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Effect of endothelin-1 (1-31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells

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1 We have previously found that human chymase cleaves big endothelins (ETs) at the Tyr^{31}-Gly^{32} bond and produces 31-amino acid ETs (1-31), without any further degradation products. In this study, we investigated the effect of synthetic ET-1 (1-31) on the proliferation of cultured human coronary artery smooth muscle cells (HCASMCs).

2 ET-1 (1-31) increased [^{3}H]-thymidine incorporation and cell numbers to a similar extent as ET-1 at 100 nM. This ET-1 (1-31)-induced [^{3}H]-thymidine uptake was not affected by phosphoramidon, an inhibitor of ET-converting enzyme. It was, however, inhibited by BQ123, an endothelin ET_A receptor antagonist, but not by BQ788, an endothelin ET_B receptor antagonist.

3 By using an in-gel kinase assay, we demonstrated that ET-1 (1-31) activated extracellular signal-regulated kinase 1/2 (ERK1/2) in a concentration-dependent manner (100 pM to 1 μM) in HCASMCs. ET-1 (1-31)-induced ERK1/2 activation was inhibited by BQ123, but not by BQ788 and phosphoramidon. Inhibition of protein kinase C (PKC) and ERK kinase also caused a reduction of ET-1 (1-31)-induced ERK1/2 activation, whereas tyrosine kinase inhibition had little effect.

4 Gel-mobility shift analysis revealed that the ERK1/2 activation was followed by an increase in transcription factor activator protein-1 DNA binding activity in HCASMCs.

5 Our results strongly suggest that ET-1 (1-31) itself stimulates HCASMC proliferation probably through endothelin ET_A or ET_A-like receptors. The underlining mechanism of cell growth by ET-1 (1-31) may be explained in part by PKC-dependent ERK1/2 activation. Since human chymase has been proposed to play a role in atherosclerosis, ET-1 (1-31) may be one of the mediators.

Keywords: Endothelin-1 (1-31); human chymase; extracellular signal-regulated kinase; protein kinase C; coronary artery smooth muscle cell

Introduction

Endothelin-1 (ET-1) is a 21-amino acid polypeptide which exhibits various physiological actions, such as vascular contraction (Yanagisawa *et al*., 1988), cardiac hypertrophy (Arai *et al*., 1995) and mitogenesis (Chua *et al*., 1992). Human ET-1 is generated from the 38-amino acid precursor, big ET-1, through cleavage of the Trp^{21}-Val^{22} bond *via* the action of a membrane-bound metalloprotease, ET-converting enzyme (ECE) (Shimada *et al*., 1994). Although ECE was originally shown to be a membrane-bound metalloprotease (Shimada *et al*., 1994), several other metalloproteases have also been postulated to catalyze the formation of ET-1 from big ET-1 (Matsumura *et al*., 1990). Rat mast cell chymase has also been reported as a putative converter of big ET-1 to ET-1 (Wypij *et al*., 1992).

We have recently found that human mast cell chymase, unlike rat mast cell chymases, selectively cleaves big ETs at the Tyr^{31}-Gly^{32} bond to produce novel trachea-constricting 31-amino acid ETs, ETs (1-31), without any further degradation products (Nakano *et al*., 1997). ET-1 (1-31) has been shown to cause contraction of rabbit afferent arterioles (Tamaki *et al*., 1997) as well as to cause an increase in intracellular free Ca^{2+} concentration in human coronary artery smooth muscle cells (HCASMCs) (Yoshizumi *et al*., 1998). Furthermore, ETs (1-31) have been isolated from various human organs, such as lung and heart (Okishima *et al*., manuscript in preparation).

Chymase has been suggested to play a role in human atherosclerosis because chymase-containing mast cells were found to be rich in atherosclerotic plaques of human carotid arteries (Jeziorska *et al*., 1997). Moreover, it has been reported that chymase reduces the cholesterol efflux-inducing ability of serum which may promote atherogenesis (Lindstedt *et al*., 1996). Although these studies suggest the involvement of chymase in atherosclerosis, the key molecule which directly relates to atherogenesis has not yet been elucidated. ET-1 (1-31) may be a candidate for this substance. Since ET-1 has been shown to cause proliferation of vascular smooth muscle cells (VSMCs) (Chua *et al*., 1992; Janakidevi *et al*., 1992; Kanse *et al*., 1995), we hypothesized that ET-1 (1-31) is an alternative mitogen for VSMCs and relevant to atherogenesis.

In the present study, we examined the effect of synthetic ET-1 (1-31) on cultured HCASMC proliferation. Results suggest that ET-1 (1-31) induces HCASMC proliferation and is almost equipotent to ET-1. Furthermore, ET-1 (1-31) activates extracellular signal-regulated kinases (ERKs) belonging to a subfamily of the mitogen-activated protein kinase (MAPK) family (Davis, 1993; Nishida & Gotoh, 1993). ET-1 (1-31) also activates DNA binding of activator protein-1 (AP-1) from HCASMC nuclear extracts.

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Methods

Cell preparation and culture

Human coronary artery smooth muscle cells (HCASMCs) at passage 4 were obtained as a commercially available product from Clonetics Corp. (San Diego, CA, U.S.A.). Cells were plated in 25-cm^2 tissue culture flasks at a density of 5×10^3 cells cm^{-2} in MCDB131 medium supplemented with 5% heat-inactivated fetal calf serum, 0.5 ng ml^{-1} epidermal growth factor, 1 ng ml^{-1} basic fibroblast growth factor, 5 μg ml^{-1} insulin, 50 μg ml^{-1} gentamicin, and 0.25 μg ml^{-1} amphotericin B. The cells were incubated at 37°C in 5% CO_2 and the medium was replaced every other day until the cells were 60–80% confluent. The cells were then removed from the flasks with 0.025% trypsin plus 0.01% EDTA and seeded onto 24-well culture plates (Coster Corp., Cambridge, MA, U.S.A.) for experiments of [^{3}H]-thymidine incorporation and cell counting. For experiments of in-gel kinase assay or gel mobility shift assay, the cells were seeded onto 35-mm or 100-mm tissue culture dishes (Coster Corp.), respectively. All experiments were performed with the cells in passage 5–15 and at 1–2 days post-confluency except for cell counting.

