

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

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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.¹⁾ ET-1 is mainly synthesized and secreted from cultured porcine endothelial cells, and it is known that ET-1 stimulates cell proliferation.²⁾ The effects of ET-1 are mediated *via* two subtypes of endothelin receptors, endothelin A (ET_A)³⁾ and endothelin B (ET_B)⁴⁾ 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.³⁾ The ET_A receptor is located predominantly on vascular smooth muscle cells (SMCs). It is a classical heptathetical 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^{5,6)} and that ET_A receptor antagonists or endothelin-converting enzyme inhibitors suppress the formation of atheroma.⁷⁾ 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-

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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),⁸⁾ insulin-like growth factor (IGF),⁹⁾ interleukin-1 (IL-1),¹⁰⁾ epidermal growth factor (EGF),¹¹⁾ glucocorticoid,¹²⁾ and c-guanosine 5'-monophosphate (c-GMP).¹³⁾

Abnormal metabolism of elastin has been associated with hypertension¹⁴⁾ and atherosclerosis,¹⁵⁾ 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] deoxycytidine 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.¹⁶⁾ They were seeded at a density of 2×10^6 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 \pm 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 statis-

tical 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 μ M BQ123, as an inhibitor of endothelin receptor A (ET_A), in DMEM containing 0.5% dialyzed FBS for 30 min and then treated with a combination of 10 μ M BQ123 and 10⁻⁷ M ET-1 in DMEM containing 0.5% dialyzed FBS for 24 hr. The cells were labeled with 25 μ Ci/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 μ l 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 Laboratoire, 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 procedure¹⁷⁾ and after being adjusted to a concentration of 2 μ g/ μ l, 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 \times 0.15 M sodium chloride and 0.015 M sodium citrate (SSC), 5 \times Denhardt's solution, 0.1% SDS, and 250 μ g/ml t-RNA. The following cDNA probes which were radioactively labeled by random priming (Amersham, U.K.) to specific activity of $\sim 10^8$ dpm/ μ g DNA were used: chicken elastin (pTE2),¹⁸⁾ and β -actin (pA1).¹⁹⁾ 300 bp of Chick LO cDNA²⁰⁾ were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using a sense primer (5'-ACGGACGATAACCCCTACTACAAC-3') and an anti-sense primer (5'-CGCACTATGTTGTTGGAG-

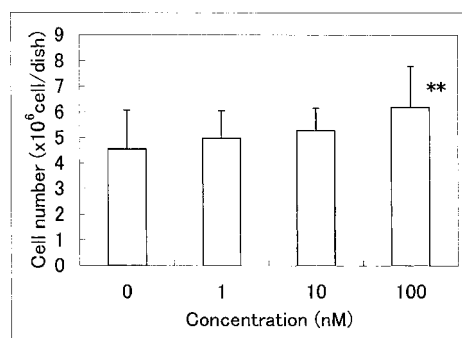


Fig. 1. Effect of Endothelin-1 on Cell Proliferation

SMCs were plated at a density of 2×10^6 cells/35 mm-diameter Petri dish and cultured in DMEM supplemented with 10% FBS. 80% of confluence SMCs were treated for 24 hr with 0, 1, 10, 100 nM of endothelin-1 in FBS-free DMEM, and then were harvested by trypsin. Cell number was counted by Beker-Turk hemocytometer. Each value indicates the means \pm S.E. from triplicate experiments; ** $p < 0.01$ (comparison with control).

TAATCAG-3'). The filters were washed for 30 min at a stringency of $1 \times \text{SSC}/0.1\%$ SDS followed by $0.1 \times \text{SSD}/0.1\%$ 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 va-

line, 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 ET_A Blockers on ET-1-Induced Elastin Synthesis

In order to determine whether the inhibition of tropoelastin synthesis by ET-1 is dependent on ET_A 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 ET_A, 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 potentially prevented the inhibitory effect of ET-1 on tropoelastin synthesis (Fig. 5).

