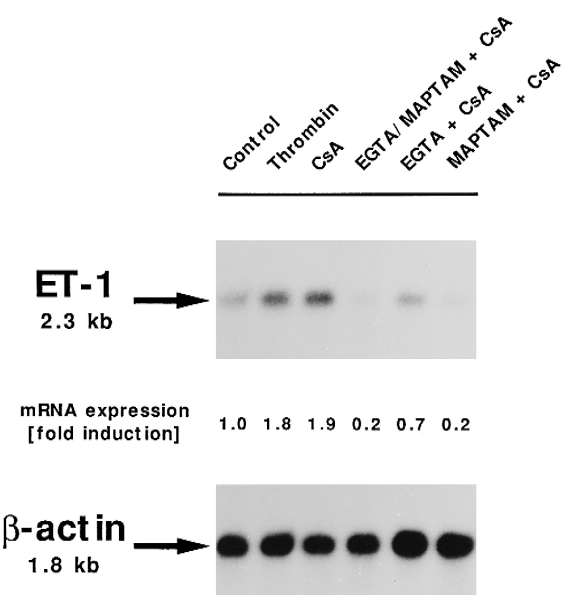

Fig. 6. Northern blot analysis hybridized with endothelin-1 cDNA and normalized to corresponding β-actin signal expression in human umbilical vein endothelial cells exposed to Ca^{2+} chelators EGTA (1 mM/30 s) or MAPTAM (100 mM/15 min) as outlined in the legend to Fig. 7, before stimulation with cyclosporin A (CsA, 5 μM/30 min) was performed. For comparison, unstimulated controls as well as stimulation with Thrombin (4 U/30 min) and with cyclosporin A alone (CsA) are shown. Data of densitometric analysis for endothelin-1 mRNA expression, normalized for β-actin mRNA expression are shown underneath each lane.