Determination of [^{3}H]-thymidine incorporation

Confluent HCASMCs in 24-well culture plates were made quiescent by placing them in serum-free medium supplemented with insulin (1 μM) and transferrin (5 mg ml^{-1}) for 2 days. They were then stimulated for 24 h with 100 nM ET-1 (1-31) or ET-1. The stimulated cells were pulsed with 1 μCi ml^{-1} [^{3}H]-thymidine during the last 8 h of culture. Cells were washed once with PBS, and twice with ice-cold 5% trichloroacetic acid (TCA) to remove the unincorporated [^{3}H]-thymidine, then solubilized in 100 μl 0.25 N NaOH in 0.1% SDS and neutralized. Aliquots of samples were added to 10 ml of scintillation fluid and counted (Aloka 703, Tokyo, Japan).

Determination of cell number

Subconfluent HCASMCs in 24-well culture plates were made quiescent by placing them in serum-free medium supplemented with insulin (1 μM) and transferrin (5 mg ml^{-1}) for 2 days. Mitogenic stimulation of HCASMCs was performed as for [^{3}H]-thymidine uptake. Cell numbers were determined at indicated time points. Monolayers were washed twice in PBS, and HCASMCs were trypsinized for 20 min at 37°C and transferred into 10 ml of MCDB131 medium for counting in a Coulter counter (Coulter Electronics, Krefeld, Germany). For each value, three wells were counted twice.

Protein extraction for protein kinase assay

Confluent HCASMCs in 35-mm tissue culture dishes were made quiescent by placing them in serum-free medium supplemented with insulin (1 μM) and transferrin (5 mg ml^{-1}) for 2 days and harvested after stimulation with ET-1 (1-31) or ET-1 at indicated time points in 100 μl lysis buffer (20 mM HEPES (pH 7.2), 25 mM NaCl, 2 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 0.2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 60 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin, and 0.1% Triton X-100). After incubation at 4°C for 30 min, the samples of HCASMC protein extract were sonicated (SONIFIER 250, Branson Ultrasonics Co., Danbury, U.S.A.) on ice for 1 min, and centrifuged at $10{,}000 \times g$ at 4°C for 30 min. The protein concentrations of the supernatants were measured with a protein assay kit (PIERCE) and stored at −80°C until protein kinase assay.

Measurement of ERK activity in HCASMCs

The assay of ERK activity was performed using an in-gel kinase method as described (Kim *et al*., 1997; Hamaguchi *et al*., 1998). Myelin basic protein (MBP) was used as ERK substrate. The samples of protein extracts (10 μg), prepared as described above, were boiled for 5 min in Laemmli sample buffer, and electrophoresed on SDS-polyacrylamide (12%) gels polymerized in the presence of 0.5 mg ml^{-1} of MBP. After electrophoresis, SDS was removed by incubation in 50 mM Tris-HCl (pH 8.0) containing 20% isopropanol for 1 h. The gels were then washed with 5 mM β-mercaptoethanol in 50 mM Tris-HCl (pH 8.0) for 1 h. To denature the protein, gels were incubated in 50 mM Tris-HCl (pH 8.0) containing 6 M guanidine-HCl and 5 mM β-mercaptoethanol for 1 h. Proteins were then renatured by incubation in 50 mM Tris-HCl (pH 8.0) containing 0.04% Tween-40 and 5 mM β-mercaptoethanol at 4°C for 12 h. To measure the ability for phosphorylation of MBP, gels were equilibrated for 1 h in kinase buffer (40 mM HEPES (pH 7.5), 0.1 mM EGTA, 20 mM $MgCl_2$, and 2 mM DTT), and then incubated at 25°C for 1 h in kinase buffer with 25 μM adenosine triphosphate (ATP) and 25 μCi [γ-^{32}P]ATP. Finally, the gels were washed extensively in 5% TCA and 1% sodium pyrophosphate several times and subjected to autoradiography. To estimate the phosphorylation of MBP, we digitized autoradiograms and measured their densities by using a bioimaging analyser (BAS-2000, Fuji photo film Co., Tokyo, Japan).

Western blot analysis

By using rabbit polyclonal ERK antibodies (polyclonal rabbit anti-ERK1 (p44ERK) IgG (c-16); polyclonal rabbit anti-ERK2 (p42ERK) IgG (c-14)), we measured ERK proteins in HCASMCs with Western blot analysis as described previously (Kim *et al*., 1998). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). HCASMC protein extract (10 μg protein), prepared as described above, were boiled for 5 min in Laemmli sample buffer, then electrophoresed on a SDS polyacrylamide gel (12%), and the separated proteins were electrophoretically transferred to Hybond-PVDF membranes (Amersham, Buckinghamshire, U.K.). Complete protein transfer to the membrane was ensured by staining the gels with Coommasie Blue. Non-specific background was blocked by incubating the membrane with 5% bovine serum albumin in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T) at 4°C overnight. The membrane was then incubated with specific ERK1 and ERK2 antibodies (1 : 3000 dilution) for 1 h at room temperature, washed four times with TBS-T, and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham, U.K.) at a dilution of 1 : 5000 in TBS-T. After a further washing with TBS-T, the membrane was treated with ECL reagent (Amersham, U.K.), and chemiluminescence was detected by exposure to Hyperfilm-ECL. The intensity of the bands was measured by Macintosh LC-III computer with an optical scanner (EPSON GT-8000, Seiko, Tokyo, Japan), using the public domain NIH Image program.

