Endothelin-1 Down-Regulates Expression of Tropoelastin and Lysyl Oxidase mRNA in Cultured Chick Aortic Smooth Muscle Cells

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(Received February 26, 2001; Accepted August 4, 2001)

Endothelin-1 (ET-1) is known as a potent stimulator of cell proliferation and as a vasoconstrictor. It is believed that ET-1 contributes to the development of arterial diseases such as atherosclerosis. In this study, we demonstrated the expression of tropoelastin and lysyl oxidase (LO) on gene levels as induced by ET-1 in cultured smooth muscle cells (SMCs). ET-1 stimulated cell proliferation in a dose–dependent manner, and the level of this proliferation increased about 1.3-fold at 100 nM of ET-1. ET-1 suppressed the tropoelastin protein synthesis in a dose–dependent and time–dependent manner. In addition, ET-1 dose–dependently suppressed the tropoelastin and LO mRNA expression. The tropoelastin and LO mRNA levels decreased to about half and 4/5, respectively, at 100 nM of ET-1. The inhibition of elastin synthesis was completely prevented by BQ123, an endothelin receptor A (ET A) antagonist. These results indicate that ET-1 can modulate the tropoelastin and LO mRNA expression via an ET A receptor in cultured SMC and that the regulator for elastin expression may play an important role in elastogenesis and SMC proliferation during the development of atherosclerosis.

Key words — elastin, lysyl oxidase, endothelin-1, smooth muscle cell, endothelin A receptor

INTRODUCTION

The regulation of cell proliferation is an important event in normal development and in pathological responses to injury. A number of growth factors and cytokines are capable of stimulating proliferation of target cells by activating their receptors. However, the presence of growth factors or cytokines and their receptors is not sufficient to induce cell proliferation. The number of the local environment, extracellular matrix and intracellular interactions can also regulate the response to a given growth factor or cytokine.

Endothelin-1 (ET-1), which is a potent vasoconstrictor, has been isolated from cultured porcine aortic endothelial cells. ET-1 is mainly synthesized and secreted from cultured porcine endothelial cells, and it is known that ET-1 stimulates cell proliferation. The effects of ET-1 are mediated via two subtypes of endothelin receptors, endothelin A (ET A) and endothelin B (ET B), receptors, which have been cloned and characterized. ET A membrane receptors have a high affinity for ET-1 and are mainly present in vascular smooth muscle cells in order to mediate vasoconstriction. The ET A receptor is located predominantly on vascular smooth muscle cells (SMCs). It is a classical heptathelical G-protein coupled receptor that activates phospholipase C to cause hydrolysis of phosphatidyl inositol and generation of cytosolic inositol triphosphate and membrane-bound diacylglycerol. It has been reported that production of ET-1 occurs in atherosclerosis and that ET A receptor antagonists or endothelin-converting enzyme inhibitors suppress the formation of atheroma. These observations suggest the possibility that ET-1 may stimulate cell proliferation and development of atherosclerosis.

The regulation of accumulation of extracellular matrices is fundamental important to mature tissues and to an understanding of connective tissue diseases such as atherosclerosis. Elastin is responsible for the characteristic elastic properties of many tissues including skin, lung and large blood vessels. Elastin is synthesized as a soluble precursor, tropoelastin, by aortic smooth muscle cells. Tropoelastin is associated with microfibrils, such as fibrillin-1, fibrillin-
2 or the microfibril associated glycoprotein (MAGP), and then cross-links with other tropoelastin molecules by lysyl oxidase (LO) to form elastic fiber. Elastin synthesis is modulated by a number of factors including transforming growth factor β (TGF-β), insulin-like growth factor (IGF), interleukin-1 (IL-1), epidermal growth factor (EGF), glucocorticoid, and c-guanosine 5’-monophosphate (c-GMP). 

