Crosstalk Between Mesangial and Endothelial Cells: Angiotensin II Down-Regulates Endothelin-Converting Enzyme 1

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Key Words

Cell communication • Endothelins • Endothelial function • Renal function

Abstract

Objective: Since mesangial and endothelial cells interact in the kidney, the present experiments were designed to analyze the ability of human mesangial cells (HMC) to modulate endothelin-1 (ET-1) synthesis by human umbilical vein endothelial cells (HuVEC). **Methods and Results:** The supernatants of HuVEC/HMC contained significantly lower amounts of ET-1 than those of HuVEC alone. This effect was not due to a decreased prepro-ET-1 mRNA expression and was only partially the consequence of HMC-dependent ET-1 degradation. Therefore, we tested the influence of the coculture on endothelin-converting enzyme-1 (ECE-1), and found a significant reduction of its mRNA and protein levels as well as a decreased activity in HuVEC/HMC as compared to HuVEC alone. Using a pharmacological blockade approach (sulotrobam, BN52021, losartan or catalase), losartan was shown to completely abolish down-regulation of ECE-1 observed in HuVEC/HMC. Angiotensin II (AngII) induced a dose and time-dependent inhibition of

ECE-1 expression in HuVEC. **Conclusions:** These results support the importance of cross-talk among different cell types in the regulation of vascular or renal function. ET-1, and particularly ECE-1, might constitute a target in this regulation. In addition, locally synthesized AngII could be one of the mediators involved in the down-regulation of ECE-1.

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Introduction

Compelling experimental and clinical evidence indicates that the endothelium plays a pivotal role in the autocrine-paracrine control of vascular structure and function in several organs. This regulatory function seems to depend on the release of different active mediators, such as prostacyclin (PGI₂), nitric oxide (NO) or endothelins. In addition, endothelial cells develop phenotypic changes in response to various stimuli that determine their interaction with surrounding cells. As a consequence, blood coagulation, platelet aggregation, vascular tone or leukocyte turn-over are regulated by endothelial cells in physiological or pathological conditions [1-3]. The importance of endothelial cells in the

maintenance of normal vascular activity cannot be understood without considering the complex nature of vessel walls, with at least three cell types: endothelial cells, smooth muscle cells and fibroblasts, embedded in a precisely defined extracellular matrix [4].

Endothelin-1 (ET-1), one of the main vasoactive factors synthesized by endothelial cells [5], plays a significant role in the regulation of vascular function and structure, as it is a potent vasoconstrictor and regulator of mitogenesis [6]. ET-1 is synthesized from a large precursor, preproET-1, by the successive action of different enzymes [7, 8]. The conversion of the inactive precursor "big endothelin" to the biologically active peptide is catalyzed *in vitro* and *in vivo* by endothelin-converting enzymes (ECEs) [6]. ECE was first purified from rat lung and subsequently found to be expressed in endothelial cells of many organs [9, 10], as well as in adrenal cells, pancreatic cells, testis and brain. Three ECEs, termed ECE-1, ECE-2 and ECE-3, which are encoded by separate genes, have been cloned and functionally characterized. Both ECE-1 and ECE-2 cleave big ET-1 more efficiently than either big ET-2 or big ET-3, whereas ECE-3, which has been purified from bovine iris [11], selectively cleaves big ET-3. ECE-1 has been shown to be mainly located on the plasma membrane, whereas the acidic pH optimum of ECE-2 suggests an intracellular localization. In contrast to ECE-2, which is expressed mainly in neural tissues, ECE-1 shows a broader tissue expression pattern [12-15].

Changes in ET-1 synthesis have been evaluated *in vitro* and *in vivo* in order to analyze the mechanisms responsible for ET-1 release as well as to explore the pathogenesis of some diseases, such as renal injury, atherosclerosis or hypertension [16-18]. However, the ability of the endothelium to synthesize ET-1 in the presence of other cell types has not been previously studied. The endothelium is surrounded by other cells and therefore, the analysis of ET-1 in this context may better reflect the peptide synthesis in basal conditions. In addition, the possible cross-talk between cells can be better evaluated. The present experiments, therefore, were aimed at analysing ET-1 synthesis in a coculture of endothelial and mesangial cells. Mesangial cells lie within the capillary network of renal glomeruli and are in close contact with glomerular endothelial cells [19]. They play an important role in the regulation of glomerular filtration rate as well as in the maintenance of normal glomerular structure [20]. In addition, they share several properties with vascular smooth muscle cells [21]. Our hypothesis was that the synthesis of ET-1 by endothelial cells might

be modulated by mesangial cells. An analysis of this interaction may contribute to explain the nature of the cross talk between different cell types at the glomerular level and, thus, at other vessel walls.

