Endothelin-1 Inhibits Endothelin-Converting Enzyme-1 Expression in Cultured Rat Pulmonary Endothelial Cells

Shojiro Naomi, MD; Taisuke Iwaoka, MD; Tumba Disashi, MD; Junnosuke Inoue, MD; Yoshie Kanesaka, MD; Hiroshi Tokunaga, MD; Kimio Tomita, MD

Background—The lung expresses large amounts of endothelin-converting enzyme-1 (ECE-1), which catalyzes a step in the biosynthesis of potent vasoactive endothelin-1 (ET-1) from the inactive intermediate big ET-1. Because there has been no report concerning a possible relationship between ET-1 and ECE-1, we investigated the effects of ET-1 on ECE-1 expression in cultured rat pulmonary endothelial cells.

Methods and Results—ECE-1 messenger RNA (mRNA) and protein expression in cultured endothelial cells were assayed by Northern and Western blotting, respectively. Incubation with ET-1 for 6 hours caused a significant decrease in ECE-1 mRNA expression. The action of ET-1 on ECE-1 mRNA expression was antagonized by pretreatment with BQ788, a specific ETB receptor antagonist, but not by pretreatment with BQ123, a specific ETA receptor antagonist. The expression of ECE-1 protein was also inhibited at 6 hours after incubation with ET-1. The effects of ET-1 on ECE-1 mRNA and protein expression were shown to be mimicked by ionomycin, a calcium ionophore, but not by 12-O-tetradecanoylphorbol 13-acetate, a protein kinase C activator.

Conclusions—The present results demonstrate that ET-1 suppressed ECE-1 protein levels by inhibiting ECE-1 mRNA expression through the ETB receptor, suggesting the existence of a feedback action of ET-1 on ECE-1 in pulmonary endothelial cells. (Circulation. 1998;97:234-236.)

Key Words: endothelin □ endothelium □ receptors

Endothelin-1 is a potent vasoconstrictor peptide produced in vascular endothelial cells and is involved in pathophysiological conditions such as acute renal failure, pulmonary hypertension, and systemic hypertension. ETA receptors are present on the vascular smooth muscle and mediate direct vasoconstriction, whereas ETB receptors are present on endothelial cells and produce vasodilation via the endothelin-induced release of nitric oxide and prostacyclin. ET-1 is initially synthesized as preproET-1, which is processed to an inactive intermediate big ET-1. Mature ET-1 is produced from big ET-1. The final step is catalyzed by the endothelin-converting enzyme. An ECE-1 has recently been cloned and is the major form of ECE in most tissues. A more recently cloned ECE-2 is a homologous protein that differs from ECE-1 in sensitivity to phosphoramidon, in optimal pH, and in tissue distribution and quantity.

The lung is an organ that abundantly expresses ECE-1 as well as preproET-1 mRNA. The conversion of big ET-1 was reported to be more efficient in the lung than in the kidney or mesentery in isolated perfusion of rats. Increased pulmonary production of ET-1 has been demonstrated in pulmonary hypertension and acute myocardial infarction. In such states, an elevation of ET-1 may not be beneficial to maintain pulmonary and systemic circulation. For the counterregulatory mechanism, ET-1 may act directly on ECE-1 expression. In the present study, we investigated the effects of ET-1 on ECE-1 expression in cultured rat pulmonary endothelial cells and evaluated whether those effects might be mediated by PKC activation or intracellular calcium accumulation.

Methods

Materials
ET-1 was purchased from Peptide Institute. TPA and ionomycin were from Sigma Chemical Co. BQ123 and BQ788 were kindly provided by Banyu Pharmaceutical Co Ltd (Tokyo). cDNA for ECE-1 representing the region between nucleotides 437 and 439 of rat ECE-1 cDNA and AEC27–121, a monoclonal antibody against rat ECE-1, were kindly donated by Dr K. Tanzawa (Sankyo Pharmaceutical Co Ltd, Tokyo).