Abnormal metabolism of elastin has been associated with hypertension and atherosclerosis, and elastin may play a key role in the development of these diseases. However, there is little report about the gene expression concerning the elastic fiber assembly in the development of atherosclerosis. Therefore, in the present study we investigated the tropoelastin and LO gene expression by ET-1 in cultured chick SMCs.

**MATERIALS AND METHODS**

**Materials** —— [3,4-3H] Valine (1.5 TBq/mmol) and [α-32P] deoxyctydine 5’-triphosphate (dCTP) (110 TBq/mmol) were supplied by Amersham. ET-1 was purchased from the Peptide Institute, Inc. (Japan), BQ123 was obtained from Alexis, and Dulbecco’s modified Eagle’s medium (DMEM), valine-free DMEM, fetal bovine serum (FBS), and dialyzed FBS were obtained from Gibco.

**Cell Culture and Proliferation** —— SMCs were isolated from 20-day-old chick embryonic aortas by serial enzyme digestion with bacterial collagenase (Sigma) and pancreatic elastase (Sigma) as previously described. They were seeded at a density of 2 x 10⁶ cells/35-mm-diameter Petri dish (Falcon Plastics) and grown to 80% of confluence in DMEM supplemented with 10% FBS. The FBS and dialyzed FBS used in this study were not heat-inactivated. SMCs grown to 80% of confluence were incubated for 24 hr in DMEM containing 0.5% dialyzed FBS to induce them to the G₀ phase (quiescent). The quiescent SMCs were cultured for 24 hr in DMEM containing 0.5% dialyzed FBS with various concentrations of ET-1 and then were harvested with 0.25% trypsin. The cell number was determined with a hemocytometer. The data are presented as a means ± standard error (S.E.). Statistically significant differences between groups were analyzed using Student’s t test, where p < 0.05 was considered statistically significant. Statview software (Abacus, Berkeley, CA, U.S.A.) was used for all statistical work.

**Metabolic Labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** —— The quiescent SMCs were treated with various concentrations of ET-1 in DMEM containing 0.5% dialyzed FBS for 24 hr or with 100 nM ET-1 in DMEM containing 0.5% dialyzed FBS for various periods of time. Quiescent SMCs were pretreated with 10 μM BQ123, as an inhibitor of endothelin receptor A (ETₐ), in DMEM containing 0.5% dialyzed FBS for 30 min and then treated with a combination of 10 μM BQ123 and 10⁻⁷ M ET-1 in DMEM containing 0.5% dialyzed FBS for 24 hr. The cells were labeled with 25 μCi/ml of [3,4-3H] valine for the last 6 hr of treatment in the valine-free DMEM. The culture medium was precipitated with ammonium sulfate (176 mg/ml) in the presence of protease inhibitor cocktails [1 mM EDTA, N-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF)]. The protein from the medium was resuspended in 35 μl of Laemmli sample buffer containing dithiothreitol (DTT) and incubated at 100°C for 5 min. The samples were electrophoresed on 4–15% SDS-polyacrylamide gels. Gels were then dried and exposed to XAR-5 X-ray film (Eastman Kodak Co., U.S.A.), and then fluorographed and scanned with a densitometer (Cliniscan, Helena Laboratorie, U.S.A.). The amount of samples applied to the gels was normalized according to cell number. The density of elastin bands per total bands was used as a measure of relative synthesis.