Materials and Methods

Materials

Angiotensin II, leupeptin, catalase, captopril, human big endothelin-1 and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO, USA). Sulotrobam was kindly provided by Boehringer Mannheim (Mannheim, Germany), losartan by Merck, Sharp & Dohme (Schuchardt, Germany) and BN 52021 by Institute Henri Beaufour (Le Plessis-Robinson, France). Acrylamide, bisacrylamide and Coomassie Blue R-250 were from Merck (Darmstadt, Germany). RPMI 1640, M199, fetal calf serum, trypsin-EDTA (0.02 %) and penicillin-streptomycin were purchased from BioWhittaker (Walkersville, Maryland). Culture plates were from Nunc (Kamstrup, Denmark). X-OMAT films were from Kodak (Rochester, NY, USA). The Supersignal detection system and NucleoBond PC 500 EF kits were from Pierce (Rockford, USA). Plates and electrophoresis equipment were from Bio-Rad (Richmond, CA, USA). The endothelin-1 ELISA system, alpha-[³²P]-dCTP and low and high molecular weight standards for electrophoresis were purchased from Amersham-Pharmacia (Buckinghamshire, UK). The monoclonal antibody against bovine ECE-1 (mAb AEC32-236) was a generous gift from Dr. Kohei Shimada (Biological Research Laboratories, Sankyo Co., Ltd. Tokyo, Japan). The monoclonal anti-THY 1.1 antibody was from Chemicon (Terencula, CA, USA).

Cell culture, glomerular isolation, and experimental design

Human umbilical vein endothelial cells (HuVEC) and human mesangial cells (HMC) were isolated according to previously published methods [22, 23]. HA-VSMC were obtained from American Type Culture Collection (VA, USA). HuVEC were grown in gelatin-coated culture plates at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂, and individual clones were subcloned and characterized by their typical cobblestone appearance, by the presence of factor VIII-related antigen and by the uniform uptake of fluorescent acetylated low-density lipoprotein, as previously described. HuVECs were fed every two days with M199 medium supplemented with 20 % fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. HMC were grown at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂, and individual clones were subcloned and characterized by reaction with a monoclonal anti-THY 1.1 antibody. HMC were fed every two days with RPMI 1640 medium supplemented with 10 % fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. HA-VSMC were fed every two days with DMEM supplemented with 10 % fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Both cell types were passaged every 5-7 days with trypsin-EDTA.

Experiments were routinely performed on confluent monolayers at passages 2-4. The studies with cocultured cells were designed as follows: HuVEC were grown in gelatin-coated 6-well plates, and HMC or HA-VSMC were grown on gelatin-coated 24 mm coverslips, until confluence. The coverslips were placed upside down over an adequate insert placed in HuVEC six-well plates, so that HuVEC and HMC/HA-VSMC were near each other (< 2 mm) but not in contact, sharing the same incubation media. Cocultured cells were maintained in M199 medium in the absence of serum, and proteins or RNA were extracted separately. To study the effect of angiotensin II (AII) added to endothelial cells, HuVEC were grown in 100 mm dishes until confluence, and were subsequently maintained in serum-free M199 medium, then proteins or RNA were extracted from these cells. Toxicity was evaluated in all experimental conditions by the trypan blue dye exclusion method and by measurement of LDH activity in the incubation media.

Renal glomeruli were isolated from normal tissue of kidneys removed due to renal cell carcinoma. Glomeruli were isolated by successive mechanical sieving (100 and 180 μ m), as previously described [24]. The final preparation was incubated in PBS at 37°C, in the presence of 1 μ M AII for 6 and 24 h, and besides in the presence or absence of 1 μ M Losartan for 24 h.

