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Effect of Endothelin-1 (1 – 31) on Human Mesangial Cell Proliferation

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ABSTRACT—It was previously found that human chymase cleaves big endothelins (ETs) at the Tyr^{31}-Gly^{32} bond and produces 31-amino acid ETs (1 – 31). In the present study, human plasma concentrations of ET-1 (1 – 31) and ET-1 were examined and the effect of synthetic ET-1 (1 – 31) on the proliferation of cultured human mesangial cells (HMCs) was investigated. The proliferative effect of ET-1 (1 – 31) was evaluated from the [^{3}H]-thymidine uptake. The activity of extracellular signal-regulated kinase (ERK) and DNA binding activity of activator protein-1 were determined by using an in-gel kinase assay and gel mobility shift assay, respectively. Immunoreactive ET-1 (1 – 31) was detectable in plasma, but the level was slightly lower than that of ET-1. ET-1 (1 – 31) increased [^{3}H]-thymidine incorporation in HMCs to a degree similar to that induced by ET-1. ET-1 (1 – 31) also activated ERK1/2. Inhibition of protein kinase C and ERK kinase caused a reduction of ET-1 (1 – 31)-induced ERK1/2 activation. The ERK1/2 activation was followed by an increase in transcription factor activator protein-1 DNA binding activity. These findings suggest that ET-1 (1 – 31) is a bioactive peptide in humans and ET-1 (1 – 31) itself stimulates HMC proliferation.

Keywords: Endothelin-1 (1 – 31), Human chymase, Extracellular signal-regulated kinase, Protein kinase C

Various vasoactive substances are known to cause a contraction or a relaxation of glomerular mesangial cells (MCs), which are one of the regulators of glomerular filtration (1 – 4). Of these substances, endothelin-1 (ET-1) is the most potent vasoconstrictor (5), which also induces contraction of MCs (6, 7) as well as their proliferation (3, 8, 9). It has also been reported that ET-1 increased production of extracellular matrix or inflammatory cytokines in the glomeruli (8, 10) and that ET-1 may be involved in the pathogenesis of glomerulonephritis. 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) (11).

It was recently reported 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 (12). ET-1 (1 – 31) has been shown to cause contraction of rabbit afferent and efferent arterioles (13) as well as causing an increase in the intracellular free Ca^{2+} concentration of human mesangial cells (HMCs) (14). It was reported that ET-1 (1 – 31) itself stimulated human coronary artery smooth muscle cell proliferation (15). Human chymase was originally shown to be an alternative angiotensin II-forming enzyme in cardiovascular tissues (16), recent studies suggest the existence of angiotensin-converting enzyme-independent angiotensin II generation in the kidney (17). It has also been reported that chymase exists in the human renal cortex, as detected from an enzymatic activity measurement (18). From these findings, it is hypothesized that ET-1 (1 – 31), which is generated by human chymase, may be an alternative mitogen for HMCs as well as angiotensin II.

After developing the enzyme immunoassay for ETs (1 – 31) (19), plasma concentrations of ET-1 (1 – 31) and ET-1 in healthy young volunteers were examined in the present study. Upon confirming that plasma concentrations of ET-1 (1 – 31) were similar to that of ET-1, the effect of synthetic ET-1 (1 – 31) on cultured HMC proliferation was examined.

MATERIALS AND METHODS

Materials

Human ET-1 and phosphoramidon (*N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-tryptophan) were

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obtained from the Peptide Institute (Osaka). ET-1 (1 – 31) was synthesizd using solid-phase procedures at the Peptide Institute. BQ123 (cyclo-(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu-)) (20) and BQ788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) (21) were from Banyu Pharmaceutical Co. (Tsukuba). [γ-^{32}P]ATP (10 mCi/mmol) was from Amersham (Tokyo). Staurosporine, genistein and PD98059 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were commercial products of reagent grade.

Measurements of plasma ET-1 (1 – 31) and ET-1

Blood samples were obtained from young, healthy volunteers (age: 25.6 ± 0.8 years, 5 males and 3 females). The blood was immediately transferred to a tube containing proteinase inhibitor cocktail (20 μM leupeptin, 20 μM benzamidine, 100 μM phenylmethylsulfonylfluoride, 20 μM chymostatin) and centrifuged at 4°C for 10 min. Plasma was separated at 4°C and kept at −80°C until assay.