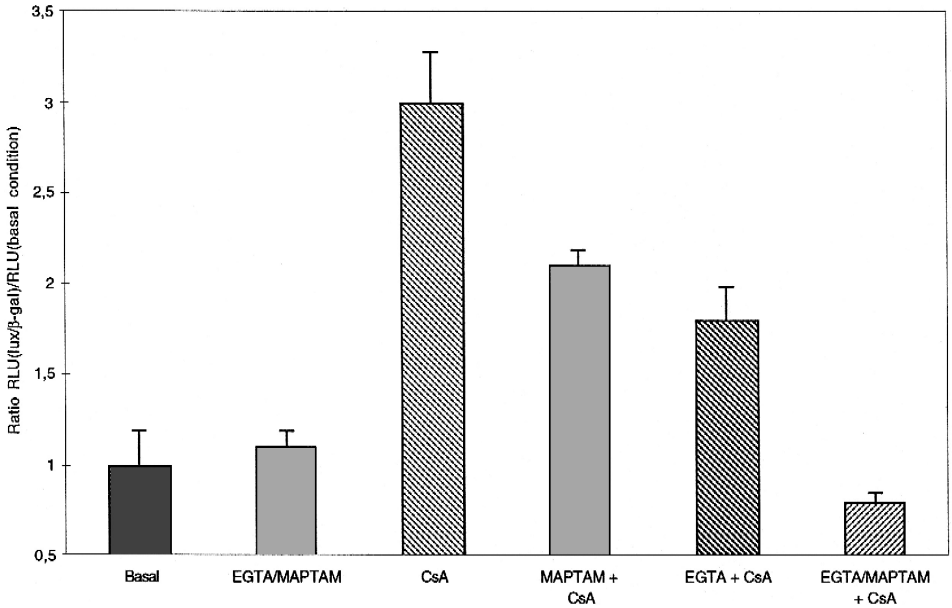

Fig. 7. Luciferase assay in human umbilical vein endothelial cells, transiently transfected with the fusion gene construct of the rat endothelin-1 promoter coupled to a luciferase reporter gene (pGL3-pr-ET-1), that had been exposed to Ca^{2+} chelators prior to cyclosporin A stimulation as indicated. Treatment conditions are as follows: MAPTAM, 100 mM/15 min, once washed and resuspended for 20 min in MAPTAM-free and serum-free medium, prior to EGTA 1 mM/30 s; cyclosporin A (CsA) 5 μM/30 min; MAPTAM 100 mM/15 min, washed once and resuspended in MAPTAM-free medium without serum for 20 min thereafter, before cyclosporin A (CsA) 5 μM/30 min was added; EGTA 1 mM/30 s prior to cyclosporin A (CsA) 5 μM/30 min; MAPTAM 100 mM/15 min, once washed and resuspended for 20 min in MAPTAM-free and serum-free medium, prior to EGTA 1 mM/30 s and subsequent addition of cyclosporin A (CsA) 5 μM/30 min. Human umbilical vein endothelial cells were then washed once and kept in serum-free medium, before luciferase assay was performed. After addition of luciferin substrate, emitted light was measured at 562 nm, corrected for background and normalized for galactosidase activity. Luciferase activity showed attenuated induction for cyclosporin A in the presence of MAPTAM or EGTA alone. When added together Ca^{2+} chelators achieved complete inhibition of cyclosporin A-induced promoter activity. Data represent five transfections each and are given as mean ± S.E.M.

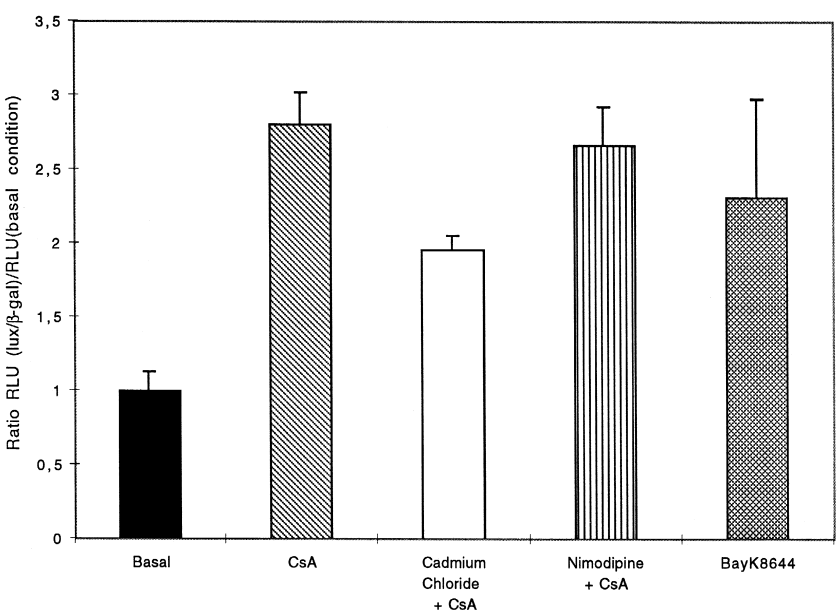

Fig. 8. Luciferase assay in human umbilical vein endothelial cells, transiently transfected with the fusion gene construct of the rat endothelin-1 promoter coupled to a luciferase reporter gene (pGL3-pr-ET-1) that had been exposed to organic and dihydropyridine Ca^{2+} channel blockers cadmium (500 μM/30 min) and nimodipine (10 μM/30 min) prior to cyclosporin A (5 μM/30 min) stimulation, and to the voltage-dependent Ca^{2+} channel activator Bay K 8644 (0.5 μM/30 min) as indicated. Human umbilical vein endothelial cells were then washed once and kept in serum-free medium, before luciferase assay was performed. After addition of luciferin substrate, emitted light was measured at 562 nm, corrected for background and normalized for galactosidase activity. Luciferase activity showed that cyclosporin A-induced promoter activity occurs by Ca^{2+} influx via voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels. Data represent five transfections each and are given as mean ± S.E.M.