Measurement of endothelin-1 in the incubation media

Confluent monolayers of HuVEC were cocultured with HMC for 24 h at 37°C. Subsequently, the supernatants were collected, lyophilized and stored at -70°C until the assay. Additionally, a fixed amount of exogenous human ET-1 (0.32 nM) was added to cultured HMC in order to study the levels of this peptide after different incubation periods. ET-1 was measured using a commercial ELISA with a 96-well microtitre plate reader. In order to generate a standard curve for ET-1, serial dilutions of ET-1 ranging from 1 – 16 fmol/well (2.49 – 39.87 pg/well) were used. A cubic-spline curve was fit to the standards and unknown values interpolated from the standard curves automatically. The cross-reactivity of the ELISA ET-1 antibody, as determined by the concentration giving 50 % B/B_{max}, was: ET-1 (100), ET-2 (>100), ET-3 (<0.001), big-ET-1 human (0.07), atrial natriuretic peptide (<0.0006).

Measurement of ECE activity

Confluent monolayers of HuVEC were cocultured with HMC for 24 h at 37°C. Membrane proteins were extracted as described for the western analysis and 30 μ g of this homogenate was incubated with Big ET-1 (100 ng) for 4 h at 37°C in 250 μ l of a reaction mixture containing 50 mmol/L Tris-HCl buffer, pH 7 [9]. The reaction was stopped by adding 600 μ l of cold ethanol (-20°C). After centrifugation at 10,000 g for 10 min, the resulting supernatant was lyophilized. The dried residues were reconstituted with assay buffer, then, ET-1 production in each sample was measured by ELISA.

Measurement of angiotensin II in the incubation media

Confluent monolayers of HuVEC were cocultured with HMC or HA-VSMC for different time periods at 37°C. Subsequently, the supernatants were collected and stored at

-70°C until the assay. AII concentration was measured by a commercial enzyme immunoassay kit (Phoenix Peptide, CA, USA), using a 96-well microtitre plate reader. To generate a standard curve for AII, serial dilutions of AII ranging from 0.04 – 25 ng/ml were used. A semi-log curve was fit to the standards and unknown values interpolated from the standard curves automatically. The cross-reactivity of the EIA was: AII (100 %), AIII (100 %), preangiotensin I (1 %), AI (1.7 %), ACTH (0 %), ET-1 (0 %), substance P (0 %), vasopressin (0 %). The intra-assay error was under 5 % and the inter-assay error was less than 14 %.

RNA isolation and Northern analysis

In order to evaluate changes in the expression of ECE-1 or prepro-ET-1 mRNA, total cellular RNA was isolated by the guanidinium lysis method, as described [25]. For Northern analysis, 10 μ g/lane of total RNA were subjected to electrophoresis in 1 % agarose gels containing 0.66 M formaldehyde and subsequently transferred to nylon filters. The filters were blocked by incubation in a hybridization solution (50% formamide, 5x Denhardt's solution, 5x SSPE, 0.5 % SDS and 100 μ g herring sperm DNA) at 42°C for 4 h and probed overnight at 42°C with labeled human probes. The hybridized filters were then washed in 0.2x SSC, 0.5 % SDS at 50°C for 30 min and subsequently exposed to X-OMAT film for 24-48 h. The human prepro-ET-1 cDNA was a 1.2 Kb fragment digested by EcoRI [26]. The human ECE-1a cDNA (798 bp fragment digested by EcoRI and XhoI) was a generous gift from Dr. Kayoko Moroi (Chiba University, Japan). The filters were stripped by boiling in 0.1 % SDS solution and reprobed with a ³²P-labeled 18S cDNA (5.8 kb fragment digested by EcoRI). The densitometric analysis of the films was performed with an Apple scanner and appropriate software. Levels of ECE-1 or prepro-ET-1 mRNA were normalized by using 18S expression within the same lane.