Gel mobility shift assay

For gel mobility shift assay, nuclear protein extracts were prepared from HCASMCs in 100-mm dishes after stimulation with ET-1 (1-31) or ET-1 at indicated time points. The samples were homogenized in 0.4 ml of 20 mM HEPES (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 20% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.2 mM DTT, 20 mM β-glycerophosphate, 0.5 mM PMSF, 60 μg ml^{-1} aprotinin, and 2 μg ml^{-1} leupeptin, incubated on ice for 15 min, and centrifuged at 15,000 r.p.m. at 4°C for 10 min. The resulting supernatant was assayed for protein concentrations and stored at −80°C until use. The procedure for gel mobility shift assay has been described previously (Kim *et al*., 1998; Hamaguchi *et al*., 1998). In brief, gel mobility shift assay of HCASMC nuclear AP-1 binding activity was performed with an oligonucleotide probe containing the AP-1 binding sequence (5′-CGCTTGATGACTCAGCCGGAA-3′) (Lee *et al*., 1987). The probe was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and purified by chromatography on a Bio-Spin column (Bio-Rad, Hercules, CA U.S.A.). For DNA-protein-binding reaction, the samples of HCASMC nuclear protein extract (10 μg protein) were incubated with 10 fmol of a ^{32}P-labeled oligonucleotide containing the consensus AP-1 binding site at room temperature for 20 min, in 20 μl of binding buffer consisting of 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 0.3 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 6% glycerol, and 2 μg of polydeoxyinosinic-deoxycytidylic acid (poly[dI-dC]; Pharmacia Biotech, Uppsala, Sweden) as a nonspecific competitor. The DNA-protein complexes were separated from free DNA probe by electrophoresis on 4% nondenaturing polyacrylamide gels in 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 0.1 mM EDTA, and 2.5% glycerol. Gels were run at 200 V at 4°C for 3 h, dried, subjected to autoradiography and analysed with a bioimaging analyser (BAS-2000).

To demonstrate the specificity of DNA-protein binding, binding reactions were performed as described above, in the presence of a 10, 50, 100 or 200 fold molar excess of a non-labeled AP-1 consensus oligonucleotide competitor or 200 fold excess of a non-labeled mutant AP-1 oligonucleotide competitor (5′-CGCTTGATGACTTGGCCGGAA-3′), and followed by electrophoresis. Furthermore, to examine the possible contribution of c-Fos or c-Jun to specific AP-1 binding activity, supershift assays were performed with rabbit polyclonal anti-c-Fos IgG raised against amino acids 128–152 of c-Fos and rabbit anti-c-Jun IgG raised against amino acids 247–263 of c-Jun (Santa Cruz Biotechnology). Anti-c-Fos IgG or anti-c-Jun IgG (each 1 μg) was added to samples after the initial binding reaction between arterial protein extracts and ^{32}P-labeled consensus AP-1 oligonucleotide, the reaction was allowed to proceed at room temperature for 1 h and then the samples were subjected to electrophoresis, as described above.

Materials

Human ET-1 and phosphoramidon (*N*-(α-Rhamnopyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-Tryptophan) were obtained from Peptide Institute (Osaka, Japan). ET-1 (1-31) was synthesized by solid-phase procedures at Peptide Institute. BQ123 (Cyclo-(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu-)) and BQ788 (*N*-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) were gifts from Banyu Pharmaceutical Co. (Tsukuba, Japan). [γ-^{32}P]ATP (10 mCi mmol^{-1}) was from Amersham. Phorbol 12-myristate 13-acetate (PMA), staurosporine, genistein, and PD98059 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were commercial products of reagent grade.

Statistics

Data are presented as means ± s.e.mean for three to five separate experiments. One-way analysis of variance was used to determine significance among groups, after which the modified *t*-test with the Bonferroni correction was used for comparison between individual groups. A value of $P<0.05$ was considered to be statistically significant.

Results

ET-1 (1-31) induced proliferation of HCASMCs similar to ET-1

Initial studies were performed to obtain information on the mitogenic effect and concentration-responses of ET-1 (1-31) on HCASMCs relative to ET-1. As shown in Figure 1, ET-1 (1-31) caused an increase in [^{3}H]-thymidine incorporation into the cells in a concentration-dependent manner (100 pM to 1 μM). This effect of ET-1 (1-31) on HCASMCs DNA synthesis was similar to that of ET-1. To determine whether the effect of ET-1 (1-31) is a receptor-mediated phenomenon, we examined the effects of ET receptor antagonists on the increase in [^{3}H]-thymidine incorporation evoked by ET-1 (1-31). Since it has been reported that there are at least two main subtypes of ET receptors, termed A-type (ET_A) and B-type (ET_B) of receptors (Watanabe *et al*., 1989), we examined the effects of a specific ET_A receptor antagonist, BQ123 (Ihara *et al*., 1992), and a specific ET_B receptor antagonist, BQ788 (Ishikawa *et al*., 1994), on the ET-1 (1-31)-induced increase in DNA synthesis. The ET-1 (1-31)-induced increase in [^{3}H]-thymidine uptake was inhibited by 100 nM BQ123, but not by 100 nM BQ788 (Figure 2). Next, to investigate the possibility that the effect of

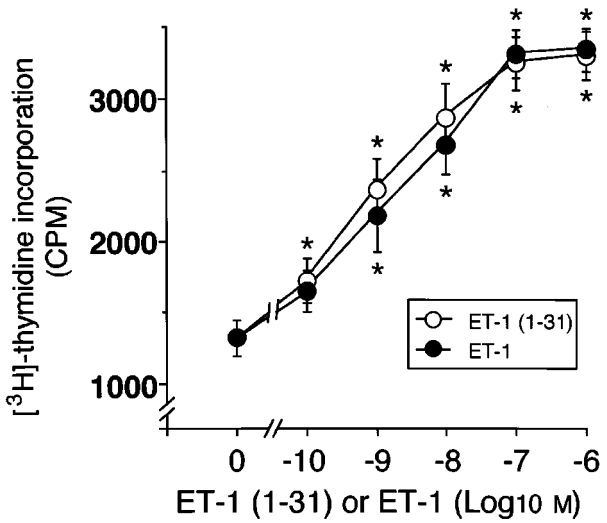

Figure 1 Concentration-response curve for the effect of ET-1 (1-31) or ET-1 on HCASMC DNA synthesis. Confluent cultured HCASMCs were growth arrested and stimulated with either ET-1 (1-31) or ET-1 at the concentrations indicated. [^{3}H]-thymidine incorporation was determined 24 h after stimulation. Radioactivity was precipitated by TCA from HCASMCs incubated under the conditions described in Methods. Values are means ± s.e.mean of five separate experiments. Significances were calculated comparing unstimulated HCASMCs to the samples incubated with ET-1 (1-31) or ET-1. *$P<0.05$.