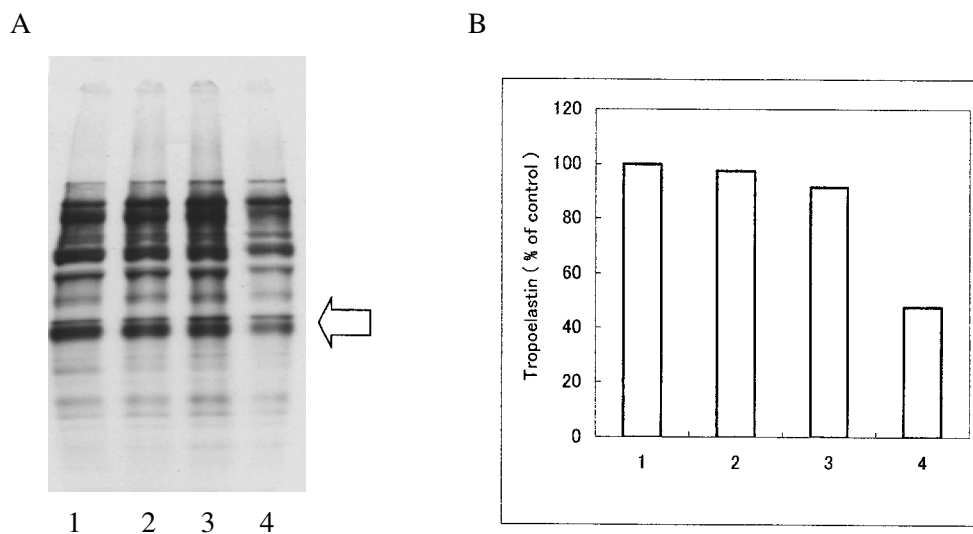


Fig. 2. Effect of ET-1 on Elastin Synthesis in Smooth Muscle Cells

(A), SMCs were treated for 24 hr with 0 (lane 1), 1 (lane 2), 10 (lane 3), 100 nM (lane 4) of ET-1 in FBS-free DMEM, then labeled with [3 H] valine for the last 6 hr of treatment. 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.

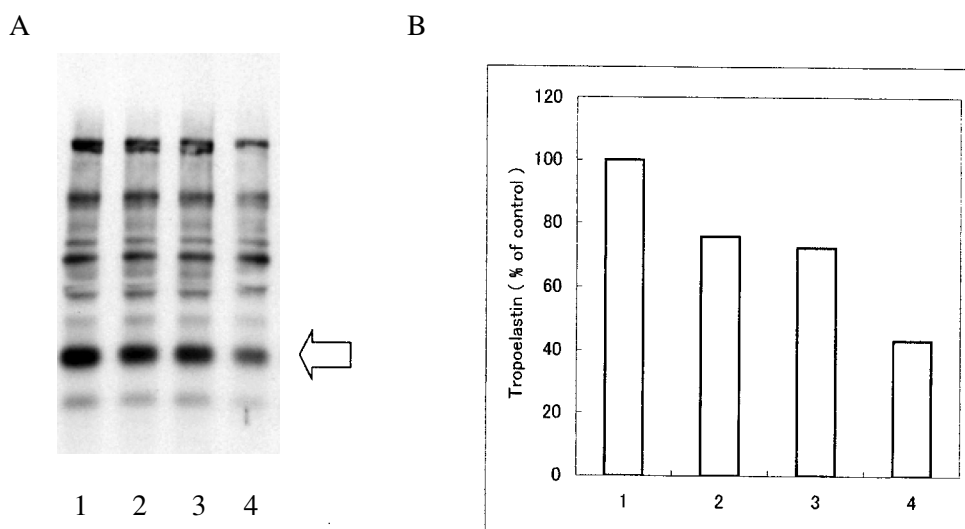


Fig. 3. Effect of ET-1 on Elastin Synthesis in Smooth Muscle Cells

(A), SMCs were treated for 0 (lane 1), 6 (lane 2), 12 (lane 3) and 24 hr (lane 4) with 100 nM endothelin-1 in FBS-free DMEM, then labeled with [3 H] valine for the last 6 hr of treatment. 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.

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),²⁷⁾ angio-

tensin II²⁸⁾ or EGF,¹¹⁾ inhibit elastin synthesis, and potent inhibitors of cell proliferation, such as minoxidil,²⁹⁾ retinoic acid³⁰⁾ or heparin,³¹⁾ 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-