**Northern Blot Analysis** —— Total RNA was isolated from cells according to a previously described procedure and after being adjusted to a concentration of 2 μg/μl, stored at ~80°C degree until use. The total RNA was denatured for 1 hr at 50°C in deionized 1 M glyoxal/10 mM phosphate buffer, pH 7.0, and electrophoresed on 1% agarose gel, then was blotted to N⁺ nylon filters (Amersham, U.K.). The membranes were hybridized for 18 hr at 42°C to 32P-labeled probes in 50% formamide, 5 x 0.15 M sodium chloride and 0.015 M sodium citrate (SSC), 5 x Denhardt’s solution, 0.1% SDS, and 250 μg/ml t-RNA. The following cDNA probes which were radioactively labeled by random priming (Amersham, U.K.) to specific activity of ~10⁹ dpm/μg DNA were used: chicken elastin (pTE2), (β-actin (pA1)). 300 bp of Chick LO cDNA were amplified by reverse transcriptase–polymerase chain reaction (RT–PCR) using a sense primer (5’-ACGGACGATAACCCCTACTACAACT-3’) and an anti-sense primer (5’-CGCATTAGTTGTGGAG-
TAATCAG-3'). The filters were washed for 30 min at a stringency of 1 × SSC/0.1% SDS followed by 0.1 × SSD/0.1% SDS and exposed at –80°C to X-ray films (Fuji RX, Japan) with an intensifying screen (Kodak Lanex Regular, U.S.A.). The autoradiograms were scanned with a densitometer. The density of tropoelastin and LO bands was normalized according to β-actin bands as a measure of relative expression.

RESULTS

Cell Proliferation by ET-1

To begin with, we determined the effect of ET-1 on cell proliferation. Quiescent SMCs were exposed to 1–100 nM ET-1 for 24 hr of exhibited proliferation compared to control. The effect was most pronounced at 100 nM ET-1, which resulted in 30% stimulation of proliferation (p < 0.01; Fig. 1). These results are similar to those reported for human skin fibroblasts in culture.21)

Tropoelastin Synthesis by ET-1

The changes in tropoelastin protein synthesis in SMCs brought about by drug treatment were detected by SDS-PAGE following a metabolic labeling assay with [3H] valine. Immunoblotting analysis using monoclonal antibody for tropoelastin revealed that the bands, which indicated with an arrow, are related to tropoelastin.22) Moreover, we previously reported that bands of tropoelastin were confirmed by incorporations of radiolabeled cysteine and valine, but not incorporations of radiolabeled mannose, glucose, and methionine.23) Since complete amino acid sequence deduced from chicken cDNA demonstrated that tropoelastin has no sugar moiety nor methionine residue and has two cysteine residues only near the carboxyl-terminal end,18,24,25) Confluent SMCs were cultured with FBS-free DMEM for 24 hr to induce the cells to enter the G₀ phase,26) and then the culture medium was replaced with FBS-free DMEM containing 0, 1, 10 or 100 nM of ET-1. After 18 hr, newly synthesized proteins were labeled with [3,4-3H] valine. ET-1 inhibited tropoelastin synthesis in the medium in a dose–dependent manner without changing other secreted proteins, and the level of tropoelastin synthesis was about 50% at a concentration of 100 nM of ET-1 (Fig. 2). We also determined the amount of tropoelastin synthesis induced by ET-1 in the medium in a time-dependent manner. ET-1 inhibited about 25% of tropoelastin synthesis after 6 hr of treatment and 50% after 24 hr of treatment (Fig. 3).

Expression of Tropoelastin and LO mRNA by ET-1

To examine the regulation of tropoelastin and LO mRNA expression by ET-1, confluent cultures of SMCs maintained in DMEM without FBS were incubated with various concentrations of ET-1 for 24 hr. Tropoelastin and LO mRNA levels were estimated by northern blot hybridization. ET-1 reduced elastin and LO mRNA levels in a dose–dependent manner, and the maximal inhibition was detected at a concentration of 100 nM (Fig. 4A). Quantification of tropoelastin and LO mRNA, after correction for β-actin mRNA levels, revealed that the maximal reduction in tropoelastin and LO mRNA to approximately 40% and 80% of the level in untreated control cells occurred after treatment with 100 nM of ET-1 (Fig. 4B).