Western blot analysis of immunoreactive ECE-1

HuVEC and HMC were grown in coculture and were then homogenized separately in 1 ml of homogenization buffer (20 mmol/L Tris-HCl pH 7.5, 5 mmol/L MgCl₂, 0.1 mmol/L PMSF, 20 μ mol/L Leupeptine, 20 μ mol/L Aprotinin) using a Polytron homogenizer, as described by Takahashi et al [27]. The homogenates were centrifuged at 100,000 g for 45 min. The resultant membranes were washed three times by resuspension in homogenization buffer and re-centrifugation after homogenization using a Teflon/glass homogenizer. The protein concentration in each supernatant was determined with a BCA Protein Assay kit [28] from Pierce. Proteins were separated on SDS-PAGE 6 % (30 μ g protein/lane), transferred onto nitrocellulose membranes using the Universal buffer (25 mM Tris pH 8.3, 192 mM Glycine, 20 % Methanol, 0.05 % SDS). The nitrocellulose membranes were blocked with 5 % (w/v) nonfat dry milk in PBS overnight and incubated with 10 μ g/ml of the monoclonal antibody against bovine ECE-1 (mAb AEC32-236). After washing in TTBS (20 mmol/L Tris-HCl, 0.9 % NaCl, 0.05 % Tween 20), the blots were incubated with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. Both antibodies were diluted in TTBS and incubated for 1 h at room

temperature. Blots were reblotted with a monoclonal anti- β -Tubulin antibody (Sigma) in order to normalize ECE-1 levels. The immunoreactive bands were visualized with the SuperSignal detection system after 30 sec of exposure to X-OMAT film. The following standards, glutamic dehydrogenase 53,000, albumin 67,000, transferrin 76,000, phosphorylase b 94,000, β -galactosidase 116,000, macroglobulin 170,000 and myosin 212,000, were electrophoresed to adequately locate the proteins.

Statistical analysis

In each case, the data shown represent the mean $\pm$ SEM of a variable number of experiments (see Figure Legends) and are usually expressed as a percentage of the control values. Since the number of data in each distribution was never over ten, non-parametric statistics, particularly the Wilcoxon (two groups) or Friedman (more than two groups) tests, were selected to compare the paired results from the different experimental groups. For the sake of clarity, the control values are represented as 100 %. A $p < 0.05$ was considered statistically significant.

Results

The concentration of immunoreactive ET-1 in the supernatants of HuVEC alone or HuVEC cocultured with HMC is shown in Figure 1, panel A. In the case of HuVEC alone, the mean ET-1 concentration was 99.11 ± 5 pg/ml, whereas no significant amount of ET-1 was detected in the incubation media of HMC (2.85 ± 2 pg/ml). In HuVEC and HMC cocultures, the presence of HMC induced a significant reduction in ET-1 concentration in the incubation media (42.53 ± 10 pg/ml).

In order to analyze the mechanisms implicated in this decreased ET-1 release, three types of experiments were performed. First, serum-deprived HMC were incubated with a fixed amount of exogenous ET-1 (0.32 nM). After a 24 h incubation, a significant reduction of immunoreactive ET-1, 22 ± 4 % ($n=6$), was detected in the wells containing these cells. In a second experiment, prepro-ET-1 mRNA expression was analyzed by Northern blot in HuVEC, in the presence or absence of HMC. As shown in Figure 1 (panel B), an increase in the steady-state levels of prepro-ET-1 mRNA was observed in the cocultured cells. A third experiment was aimed at testing the importance of ECE in the genesis of the observed effect by measuring ECE activity (Figure 2, panel A), ECE-1 content (Figure 2, panel B) and ECE-1 mRNA expression (Figure 2, panel C) in HuVEC alone or cocultured with HMC. In each case, a significant reduction in endothelial ECE was observed in the coculture

Fig. 1. Endothelin-1 (ET-1) synthesis in cocultures of human mesangial (HMC) and human umbilical vein endothelial (HuVEC) cells. A) ET-1 content in the supernatants of the culture media. The results shown are the mean of 10 independent experiments. B) Prepro-ET-1 (ppET-1) mRNA expression. A representative Northern-blot experiment, including the 18S mRNA expression (top), as well as the densitometric analysis of 4 independent experiments (bottom) are shown. Hu: HuVEC cultured alone. Hu+: HuVEC cocultured with HMC. Results (mean $\pm$ SEM) are expressed as % of Hu values. * $p < 0.05$ vs Hu.