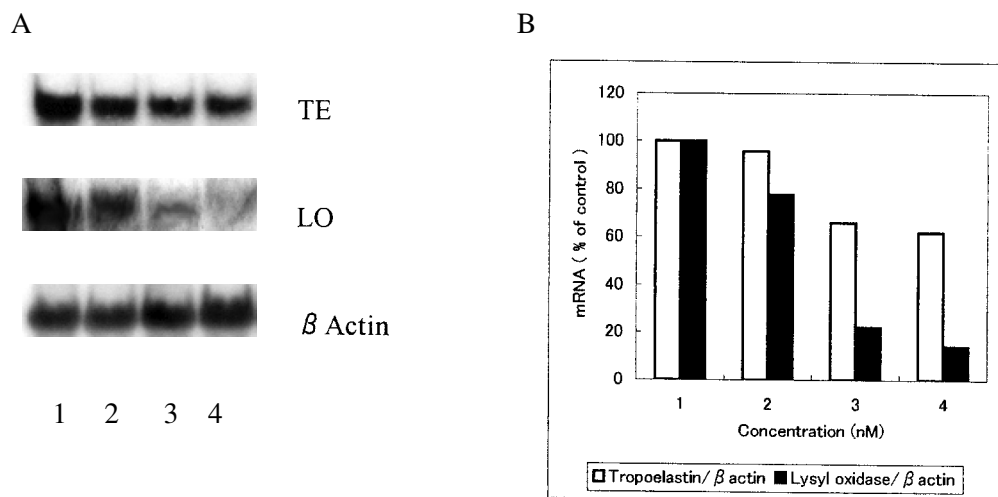


Fig. 4. Effect of ET-1 on Tropoelastin (TE), LO and β -Actin mRNA Levels in SMCs

(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 32 P-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.

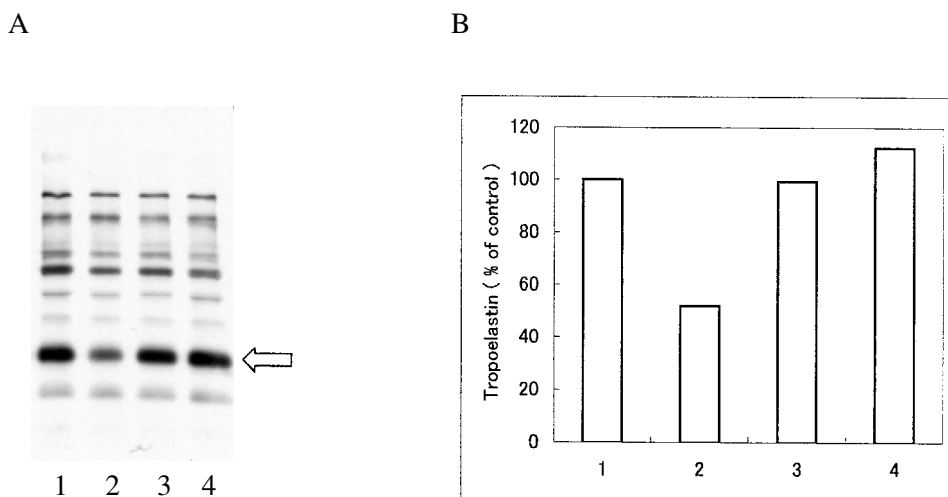


Fig. 5. Effect of BQ123 on ET-1-Mediated Elastin Suppression

(A), SMCs were treated with vehicle (lane 1), 100 nM of ET-1 (lane 2), 10 μ M of BQ123 (lane 3) or combination of 100 nM of ET-1 and 10 μ M of BQ123 (lane 4) for 24 hr in the absence of FBS, and labeled [3 H] 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.

sion, and BQ 123, a specific ET_A receptor antagonist,³² can effectively prevent the effect of ET-1 on tropoelastin synthesis. ET-1 binds to ET_A receptors on the cell surface, and these receptors are classical heptathelical 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 re-

ported 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* ET_A receptors.

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

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 -amino adipic- δ -semialdehyde. Subsequent spontaneous condensations of the aldehyde residues produce the cross-linkages that account for the stability of these fibrous proteins.³⁴⁾ The expression of the LO gene is regulated at transcriptional or post-transcriptional levels by a number of agents or conditions, or both, relevant to the normal and/or diseased arterial wall. LO expression is significantly up-regulated by TGF- β 1,^{35,36)} platelet-derived growth factor (PDGF)³⁷⁾ and cAMP.³⁸⁾ It has been reported that cAMP inhibits the proliferation of vascular smooth muscle cell (VSMC).³⁹⁾ LO expression is significantly stimulated upon reduction of SMC proliferation in culture either by serum starvation or by the addition of TGF- β 1.³⁵⁾ In the present study, ET-1 inhibited LO mRNA expression in a dose-dependent manner. Together with these reports, our results may suggest that modulation of LO mRNA expression is inversely associated with cell proliferation.

It has been reported that amounts of desmosine or isodesmosine, which are cross-linking amino acids in elastic fiber, decrease in human atherosclerotic plaques.^{40,41)} ET-1 is known as an activator of atherosclerosis. Therefore, the results of this paper suggest that inhibition of tropoelastin and LO mRNA expression by ET-1 may cause inhibition of the elastin cross-linking processes in the aorta and result in a destabilization of the aortic wall such as occurs in the disease of atherosclerosis.

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