ET-1 (1-31) is due to further degradation of ET-1 (1-31) to ET-1 by ECE in the medium or in the cells, we examined the effect of an inhibitor of ECE, phosphoramidon (Matsumura *et al*., 1991), on the ET-1 (1-31)-induced increase in [^{3}H]-thymidine uptake. The increase in [^{3}H]-thymidine uptake evoked by ET-1 (1-31) was not affected by 1 μM phosphoramidon (Figure 2). Counts of HCASMCs incubated with ET-1 (1-31) (100 nM) or ET-1 (100 nM) were determined on days 1, 3 and 5 after stimulation (Figure 3). ET-1 (1-31) led to a 1.54 fold increase in the HCASMC count on day 5 which was similar in extent to the effect of ET-1 at the same concentration. This roughly correlated with the results of DNA synthesis (Figures 1 and 3).

Time course of ERK activity in HCASMCs after ET-1 (1-31) stimulation

As indicated by the autoradiograms in Figure 4, ERKs in HCASMCs were composed of two isoforms, ERK1 (p44ERK) and ERK2 (p42ERK). After stimulation by ET-1 (1-31), ERK1 and ERK2 activities rapidly increased by 6.88 and 6.56 fold ($P<0.05$), respectively, at 5 min and peaked (7.28 and 7.15 fold, respectively; $P<0.05$) at 10 min (Figure 4A). Thereafter, the activities of both ERKs rapidly declined, returning to the baseline control value at 60 min after stimulation. The time course for the ERK activation induced by ET-1 was similar to that by ET-1 (1-31) (Figure 4B).

Concentration-response curve for ET-1 (1-31)-induced ERK activation in HCASMCs

We next examined ERK activation at different concentrations of ET-1 (1-31) by using the time points of maximum response for ERKs (10 min) and compared it to that of ET-1. As shown in Figure 5A, ET-1 (1-31) caused an increase in ERK activity in a concentration-dependent manner (from 100 pM to 1 μM). The concentration-response curve for ERK activation induced by ET-1 was compatible to that induced by ET-1 (1-31) (Figure 5B). These results are similar to the results of DNA synthesis evoked by ET-1 (1-31) and ET-1 (Figures 1 and 5).

Effects of PKC inhibition, MEK inhibition and tyrosine kinase inhibition on ET-1 (1-31)-induced ERK activation

To investigate the involvement of PKC in ET-1 (1-31)-induced ERK activation, the effects of staurosporine, as a PKC inhibitor, and downregulation of PKC by pretreatment with PMA for 24 h were examined. As shown in Figure 6, 1 μM staurosporine or 100 nM pretreatment by PMA strongly inhibited the ET-1 (1-31)-induced ERK activation. PD98059 at 50 μM, a selective inhibitor of MAPK kinase or ERK kinase (MEK) which activates ERKs by phosphorylation on both threonine and tyrosine residues, also inhibited the ERK activation induced by ET-1 (1-31). However, pretreatment with genistein at 10 μM, a chemical inhibitor of tyrosine kinase, had little effect on ET-1 (1-31)-induced ERK activation.

Effect of ET-1 (1-31) on DNA binding activity of AP-1 from HCASMCs

Figure 7 shows DNA binding activities of AP-1 from the nuclear extracts of HCASMCs after 2 h treatment with 100 nM ET-1 (1-31) or ET-1. As shown in the left panel of Figure 7A, the incubation of consensus AP-1 oligonucleotide with HCASMC nuclear extracts resulted in the formation of the broad shift band of AP-1 complexes. This shifted band was found to have specific binding for AP-1, because the addition of unlabeled AP-1 consensus oligonucleotide resulted in a decrease in the formation of AP-1 complexes in a concentration-dependent manner, but the addition of excess amounts of unlabeled mutant AP-1 oligonucleotide did not affect the AP-1 complexes. Furthermore, the addition of anti-c-Fos antibody and anti-c-Jun antibody, to the binding reaction produced

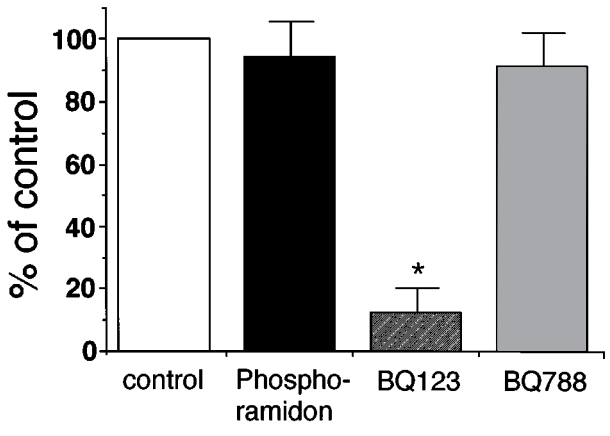

Figure 2 Effects of phosphoramidon (10 μM), BQ123 (100 nM) or BQ788 (100 nM) on ET-1 (1-31)-induced increases in DNA synthesis in HCASMCs. Confluent cultured HCASMCs were growth arrested and stimulated with ET-1 (1-31) in the presence or absence of the indicated drugs. Values are expressed as percentage of control (means$\pm$s.e.mean of five separate experiments). The control value is the radioactivity obtained by the stimulation of 100 nM ET-1 (1-31) for 24 h. Each drug was added to the incubation medium throughout ET-1 (1-31) stimulation in each experiment. *$P<0.05$ from the control value.