conditions. A similar effect was detected when HuVEC were cocultured with HA-VSCM (Figure 3)

In order to test the relevance of soluble mediators in the genesis of the ECE down-regulation, different pharmacologic antagonists were added to the HuVEC-HMC coculture, and ECE-1 protein content in HuVEC was measured. The antagonists used included catalase, a hydrogen peroxide scavenger, and losartan, BN 52021 and sulotrobam, receptor blockers for the AII type 1 receptor, platelet-activating factor, and thromboxane A_2 , respectively. These antagonists did not modify the basal ECE-1 content in HuVEC (data not shown). However,

Fig. 2. Endothelin-converting enzyme (ECE) in cocultures of human mesangial (HMC) and human umbilical vein endothelial (HuVEC) cells. A) ECE activity in endothelial cells. Results are the mean of 6 independent experiments. B) ECE-1 protein content in HuVEC. A representative Western-blot experiment including the tubulin levels (top), as well as the densitometric analysis of 4 independent experiments (bottom) are shown. C) ECE-1 mRNA expression in HuVEC. A representative Northern-blot experiment including the 18S mRNA expression (top) as well as the densitometric analysis of 4 independent experiments (bottom) are shown. Hu: HuVEC cultured alone. Hu+: HuVEC cocultured with HMC. Results (mean $\pm$ SEM) are expressed as % of Hu values. * p < 0.05 vs Hu.

Fig. 3. Endothelin-converting enzyme-1 (ECE-1) protein content in human umbilical vein endothelial cells (HuVEC) after the culture of these cells with human vascular smooth muscle cells (HA-VSMC). A representative Western-blot experiment with the densitometric analysis of 4 independent experiments are shown. Hu: HuVEC cultured alone. Hu+: HuVEC cocultured with HA-VSMC. Results (mean $\pm$ SEM) are expressed as a % of Hu values. * p < 0.05 vs Hu.

losartan completely abolished the HMC-dependent inhibition of ECE-1 content in HuVEC, suggesting that AII could be involved in the genesis of this effect (Figure 4, panel A). In addition, this effect was blocked not only by losartan, but also by captopril, an angiotensin-converting enzyme inhibitor (Figure 4, panel B).

In order to confirm the relevance of AII in the regulation of ECE-1, two types of experiments were performed. First, we demonstrated “*de novo*” synthesis of AII in both types of cocultures (Figure 5). Second, HuVEC were incubated with different AII concentrations for different time-periods and ECE-1 content was measured. The results are shown in Figure 6. A dose- and time-dependent inhibition of ECE-1 content was confirmed in the presence of AII, an effect which was blocked when HuVEC were preincubated with losartan (Figure 7, panel A). Similar results were observed for ECE-1 mRNA, evaluated by Northern blot (Figure 7, panel B): AII induced a significant decrease of the steady-state mRNA levels, which was blocked by losartan. The

possible physiological relevance of this AII-dependent ECE-1 inhibition was tested in freshly isolated human glomeruli. AII (1 μ M) inhibited ECE-1 protein content in

Fig. 4. Endothelin-converting enzyme-1 (ECE-1) protein content in a coculture of human mesangial (HMC) and human umbilical vein endothelial (HuVEC) cells in the presence of different pharmacological antagonists and after angiotensin II blockade. Panel A) Catalase (Cat, 80 U/ml), losartan (Los, 1 μ M), BN 52021 (BN, 50 μ M) or Sulotrobam (Sul, 5 μ M) were added to the cells for 24 h, and the ECE-1 protein content in HuVEC was evaluated. * p < 0.05 vs Hu and Los. Panel B) Losartan (Los, 1 μ M) or Captopril (Cap, 100 μ M) were added to the cells for 24 h, and the ECE-1 protein content in HuVEC was evaluated. * p < 0.05 vs other groups. A representative Western-blot experiment including the tubulin levels (top), as well as the densitometric analysis of 4 independent experiments (bottom) are shown in both panels. Hu: HuVEC cultured alone. Hu+: HuVEC cocultured with HMC. Results (mean $\pm$ SEM) are expressed as a % of Hu values.