Extraction was performed by absorption on SepPak C18 cartridges (Waters, Milford, MA, USA). SepPak C18 cartridges were preactivated by successive washes with 8 ml of ethanol, 8 ml of distilled water and 8 ml of 0.1% trifluoroacetic acid (TFA). A 2-ml aliquot of the plasma sample acidified with 6 ml of 10% acetic acid was then applied to the column. The columns were washed with 9 ml of distilled water and the absorbed materials were eluted with 6 ml of 60% acetonitrile/0.1% TFA. The eluate was dried using a SpeedVak concentrator and redissolved in 160 μl of dimethyl sulfoxide. The redissolved sample was diluted with 640 μl of assay phosphate-buffered saline (containing 1% BSA and 0.05% Tween 20). The reconstituted plasma samples (100 μl) were analyzed in duplicate. The enzyme immunoassay of the reconstituted plasma ETs was performed with a sandwich enzyme immunoassay kit for ET-1 (1 – 31) and ET-1 using specific antibodies for ET-1 (1 – 31) and ET-1 as described previously (Immuno-Biological Laboratories Co., Fujioka) (19). The detection limit of the assay was considered to be 0.1 pg/ml or higher, and no cross reactivity was observed between ET-1 (1 – 31) and ET-1 antibodies. The recovery rates of ET-1 (1 – 31) and ET-1, analyzed by the addition of internal ET derivatives, were 81.9 ± 14.2% and 87.6 ± 11.8%, respectively.

Cell preparation and culture

Human mesangial cells (HMCs) at passage 3 were obtained as a commercially available product from Clonetics Corp. (San Diego, CA, USA). Cells were plated in 25-cm^2 tissue culture flasks at a density of 5×10^3 cells/cm^2 in CCMD180 medium supplemented with 5% heat-inactivated fetal calf serum and 50 μg/ml gentamicin. 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, USA) for experiments on [^{3}H]-thymidine incorporation. For experiments involving in-gel kinase assay or gel mobility shift assay, the cells were seeded onto 35-mm and 100-mm tissue culture dishes (Coster Corp.), respectively. All experiments were performed with the cells from passage 5 – 15 and at 24 – 48 h post-confluency except for [^{3}H]-thymidine incorporation.

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

Subconfluent HMCs in 24-well culture plates were made quiescent by placing them in serum-free medium supplemented with insulin (1 μM) and transferrin (5 μg/ml) for 48 h. They were then stimulated for 24 h with 100 pM to 100 nM of ET-1 (1 – 31) or ET-1. The stimulated cells were pulsed with 1 μCi/ml [^{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, and then they were 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).

Protein extraction for protein kinase assay

Confluent HMCs in 35-mm tissue culture dishes were made quiescent by placing them in serum-free medium supplemented with insulin (1 μM) and transferrin (5 μg/ml) for 48 h and harvested after stimulation with ET-1 (1 – 31) or ET-1 at the 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 PMSF, 60 μg/ml aprotinin, 2 μg/ml leupeptin and 0.1% Triton X-100). After incubation at 4°C for 30 min, the samples of HMC protein extract were sonicated (SONIFIER 250; Branson Ultrasonics Co., Danbury, UK) on ice for 1 min and then centrifuged at 10,000 × *g* at 4°C for 30 min. The protein concentrations of the supernatants were measured using a protein assay kit (Pierce, Rockford, IL, USA) and stored at −80°C until the protein kinase assay.

Measurement of ERK activity in HMCs

The assay of ERK activity was performed using an in-gel kinase method as previously described (22, 23). Myelin basic protein (MBP) was used as an ERK substrate. The samples of protein extracts (10 μg), prepared as described above, were boiled for 5 min in Laemmli sample buffer and then electrophoresed on SDS-polyacrylamide (12%) gels polymerized in the presence of 0.5 mg/ml 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, autoradiograms were digitized and their densities were measured using a bioimaging analyzer (BAS-1500; Fuji Photo Film Co., Tokyo).