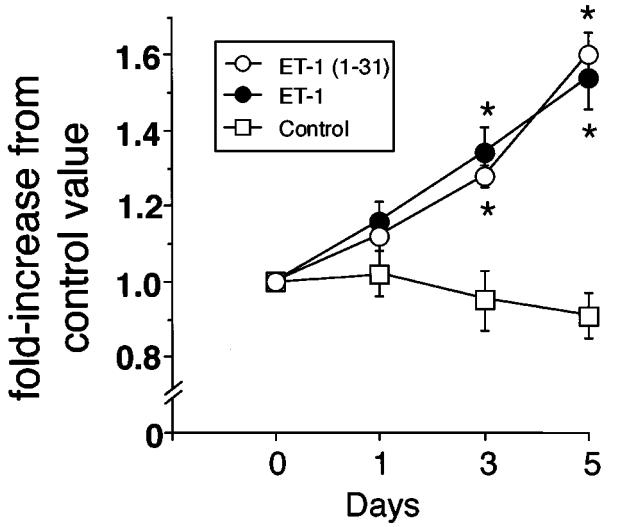

Figure 3 Effects of ET-1 (1-31) or ET-1 on HCASMC proliferation. Subconfluent cultured HCASMCs were stimulated with 100 nM ET-1 (1-31) or ET-1. Cell counts of HCASMCs were determined at days 1, 3 and 5 after stimulation. Data were corrected for replication of unstimulated HCASMCs and the index of cell number indicated as the x fold increase over the baseline values of HCASMCs. Values are means$\pm$s.e.mean of five separate experiments. Significances were calculated comparing unstimulated HCASMCs to the samples incubated with ET-1 (1-31) or ET-1. *$P<0.05$.

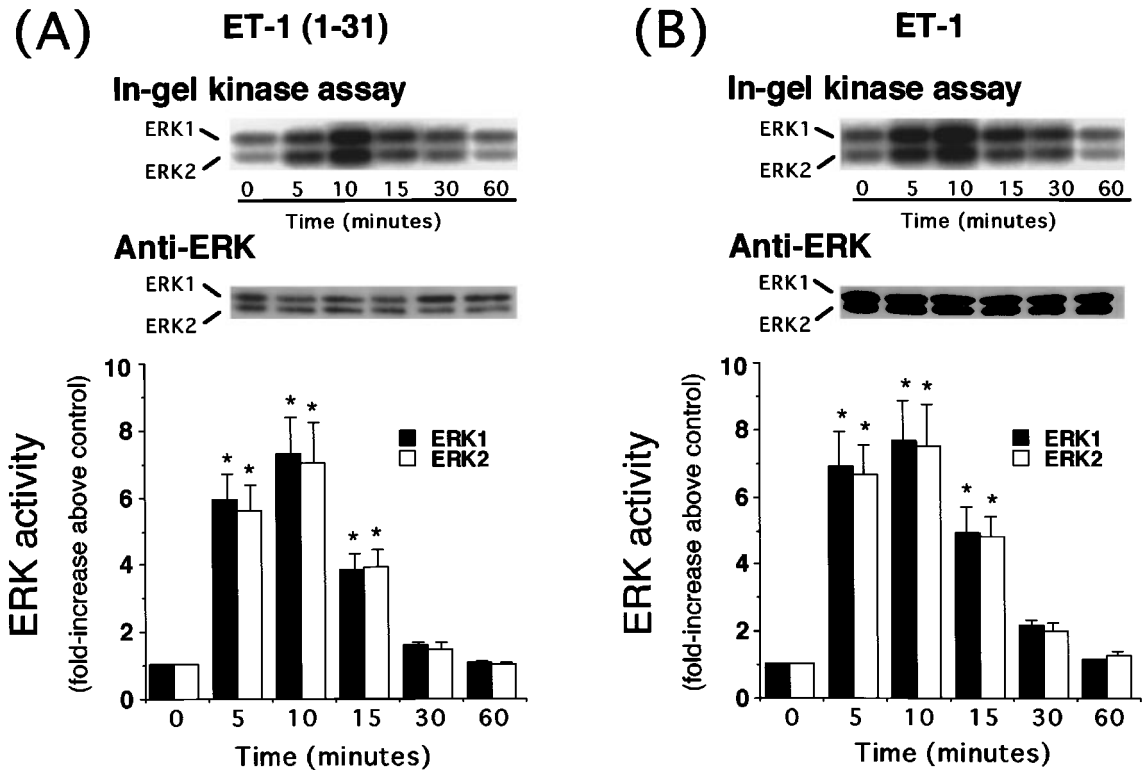

Figure 4 Time course of the induction of ERK1 (p44ERK) and ERK2 (p42ERK) by ET-1 (1-31) (A) or ET-1 (B). Confluent cultured HCASMCs were growth arrested and stimulated with 100 nM ET-1 (1-31) or ET-1 for the times indicated. Upper panels indicate representative autoradiograms showing the activity of ERK1/2 from the samples after stimulation by ET-1 (1-31) (A) or ET-1 (B), determined by in-gel kinase assays as described in Methods. Middle panels are Western blots showing the existence of ERK1/2 in HCASMC extracts using anti-ERK1/2 antibodies. The mean value of ERK activity from non-stimulated cells (0 min) is defined as 1 in the graphs in the lower panels. Values are means ± s.e.mean of three separate experiments. $*P<0.05$ from the control value (0 min).

supershifted complexes. As shown in Figure 7B, ET-1 (1-31) treatment for 2 h increased AP-1 binding activity in HCASMCs by 1.63 fold compared with the control. This increase in AP-1 DNA binding activity by ET-1 (1-31) was almost equipotent to that by ET-1. Moreover, the ET-1 (1-31)-induced increase in AP-1 binding activity was inhibited by co-incubation with 100 nM BQ123, as well as with 1 μM staurosporine or 50 μM PD98059, but not with 100 nM BQ788 (Figure 7B).