Culture of Rat Endothelial Cells
The SV40-transformed rat vascular endothelial cell line in lung, TRLEC-03, was established at the Institute of Cytosignal Research, Inc (Tokyo, Japan) and was kindly donated by Dr S. Tsurufuji. TRLEC-03 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO2. The cells used in the study were taken from the 5th to 20th passages. Confluent cells (107) in 100-mm collagen-coated dishes were preincubated in serum-free RPMI overnight. Those cells received 8 mL of fresh, serum-free RPMI before application of the PKC activator TPA.
or the calcium ionophore ionomycin. ET-1 (10^{-7} mol/L) was applied at time zero with or without pretreatment of the ETA receptor antagonist (BQ123) or ETB receptor antagonist (BQ788) for 30 minutes. Samples were obtained for RNA or protein analysis at 1, 3, 6, and 20 hours. At each time point, samples were also obtained from cells not exposed to the interventional compounds as time controls.

**Northern Blot Analysis**

Total RNA was isolated from endothelial cells with the acid guanidinium thiocyanate–phenol-chloroform method. Total RNA (20 μg) was size fractionated by 1.2% agarose gel electrophoresis and transferred to a nylon membrane. The rat ECE-cDNA probe was labeled with [32P]dCTP (3000 Ci/mmol, Amersham International PLC) by the random primed labeling method. The RNA immobilized on the membrane was hybridized with the labeled probes in the presence of 50% formamide, 5× Denhardt’s solution, 100 μg/mL salmon sperm DNA, 0.5% SDS, and 5× SSPE buffer (1× SSPE buffer: 150 mmol/L NaCl, 10 mmol/L NaHPO 4, 1 mmol/L EDTA, pH 7.4) for 20 hours at 42°C. Autoradiography was performed with an intensifying screen at ~80°C. The results were quantified by densitometric scanning. To control for variability in the loaded quantity of RNA, membranes were probed with GAPDH cDNA and used to normalize ECE-1 mRNA.

**Western Blot Analysis**

The confluent cells were collected with scrapers. After centrifugation, precipitated cells were homogenized in 10 vol of homogenization buffer (20 mmol/L Tris/HCl, pH 7.5, 5 mmol/L MgCl 2, 0.1 mmol/L PMSF, 20 μmol/L pepstatin A, and 20 μmol/L leupeptin) by use of a Polytron homogenizer. The homogenates were centrifuged at 1000 g for 10 minutes. The supernatants were centrifuged at 100 000 g for 30 minutes at 4°C. The membrane proteins (1 μg) obtained were subjected to a 4% to 20% gradient SDS–polyacrylamide gel. The separated proteins were loaded quantity of RNA, membranes were probed with GAPDH cDNA and used to normalize ECE-1 mRNA.

**Selected Abbreviations and Acronyms**

- ECE-1 = endothelin-converting enzyme-1
- ET-1 = endothelin-1
- ETA = endothelin A
- ETB = endothelin B
- PKC = protein kinase C
- TPA = 12-O-tetradecanoylphorbol 13-acetate
- TRLEC-03 = SV40-transformed rat vascular endothelial cell line in lung

Figure 1. Northern blot analysis demonstrating effects of ET-1 on ECE-1 mRNA expression in cultured endothelial cells. A, Representative northern blot analysis (top) of ECE-1 and GAPDH mRNA. Lanes 1, 2, 4, 6, and 8 show control cells (C) before and after incubation for 1, 3, 6, and 20 hours. Lanes 3, 5, 7, and 9 show cells (ET-1) incubated with ET-1 (10^{-7} mol/L) for 1, 3, 6, and 20 hours. Graph (bottom) shows abundance of ECE-1 mRNA relative to GAPDH mRNA as corrected for respective time controls (100%). *P<.05 compared with respective time controls. B, The effect of either the ETA or ETB receptor antagonist on ET-1–induced suppression of ECE-1 mRNA. Bar graph shows ECE-1 mRNA abundance relative to GAPDH mRNA. Values are expressed as percent of time controls (100%). †P<.05 compared with controls. ‡P<.05 compared with respective time controls. C, Effect of ionomycin on the expression of ECE-1 mRNA. Graph shows abundance of ECE-1 mRNA relative to GAPDH mRNA as corrected for respective time controls (100%). *P<.05, **P<.01 compared with respective time controls.