chelate intracellular or extracellular Ca^{2+}, before they were challenged with cyclosporin A (Fig. 7). Luciferase activity revealed attenuated response of endothelin-1 promoter activity in EGTA- as well as in MAPTAM-pretreated conditions compared to cyclosporin A-challenged human umbilical vein endothelial cells in the presence of $[Ca^{2+}]_i$. EGTA treatment did not prove to be significantly more effective in reducing cyclosporin A-stimulated endothelin-1 promoter activity than chelation of intracellular Ca^{2+} by MAPTAM. When both substances, EGTA and MAPTAM, were added together to virtually abolish extracellular and intracellular Ca^{2+} concentrations, prior to cyclosporin A challenge, endothelin-1 promoter activity was indistinguishable from basal conditions. Neither EGTA nor MAPTAM, when added together, affected constitutive endothelin-1 promoter activity.

Two different classes of Ca^{2+} channel blockers, nimodepine (10 μM) and cadmium (500 μM) attenuated cyclosporin A-stimulated endothelin-1 promoter activity, while a dihydropyridine Ca^{2+} channel agonist, 1,4-dihydro-2,6-dimethyl-5-nitro-4[2′-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (Bay K 8644, 0.5 μM) stimulated promoter activity similar to cyclosporin A (Fig. 8). We conclude that inward $[Ca^{2+}]_i$ currents, that are crucial in cyclosporin A-stimulated endothelin-1 promoter activity, preferably act through voltage-operated dihydropyridine-sensitive Ca^{2+} channels in human umbilical vein endothelial cells.

4. Discussion

To date, the role of endothelin-1 in cyclosporin A-dependent hypertension remains speculative and bases mainly on data gathered in vitro and in animals (Kon et al., 1990; Bunchman and Brookshire, 1991; Haug et al., 1995a; Abassi et al., 1996; Shiraishi et al., 1997), whereas studies on the exact molecular mechanisms are missing. We have already shown enhanced prepro endothelin-1 mRNA induction upon cyclosporin A stimulation in human umbilical vein endothelial cells (Marsen et al., 1996), and have speculated that constant activation of at least two Ca^{2+}/calmodulin-dependent signaling pathways via calcineurin and via Ca^{2+}/calmodulin-dependent kinases contribute to prepro endothelin-1 gene regulation (Marsen et al., 1995b). To address endothelin-1 gene transcriptional activity upon cyclosporin A stimulation in detail and to overcome possible shortcomings of nuclear run on analysis, we constructed an experimental model in human umbilical vein endothelial cells by expressing the rat endothelin-1 promoter coupled to luciferase as reporter gene. This approach allowed us to utilize an established cell model as well as its native second messengers, but to dissect the signaling pathways and focus onto promoter-specific mechanisms. The present report, for the first time, provides conclusive evidence for cyclosporin A-dependent induction of the prepro endothelin-1 gene by direct stimulation of the endothelin-1 promoter in human endothelial cells. Transcription of the prepro endothelin-1 gene by cyclosporin A depends on the presence of extracellular as well as intracellular Ca^{2+} and on transmembranous Ca^{2+} traffic via voltage-operated dihydropyridine-sensitive Ca^{2+} channels. With these data on hand, we are now able to confirm the importance of prepro endothelin-1 in cyclosporin A-mediated arterial hypertension and to conclude on the molecular mechanism of this pharmacological side-effect.