Discussion

We have previously found that human mast cell chymase specifically converts big ETs to novel vasoactive 31-amino acid peptides, ETs (1-31), which are longer than the well-known 21-amino acid ETs (Nakano *et al*., 1997). It has also been reported that a serine protease in human lungs hydrolyzes big ET-1 to a fragment of ET-1 (1-31) which has contractile activity in pulmonary artery (Hanson *et al*., 1997). We also found that ET-1 (1-31) contracted rabbit afferent arterioles (Tamaki *et al*., 1997) or increased the intracellular free Ca^{2+} concentration in HCASMCs (Yoshizumi *et al*., 1998).

Recent accumulating evidence has revealed a pathophysiological role for chymase which relates to atherosclerosis (Jeziorska *et al*., 1997; Lindstedt *et al*., 1996). It has been reported that chymase-containing mast cells accumulate in the shoulder region of coronary atheromas and this may be a trigger for atheromatous rapture (Kaartinen *et al*., 1994). In addition, infiltration by mast cells of the ruptured site of coronary atheromas has been observed in patients who died of myocardial infarction (Kovanen *et al*., 1995). These studies suggest a role for human mast cell chymase in atherosclerosis. However, the underlying mechanism which results in chymase-related atherogenesis has not been clarified. Since ET-1 has been suggested to play a role in the pathogenesis of atherosclerosis (Chua *et al*., 1992; Weissberg *et al*., 1990; Komuro *et al*., 1989), ET-1 (1-31), produced by human mast cell chymase, may also be involved. Therefore, we investigated the effect of synthetic ET-1 (1-31) on HCASMC proliferation leading to atherosclerosis.

As shown in Figure 1, the results revealed that the activity of ET-1 (1-31) to promote DNA synthesis in HCASMCs was almost equivalent to that of ET-1. Moreover, ET-1 (1-31) stimulated cell proliferation at 3–5 days incubation similar to ET-1 (Figure 3). These results suggest that ET-1 (1-31) has a potency to induce cell growth as well as ET-1. It is important to elucidate whether the effect of ET-1 (1-31) on HCASMCs is a result of its extracellular conversion to ET-1, or whether ET-1 (1-31) itself acts directly on the cells. As shown in Figure 2, phosphoramidon, an inhibitor of metalloendopeptidases and ECE (Matsumura *et al*., 1991), at a concentration of 1 μM had almost no effect on the increase in [^{3}H]-thymidine incorporation elicited by ET-1 (1-31), although phosphoramidon at the same concentration effectively inhibits the proliferating activity of big ET-1 (data not shown). These results are consistent with the findings in the previous report that ECE requires the C-terminal structure of big ET-1 for enzyme recognition and is

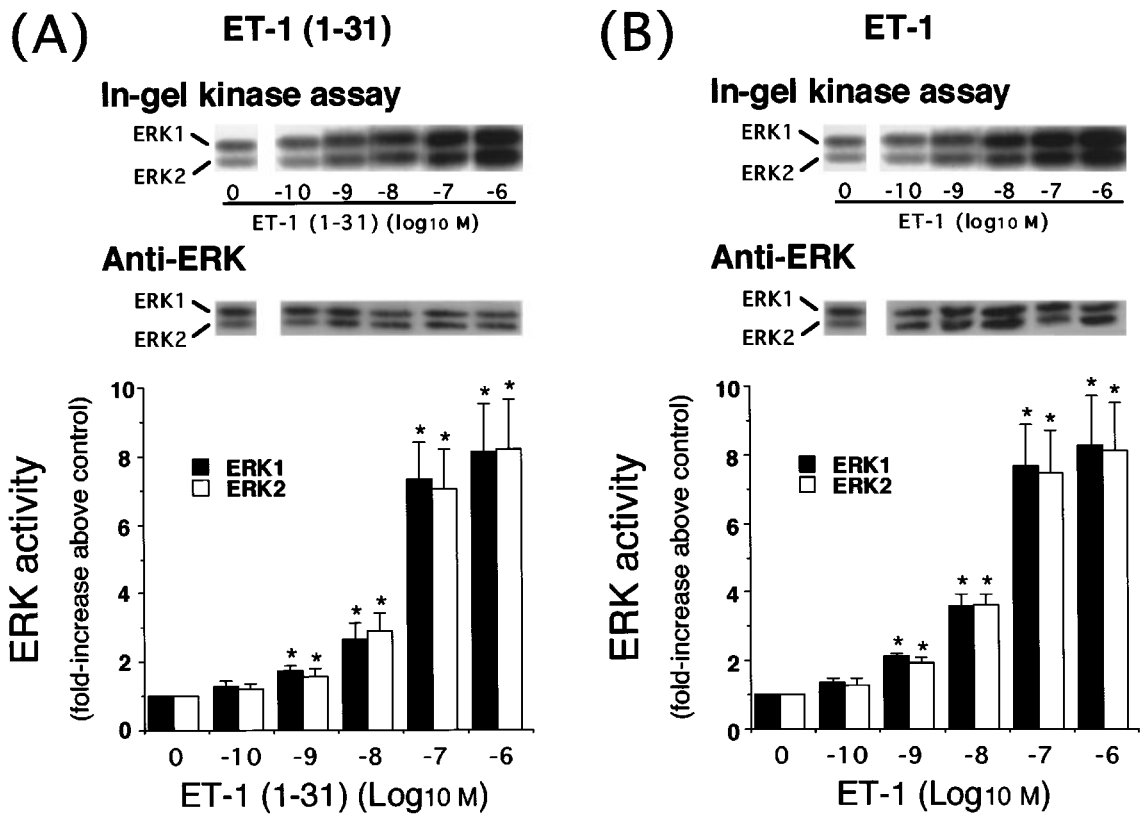

Figure 5 Concentration-response curve for the induction of ERK1 (p44ERK) and ERK2 (p42ERK) by ET-1 (1-31) (A) or ET-1 (B). Confluent cultured HCASMCs were growth arrested and stimulated with 100 nM ET-1 (1-31) or ET-1 for 10 min. Upper panels indicate representative autoradiograms showing the activity of ERK1/2 from the samples after stimulation by ET-1 (1-31) (A) or ET-1 (B), determined by in-gel kinase assays as described in Methods. Middle panels are Western blots showing the existence of ERK1/2 in HCASMC extracts using anti-ERK1/2 antibodies. The mean value of ERK activity from non-stimulated cells (without agonists) is defined as 1 in the graphs in the lower panels. Values are means ± s.e.mean of three separate experiments. $^*P<0.05$ from the control value (without agonists).

not able to cleave ET-1 (1-31) (Xu *et al*., 1994). Taken together, the results indicate that the activity of ET-1 (1-31) to facilitate DNA synthesis is not a consequence of its conversion to ET-1 by ECE or metalloendopeptidase(s). It should be noted that ET-1 (1-31) itself has biological activity in HCASMCs, regardless of whether or not it is degraded to ET-1.