Fig. 5 Angiotensin II (AII) production in cocultures of human umbilical vein endothelial cells (HuVEC) with human mesangial (HMC), and with human vascular smooth muscle (HA-VSMC) cells. Results shown are the mean $\pm$ SEM of 5 experiments. A significant concentration of the peptide was detected after 30 min and for at least 6 hours.

a time dependent fashion (Figure 8, panel A), and this effect was blocked in the presence of Losartan (Figure 8, panel B).

Discussion

The present results clearly demonstrate that the ET-1 content in the culture media from cocultured HuVEC and HMC was significantly lower than in the supernatant from HuVEC alone, under the same experimental conditions. This decreased content could be explained by two basic mechanisms, a decreased synthesis of ET-1 by HuVEC or an increased degradation of the peptide by both cell types when cocultured. As shown in the Results section, HMC degrades a certain amount of exogenously added ET-1 (around 22 %), suggesting that an increased degradation could play a significant role in the genesis of the observed results. However, this catabolic ability cannot completely explain the quantitative reduction in ECE-1 protein levels observed in the cocultured cells (over 50-60 %). Thus, the possibility that ET-1 synthesis by these cells could be decreased was explored. Interestingly, HuVEC incubated with HMC showed an augmented expression of prepro-ET-1 mRNA, however, ECE activity was reduced. These changes in ECE activity were the consequence of a decrease in ECE-1 protein content, probably as a result of a decreased expression of its mRNA.

Fig. 6. Effect of angiotensin II (AII) on endothelin-converting enzyme-1 (ECE-1) steady-state protein content in human umbilical vein endothelial cells (HuVEC). HuVEC were incubated with different doses of AII for 24 h (A) or with 1 μ M AII for different time periods (B), and steady-state ECE-1 protein levels were evaluated by Western-blot. A representative Western-blot experiment including the tubulin levels is shown at the top of each panel. The results of the densitometric analysis are expressed as a % of the control values (C), and are the mean $\pm$ SEM of 5 independent experiments. * p <0.05 vs C.

These results stress three important facts. First, the availability of ET-1 in the extracellular compartment decreases when HuVEC are cocultured with HMC. This suggests that tissular levels of bioactive ET-1 may be regulated not only by cell synthesis but also by the complex

Fig. 7. Endothelin-converting enzyme-1 (ECE-1) protein and mRNA expression in human umbilical vein endothelial cells (HuVEC), in the presence or absence of the AT1 receptor antagonist, Losartan. Cells were incubated for 24 h with losartan (Los, 1 μ M), 1 μ M AII or Los plus AII (Los was added 10 min before AII), and the ECE-1 protein content (panel A) or ECE-1 mRNA expression (panel B) were evaluated in HuVEC. A representative Western-blot experiment including the tubulin levels (panel A) or Northern-blot experiment including the 18S mRNA expression (panel B) are shown at the top of each panel. Results (mean $\pm$ SEM) are expressed as a % of the control values(C). * p < 0.05 vs C and Los.

interaction with surrounding cells. Secondly, HMC act not only as target cells for ET-1 [29, 30], but also as a scavenger system for this protein. This effect could

Fig. 8. Effect of angiotensin II (AII) on endothelin-converting enzyme-1 (ECE-1) steady-state protein content in freshly isolated human glomeruli. Glomeruli were incubated for 6 or 24 h with 1 μ M AII (panel A) and for 24 h with 1 μ M AII with or without 1 μ M Losartan (Los) (panel B), and ECE-1 protein levels were evaluated by Western-blot. A representative Western-blot is shown at the top of each panel. The results of the densitometric analysis are expressed as a % of the control values (C) and are shown at the bottom of each panel. The results are the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs C.

partially explain the observed results. Finally, a decreased ECE activity is probably involved in the genesis of the ET-1 reduction, as a down-regulation of prepro-ET-1 mRNA expression cannot account for this finding.