In human umbilical vein endothelial cells, cyclosporin A may act through binding to cyclophilin and inhibition of the protein phosphatase calcineurin (Liu et al., 1991). Presented evidence on augmented cyclosporin A-dependent transcriptional activity of prepro endothelin-1 emphasizes the critical role of possible transcription factors involved. In pancreatic islet cells, cyclosporin A has previously been demonstrated to inhibit cAMP-responsive element (CRE)-mediated gene transcription in response to transmembranous Ca^{2+}-influx, which could in part be overcome by calcineurin overexpression (Schwaninger et al., 1993). These data stand in contrast to our results, however, at present no data exist on CRE-mediated prepro endothelin-1 gene transcriptional activity, and although the prepro endothelin-1 promoter expresses CRE binding protein (CREB), other transcription factors have been demonstrated for the prepro endothelin-1 gene as well (Inoue et al., 1989). Moreover, it remains questionable, whether cyclosporin A induces the prepro endothelin-1 gene selectively via transcription factor activity, or whether other signaling mechanisms exert a crucial and eventually more critical role. Activation of a target protein at receptor level, resulting in phospholipase C stimulation and potentiation of inositol trisphosphate with subsequent Ca^{2+} release as been suggested as a potential mode of action (Lo Russo et al., 1997b) has to be rejected on the basis of our results.

Presented evidence emphasizes a role for the presence of extracellular and intracellular Ca^{2+} and transmembrane Ca^{2+} currents via voltage-operated dihydropyridinesensitive Ca^{2+} channels involved in cyclosporin A-dependent transcription of prepro endothelin-1 in human umbilical vein endothelial cells. Upon stimulation with cyclosporin A, endothelin-1 is suspected to serve as a mediator to induce cytosolic-free Ca^{2+} $[Ca^{2+}]_i$ (Lo Russo et al., 1997a; Meyer-Lehnert et al., 1997). However, as this effect is not related to cyclophilin or calcineurin and can also be induced by direct stimulation of G proteins, it is speculated that cyclosporin A increases the expression of receptors, resulting in a potentiation of $[Ca^{2+}]_i$ upon stimulation with the constitutively expressed vasoactive peptide (Lo Russo et al., 1997a,b). Reverse transcription polymerase chain reaction (RT-PCR) analysis supports this hypothesis, demonstrating endothelin ET_A receptor mRNA upregulation in vascular smooth muscle cells of cyclosporin A-treated rats (Iwai et al., 1995). On the other

hand, glomerular mRNA expression for either endothelin ET_A and endothelin ET_B receptor was not affected by cyclosporin A in rats, while in the medulla endothelin ET_B receptor mRNA increased (Iwasaki et al., 1994). Furthermore, in humans, evidence for this speculative mechanism must be rejected. Demonstration of endothelin ET_A receptor down-regulation in renal transplant recipients favours receptor escape phenomenons rather than increased expression (Karet and Davenport, 1996).

The contribution of extracellular and intracellular Ca^{2+} in our hands must be assumed as direct sensitization of promoter activity via Ca^{2+} signaling. The exact position of Ca^{2+}-dependent kinases in the intracellular signaling cascade, however, remain to be elucidated. Our data do not address these kinases upon stimulation with cyclosporin A and do not preclude on their distinct molecular actions in endothelial cells. Still, the importance of Ca^{2+} signaling in prepro endothelin-1 gene induction is undoubted. Endothelin-1 mRNA is induced via Protein Kinase C activation and cytosolic-free $[Ca^{2+}]_i$ mobilization as well as Ca^{2+}-dependent kinases after phosphoinositide breakdown (Emori et al., 1989; Inoue et al., 1989; Yanagisawa et al., 1989; Marsen et al., 1996). The increase of cytosolic-free $[Ca^{2+}]_i$ upon cyclosporin A exposure in our hands is crucial to stimulate the promoter, however, cyclosporin A is not as strong a Ca^{2+}-mobilizing agent as other peptides are. Still, compared to Thrombin, which is more potent to liberate Ca^{2+}, cyclosporin A comparably induces the prepro endothelin-1 mRNA, while it has even stronger effects on the endothelin-1 promoter stimulation. Cyclosporin A may therefore, via altered prepro endothelin-1 gene expression, essentially contribute to cyclosporin A-dependent hypertension. The clinical impact of our findings, however, clearly depends on the availability of endothelin receptor antagonists or endothelin converting enzyme inhibitors. They may prevent the dominant side effect, hypertension, of the immunosuppressor drug in everyday clinical use.

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