The above findings surmise the hypothesis that ET-1 (1-31) binds to its receptor(s) without further proteolytic degradation and induces a proliferation of HCASMCs. As shown in Figure 2, the cell proliferating effect of ET-1 (1-31) was inhibited by 100 nM of BQ123, but not by 100 nM of BQ788, known inhibitors of ET_A and ET_B receptors, respectively. Although we have no evidence that the receptor of ET-1 (1-31) is identical to that of ET-1, the results suggest that the cell response induced by ET-1 (1-31) is mediated through ET_A or ET_A-like receptors. Further studies are needed to clarify what types of receptors are involved in the ET-1 (1-31)-induced phenomenon.

The intracellular signal transduction pathways activated by ET receptors have been investigated. ETs have been shown to cause an increase in inositol 1,4,5-trisphosphate production (Xuan *et al*., 1994), to mobilize Ca^{2+} from intracellular and extracellular sources (Gardner *et al*., 1992), and to activate protein kinase C (Griendling *et al*., 1989) in VSMCs. In mitogenesis, accumulating evidence shows that the activation of ERKs, belonging to the MAPK family, plays a critical role in the proliferation of a variety of cells induced by ET-1 (Cazaubon *et al*., 1993; Wang *et al*., 1994; Cadwallader *et al*., 1997). However, to our knowledge, there have been no reports concerning the effect of ET-1 on ERKs in HCASMCs. Therefore, we investigated the effect of ET-1 (1-31) on ERK activity in HCASMCs and compared it to that of ET-1. As shown in Figure 4A, application of ET-1 (1-31) to HCASMCs resulted in a rapid and significant activation of ERK1 and ERK2. It should be noted that the potency of ET-1 (1-31) in activating ERKs was similar to that of ET-1 (Figure 5). In our previous studies, ET-1 (1-31) was ten times less potent than ET-1 in tracheal smooth muscle constriction (Nakano *et al*., 1997) and in increasing the intracellular free Ca^{2+} concentration of HCASMCs (Yoshizumi *et al*., 1998). One explanation for the discrepancy in the potencies of ET-1 (1-31) and ET-1 between our previous and present studies, is the existence of specific receptor(s) for ET-1 (1-31). In any way, the potency of ET-1 (1-31) as a mitogen for HCASMCs is more notable than its ability as a vasoconstrictor.

Next, the signaling cascade from the extracellular stimuli to intracellular response induced by ET-1 (1-31) in HCASMCs was examined. A lot of information has been accumulated suggesting that the ET signal transduction pathway leading to ERK activation requires PKC, since depletion of PKC significantly inhibited ERK activation stimulated by ET (Malarkey *et al*., 1995). Our results shown in Figure 6 also revealed that ET-1 (1-31)-induced ERK activation was dependent on PKC because PKC-depletion by 24 h of PMA treatment (100 nM) or pharmacological PKC inhibition by

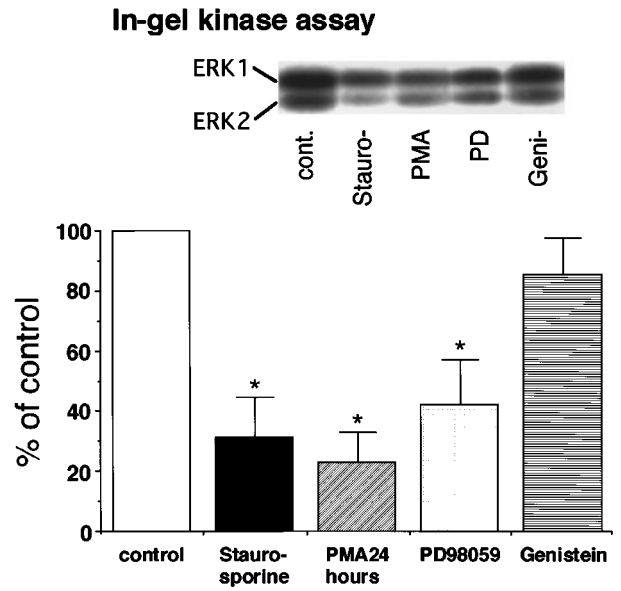

Figure 6 Effects of PKC inhibitor and PKC-downregulation, MAPK kinase or ERK kinase (MEK) inhibitor or tyrosine kinase inhibitor on ERK activation by ET-1 (1-31). Staurosporine (1 μM), PD98059 (50 μM), or genistein (10 μM) were added to the incubation medium 30 min prior to ET-1 (1-31) stimulation. Upper panel indicates representative autoradiograms showing the activity of ERK1/2 from the samples as described in Figures 4 and 5. For PKC depletion from the cells, confluent HCASMCs were treated with 100 nM PMA for 24 h prior to stimulation. Values are expressed as percentage of control (means$\pm$s.e.mean of three separate experiments). The control value is the ERK activity (sum of the activities of ERK1 and ERK2) obtained by the stimulation of 100 nM ET-1 (1-31) for 10 min. *$P<0.05$ from the control value.