Effect of ETA Blockers on ET-1-Induced Elastin Synthesis

In order to determine whether the inhibition of tropoelastin synthesis by ET-1 is dependent on ETA or not, confluent cultures of SMCs were treated with ET-1 (100 nM) alone or in combination with BQ-123 (10 µM), as a specific inhibitor of ETA, in DMEM without FBS. In these cells, treatment with BQ-123 alone had no effect on tropoelastin synthesis. However, exposure of SMCs to BQ-123 potently prevented the inhibitory effect of ET-1 on tropoelastin synthesis (Fig. 5).
DISCUSSION

SMC proliferation is a key event in the development of atherosclerosis. Several studies have revealed that elastin synthesis is inversely related to cell proliferation. It has been reported that potent stimulators of cell proliferation, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), inhibit elastin synthesis, and potent inhibitors of cell proliferation, such as minoxidil, retinoic acid or heparin, stimulate elastin synthesis. In this study, our results show that ET-1 stimulates SMC proliferation and reduces tropoelastin synthesis in mRNA levels as previously reported.

ET-1 reduces tropoelastin and LO mRNA expres-
sion, and BQ 123, a specific ET₄ receptor antagonist,³² can effectively prevent the effect of ET-1 on tropoelastin synthesis. ET-1 binds to ET₄ receptors on the cell surface, and these receptors are classical heptathelial G-protein coupled receptors that activate phospholipase C to cause hydrolysis of phosphatidyl inositol and generation of cytosolic inositol triphosphate and membrane-bound diacylglycerol, which accelerate protein kinase C (PKC) activity and intracellular Ca²⁺ concentration.³³ It has been reported that a PKC activator, such as TPA, reduces elastin expression by a posttranscriptional mechanism. It has also been postulated that TPA may control the tropoelastin mRNA via unique cis-acting sequences of the 3′ untranslated region (3′UTR).²⁷ We have demonstrated that ET-1-induced reduction of elastin expression is mediated by the activation of PKC activity via ET₄ receptors.

LO is a key participant in the accumulation of insoluble fibers of elastin and collagen by virtue of

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**Fig. 4.** Effect of ET-1 on Tropoelastin (TE), LO and β-Actin mRNA Levels in SMCs

(A), RNA was extracted from SMCs treated for 24 hr with 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 nM (lane 4) of ET-1. Ten micrograms RNA was resolved on 1% agarose gel electrophoresis, blotted onto membranes and hybridized with ³²P-labeled chick elastin, LO and β-actin cDNA probes. The filters were washed and visualized by autoradiography. (B), The autoradiograms were quantitated with a scanning densitometer. Open bars represent the quantitative tropoelastin mRNA. Black bars represent the quantitative LO mRNA. Each value indicates the average from triplicate experiments.

**Fig. 5.** Effect of BQ123 on ET-1-Mediated Elastin Suppression

(A), SMCs were treated with vehicle (lane 1), 100 nM of ET-1 (lane 2), 10 µM of BQ123 (lane 3) or combination of 100 nM of ET-1 and 10 µM of BQ123 (lane 4) for 24 hr in the absence of FBS, and labeled [³H] valine for the final 6 hr. The proteins from culture medium analyzed by 2–15% SDS-PAGE, and visualized by fluorography. (B), The arrow indicates the position of tropoelastin. The fluorograms were quantitated with a scanning densitometer. Each value indicates the average from triplicate experiments.
its role in the initiation of the covalent cross-linkages between and within individual molecules comprising these fibers. This enzyme oxidizes specific lysine residues within these matrix molecules into peptidyl-α-aminoadipic-δ-semialdehyde. Subsequent spontaneous condensations of the aldehyde residues produce the cross-linkages that account for the stability of these fibrous proteins. ET-1 is known as an activator of elastin cross-linking processes in the aorta and result in a destabilization of the aortic wall such as occurs in the disease of atherosclerosis. 40,41 It is has been reported that ET-1 inhibited LO mRNA expression in a dose–dependent manner. Together with these reports, our results suggest that inhibition of tropoelastin and LO mRNA expression by ET-1 may cause inhibition of the elastic fiber, decrease in human atherosclerosis.

Acknowledgements This work was supported by the Ministry of Education, Science, Sports, and Culture, Japan. We thank Kayo Tomioka, Saori Yoshioka, Rie Kobayashi, and Atsushi Ito for technical assistance.

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