**Results**

ECE-1 mRNA levels were measured at different time points up to 20 hours after the addition of ET-1 (10^{-7} mol/L). Fig 1A shows that the ECE-1 message that was normalized as a ratio of ECE-1 mRNA to GAPDH mRNA decreased after 6 hours of exposure to ET-1 (P<.05 versus control; n=5). We tested whether the inhibitory effect of ET-1 on ECE-1 mRNA expression was mediated by either the ETA or ETB receptor. Fig 1B shows that ET-1 inhibited ECE-1 mRNA to 62±10% of control levels (P<.05 versus control; n=5) at 6 hours after incubation. ECE-1 mRNA expression by ET-1 remained suppressed to 57±10% of control (P<.05 versus control; n=5) by preincubation with BQ123 (10^{-6} mol/L). Preincubation with BQ788 (10^{-6} mol/L) abolished ET-1–induced inhibition in ECE-1 mRNA expression to 85±12% of control (P=NS versus control; n=5). TPA (5×10^{-6} mol/L) did not alter ECE-1 mRNA expression up to 20 hours after incubation (data not shown). Ionomycin (5×10^{-6} mol/L) decreased ECE-1 mRNA expression at 3, 6, and 20 hours' incubation to 79±7%, 39±5%, and 44±12% of control, respectively (Fig 1C).

As shown in Fig 2A, ECE-1 protein level decreased to 71±12% of control (P<.05 versus control; n=6) after incubation with ET-1 for 6 hours. ECE-1 protein level did not change significantly after 6 hours' incubation with TPA (Fig 2B). As with the action on ECE-1 mRNA expression, ionomycin inhibited the ECE-1 protein level to 73±11% of control (P<.05 versus control; n=8) at 6 hours after the incubation (Fig 2B).

**Discussion**

We used SV40-transformed rat pulmonary endothelial cells in which the mRNA and protein for ECE-1 were abundantly expressed. To prove the existence of a feedback action between ECE-1 and ET-1, we first obtained data showing that ECE-1 mRNA expression was suppressed at 6 hours after treatment with ET-1. Similar suppression occurred in the ECE-1 protein level at
ET-1–induced intracellular signaling. Uchida et al.8 studied the activation of PKC is one of the important components caused by ET-1 on the regulation of ECE-1 expression. The specific ETB receptor antagonist BQ788 antagonized the inhibitory action of ET-1 on ECE-1 mRNA expression. These results are the first to demonstrate that ET-1 can affect ECE-1 expression through the ETB receptor.

The ETB receptor has been reported to be coupled to phospholipase C and could mediate phosphoinositide hydrolysis, thereby inducing elevation of cytosolic Ca\(^{2+}\) in endothelial cells.7 We showed that ionomycin suppressed mRNA and protein expression for ECE-1. Thus, ionomycin mimics the action of ET-1 on ECE-1 expression, supporting the idea that elevation of cytosolic Ca\(^{2+}\) may be responsible for the intracellular signaling caused by ET-1 on the regulation of ECE-1 expression. The activation of PKC is one of the important components of ET-1–induced intracellular signaling. Uchida et al.8 studied the regulation of ECE-1 mRNA in bovine glomerular endothelial cells. They found that TPA increased the expression of ECE-1 mRNA. However, they did not measure the ECE-1 protein level. This discrepancy may be explained by the difference in cell type used. The AP-1 site, which mediates the transcriptional response from TPA, has been found in the human promotor regions of the ECE-1 isoyme ECE-1a gene but not in the promotor of the ECE-1b gene.9 TRLEC-03 cells possess only ECE-1b.10 Although the promotor region of rat ECE-1 has not been analyzed, our data suggest that AP-1 sites on the promotor region of ECE-1b either do not exist as they do in humans or are not involved in operating the transcriptional response of rat ECE-1b. Collectively, our results indicate that the inhibitory action of ET-1 on ECE-1 may not be mediated by the activation of PKC in TRLEC-03 cells.

Miyauchi et al.11 reported that in patients with chronic hemodialysis, strikingly elevated big ET-1 levels with only slightly elevated ET-1 levels in plasma are observed. Recently, it has been reported that rats with hypertension induced by hepatic overexpression of human preproET-1 have a high big ET-1 to mature ET-1 ratio.12 These findings suggest that under certain conditions in which big ET-1 is elevated, ECE-1 can act as a rate-limiting enzyme in the conversion of big ET-1 to mature ET-1 in plasma.

The present results have demonstrated that ET-1 inhibits the level of ECE-1 protein by suppressing the expression of ECE-1 mRNA through the ETB receptor in cultured pulmonary endothelial cells. Future analysis of the feedback system in certain pathophysiological conditions with elevated ET-1 levels should prove that it plays an important role in the regulation of ECE-1 expression by ET-1 in vivo.

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