At this point, some methodological aspects should be pointed out. Although previous studies have

demonstrated ET-1 synthesis by HMC [31-34], in our experimental conditions, HMC synthesized minimal amounts of ET-1. In fact, no immunoreactive ET-1 was detected in the supernatants of HMC alone and only a minimal expression of prepro-ET-1 mRNA appeared in these cells, independently of the presence or absence of HuVEC. This may be explained by the resting state of the cells, since they were serum-deprived for 24 h. Hughes et al. previously published similar results [31]. In this context, a possible contribution of HMC-derived ET-1 to the changes in the protein content in coculture supernatants can be excluded. On the other hand, the lack of ability of HMC to synthesize ET-1 allowed us to perform the analysis of protein degradation previously mentioned. As endogenous ET-1 synthesis by HMC is negligible, the protein levels detected after 24 h can be considered a reflection of the exogenously added ET-1 remaining in the incubation media.

An alternative mechanism for the ET-1 reduction in supernatants of cocultured cells can be proposed. HuVEC, in the presence of HMC, might also degrade increasing amounts of ET-1. This possibility, however, has not been tested as in HMC due to the interferences of endogenously synthesized ET-1. Moreover, the conversion of prepro-ET-1 to big ET-1 in HuVEC could also be inhibited by the coculture. No significant information regarding the exact mechanism responsible for this conversion has been previously published, and the present experiments have not explored this possibility.

Different questions arise from the present results. Therefore, additional experiments were aimed at analysing the mechanisms underlying the changes observed in ECE-1 protein content in HuVEC. The importance of this protease in the synthesis of ET-1 is widely recognized [10, 13], and various pharmacological inhibitors have been developed to control the deleterious effects of ET-1 in pathophysiological conditions [35]. Considering that HuVEC and HMC were not in contact but shared the incubation media, our hypothesis was that a soluble mediator synthesized by HMC could be responsible for the modulation of ECE-1. As HMC release a great amount of active metabolites [20], a rather non-specific approach was selected, in which some pharmacological antagonists, including receptor antagonists for AII, platelet-activating factor and thromboxane A_2 , as well as a hydrogen peroxide scavenger, were used. The blocking concentrations of these antagonists were selected according to concentrations previously shown to be effective [23, 36, 37]. As shown in the Results section, losartan was the most efficient antagonist among those

selected, as it completely blunted the decreased ECE-1 protein content observed in HuVEC cocultured with HMC. In consequence, AII could be proposed as one of the soluble factors responsible for ECE-1 down-regulation. Although neither HuVEC nor HMC alone produce AII, both cell types synthesize different components of the renin-angiotensin system [38-43]. In this regard, captopril, an angiotensin-converting enzyme inhibitor, reproduced the losartan effect, and it was possible to detect significant amounts of the peptide in the coculture media.

The importance of AII in the down-regulation of ECE-1 content in HuVEC was confirmed in experiments performed in this cell type. A dose and time-dependent decrease of ECE-1 protein content was observed in HuVEC treated with AII, an effect which depended on the type I-receptor. Significant inhibitory effects of AII were detected at 10 nM, a concentration greater than the previously described plasma concentration in physiological conditions [44]. However, plasmatic AII concentration does not necessarily reflect local synthesis, as the peptide is readily degraded. Previous studies from Tharaux et al [39] have suggested a link between AII and ET-1, as angiotensin-converting enzyme inhibitors decrease urinary ET-1 levels, and bosentan, a dual endothelin receptor antagonist, prevents the over-expression of collagen I induced by AII. These studies suggest a stimulatory effect of AII on ET-1 synthesis. Taking into account the present data, these "in vivo" results must be considered as the consequence of a very complex interaction between both systems, since ECE-1, responsible for the cellular release of active endothelin, is down-regulated by AII, at least in endothelial cells.

The use of a HuVEC and HMC coculture, with two types of cells from distinct anatomical structures, raises some doubts concerning the relevance of the results obtained. However, similar data were obtained when HuVEC were cocultured with vascular smooth muscle cells. In addition, the importance of the AII-dependent ECE-1 down-regulation was confirmed in more complex structures such as renal glomeruli.

Altogether, these results suggest that in vascular structures, such as the glomerular capillary network or other vascular beds, a cross-talk between resident cells is a basic mechanism for the regulation of bioactive metabolite synthesis, in normal and pathological conditions. This is the case of ECE-1, since the main enzyme responsible for the final synthesis of ET-1 is under the control of AII. The consequences of this interaction, and its importance in the biological response to various pharmacological antagonists, remain to be explored.

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