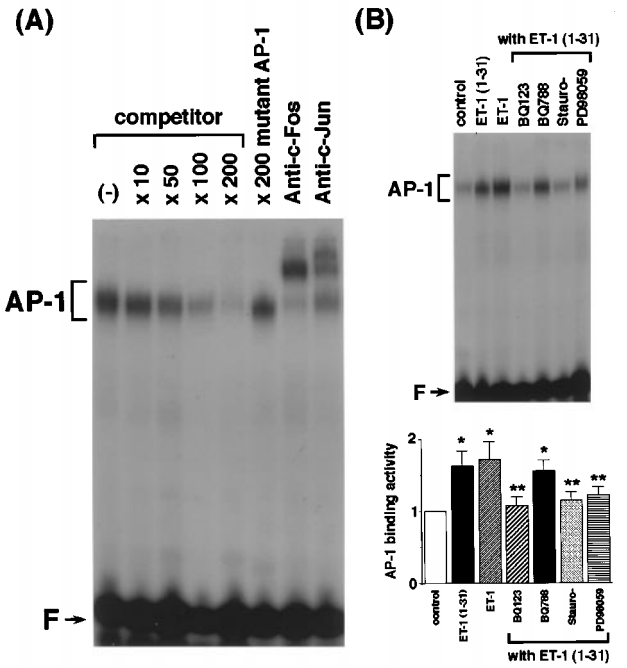

Figure 7 AP-1 DNA binding activity from HCASMC nuclear extracts stimulated with 100 nM ET-1 (1-31) for 2 h. (A) The bracket in the left panel indicates HCASMC nuclear extract AP-1 DNA binding complexes induced by ET-1 (1-31) stimulation. F, free probe. A competition assay for AP-1 was carried out in the presence of a 10-, 50-, 100- and 200 fold molar excess of unlabeled AP-1 oligonucleotide (competitor) or unlabeled 200 fold molar excess of mutant AP-1 oligonucleotide (mutant). Supershift analysis was performed with specific anti-c-Fos and anti-c-Jun antibodies. (B) Effects of ET receptor antagonists, PKC inhibitor or MEK inhibitor on ET-1 (1-31)-induced increase in AP-1 DNA binding activity from HCASMC nuclear extracts. Bar graph shows HCASMC nuclear extracts AP-1 DNA binding activities stimulated with 100 nM ET-1 (1-31) or ET-1 for 2 h. For the experiments of the inhibitors against ET-1 (1-31)-induced increase in AP-1 DNA binding activity, BQ123 (100 nM), BQ788 (100 nM), staurosporine (1 μM), and PD98059 (50 μM) were added to the incubation medium 30 min prior to ET-1 (1-31) stimulation. The control value is the AP-1 DNA binding activity obtained without ET-1 (1-31) or ET-1 which is represented as 1. Each value is expressed as the mean$\pm$s.e.mean of three separate experiments. *$P<0.05$ from the control value. **$P<0.05$ from the value with 100 nM ET-1 (1-31).

staurosporine (1 μM) significantly inhibited it. Other kinds of protein kinases known to be involved in mitogenesis, such as tyrosine kinases, would relate little to ERK activation by ET-1 (1-31), since the well known tyrosine kinase inhibitor genistein (10 μM) inhibited less ERK activation by ET-1 (1-31) than staurosporine (Figure 6). It has been indicated that ERKs are activated by MEK, which exist just upstream of the signal transduction cascade of ERK activation, by phosphorylation on both threonine and tyrosine residues of ERKs (Crews *et al*., 1992). Therefore, we examined the effect of the selective MEK inhibitor, PD98059 (Servant *et al*., 1996) on ET-1 (1-31)-induced ERK activation. As shown in Figure 6, pretreatment with 50 μM PD98059 significantly inhibited the activation. These results are consistent with the notion that MEK activity directly relates to the activation. However, mechanisms other than ERK activation cannot be ruled out because PKC inhibition and MEK inhibition both failed to complete suppression of ET-1 (1-31)-induced ERK activation. Further studies are needed to clarify the intracellular signaling pathway leading to cell proliferation induced by ET-1 (1-31).

It is believed that ERKs play a central role in the formation of transcription factor AP-1 complex (Karin, 1995). ERKs are known to induce c-*fos* mRNA expression by phosphorylating Elk-1/TCF transcription factors (Karin, 1995). Previously, ET-1 treatment has been shown to increase c-*fos* or c-*jun* mRNA in various cell types (Komuro *et al*., 1989; Simonson *et al*., 1992). Therefore, we examined the effect of ET-1 (1-31) on AP-1 DNA binding activity in HCASMCs as well as ET-1. Our present study, using gel shift analysis, provided the first evidence that AP-1 DNA binding activity, which contains c-Fos and c-Jun proteins, significantly increased on the addition of ET-1 (1-31) as well as ET-1 (Figure 7). The present finding is consistent with a report that ET-1 stimulates AP-1 DNA binding activity in rat mesangial cells (Simonson & Herman, 1993). Thus, it is likely that the activation of AP-1 is implicated in the stimulation of HCASMC growth by ET-1 (1-31).

Our present data on gel shift analysis of AP-1 activity demonstrates that the inhibition of ET_A or ET_A-like receptors, as well as the inhibition of PKC or MEK, is associated with the suppression of activation of AP-1 complex (Figure 7B). These findings suggest that the suppression of AP-1 activity by ET_A receptor antagonist is at least in part mediated by the suppression of ERK activation and that ERKs are responsible for AP-1 activation in HCASMCs, although the present work provided no direct evidence of this.

In conclusion, ET-1 (1-31) is a novel putative vasoactive peptide of the ET family that may be deeply involved in chymase-related pathophysiological processes in humans. We obtained the first evidence that ET-1 (1-31) is equipotent to ET-1 in HCASMC proliferation. The intracellular signal transduction cascade induced by ET-1 (1-31) may be explained in part by PKC-dependent activation of ERKs, a main

subgroup of the MAPK family, which is associated with the activation of transcription factor AP-1 complex. The activation of ERKs by ET-1 (1-31) may be mediated by ET_A or ET_A-like receptors. Since chymase plays a significant role in foam cell formation in human coronary atheromas (Kaartinen *et al*., 1994; Kovanen *et al*., 1995), the pathogenesis of atherosclerosis in coronary artery may be partly attributable to the effect of bioactive ET derivatives, including ET-1 (1-31), an endogenous product of human mast cell chymase.

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