Western blot analysis

Using rabbit polyclonal ERK antibodies (polyclonal rabbit anti-ERK1 (p44ERK) IgG (c-16) and polyclonal rabbit anti-ERK2 (p42ERK) IgG (c-14)), ERK proteins in HMCs were measured with Western blot analysis as described previously (24). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HMC protein extract (10 μg protein), prepared as described above, was boiled for 5 min in Laemmli sample buffer then electrophoresed on a SDS polyacrylamide gel (12%); the separated proteins were electrophoretically transferred to Hybond-PVDF membranes (Amersham Life Sciences, Buckinghamshire, England). Complete protein transfer to the membrane was verified 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 4 times with TBS-T, and then incubated with horseraddish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham) at a dilution of 1:5000 in TBS-T. After a further washing with TBS-T, the membrane was treated with ECL reagent (Amersham), and chemiluminescence was detected by exposure to Hyperfilm-ECL. The intensity of the bands was measured using a Macintosh LC-III computer with an optical scanner (EPSON GT-8000; Seiko, Tokyo), using the public domain NIH Image program.

Gel mobility shift assay

For gel mobility shift assay, nuclear protein extracts were prepared from HMCs in 100-mm dishes after stimulation with ET-1 (1–31) or ET-1 at the 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 aprotinin, and 2 μg/ml leupeptin; incubated on ice for 15 min; and then centrifuged at 15,000 rpm 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 (23, 24). In brief, the gel mobility shift assay of HMC nuclear AP-1 binding activity was performed with an oligonucleotide probe containing the AP-1 binding sequence (5′-CGCTTGA TGACTCA GCCGGAA-3′) (25). 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, Richmond, CA, USA). For the DNA-protein-binding reaction, the samples of HMC 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 using 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 analyzed with a bioimaging analyzer (BAS-1500).

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′-CGCTTGA TGACTTG GCC GGAA-3′), 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, Inc.). Anti-c-Fos IgG or anti-c-Jun IgG (each 1 μg) was added to samples after the initial binding reaction between MC nuclear 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.

Statistics

Data are presented as means ± S.D. for 3 – 5 separate experiments. One-way analysis of variance was used to determine significance among groups, after which the modified *t*-test with the Bonferroni correction were used for comparison between individual groups. A value at $P<0.05$ was considered to be significant.

RESULTS

Plasma concentrations of ET-1 (1 – 31) and ET-1

Plasma concentrations of immunoreactive ET-1 (1 – 31) and ET-1 in healthy young volunteers were 0.47 ± 0.06 pM and 0.81 ± 0.06 pM (n = 8), respectively. Plasma concentrations of immunoreactive ET-1 (1 – 31) was slightly lower than that of ET-1.

Effect of ET-1 (1 – 31) on HMCs proliferation and comparison with that of ET-1

As shown in Fig. 1, ET-1 (1 – 31) caused an increase in [^{3}H]-thymidine incorporation into the cells in a concentration-dependent manner (100 pM to 100 nM). Although this effect of ET-1 (1 – 31) on HMCs DNA synthesis was slightly less potent than that of ET-1, ET-1 (1 – 31) at concentrations greater than 1 nM induced significant increase in [^{3}H]-thymidine uptake. 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) receptors (26), we examined the effects of a specific ET_A receptor antagonist, BQ123 (20), and a specific ET_B receptor antagonist, BQ788 (21), on the ET-1

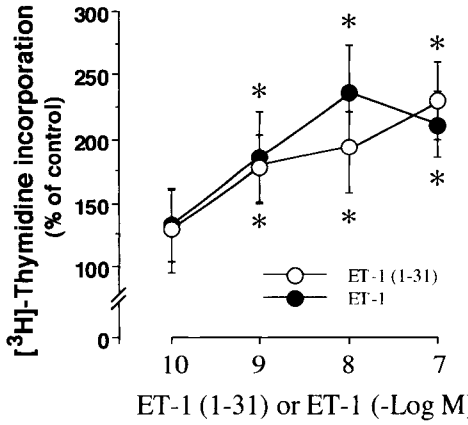

Fig. 1. Concentration-response curve for the effect of ET-1 (1 – 31) or ET-1 on HMC DNA synthesis. Subconfluent cultured HMCs were growth arrested and stimulated with either ET-1 (1 – 31) (○) or ET-1 (●) for 24 h at the concentrations indicated. Values are expressed as a percentage of the unstimulated control value (1,050 ± 103 cpm) and are means ± S.D. of 5 separate experiments. Significances were calculated comparing unstimulated HMCs with the samples incubated with ET-1 (1 – 31) or ET-1. *$P<0.05$.

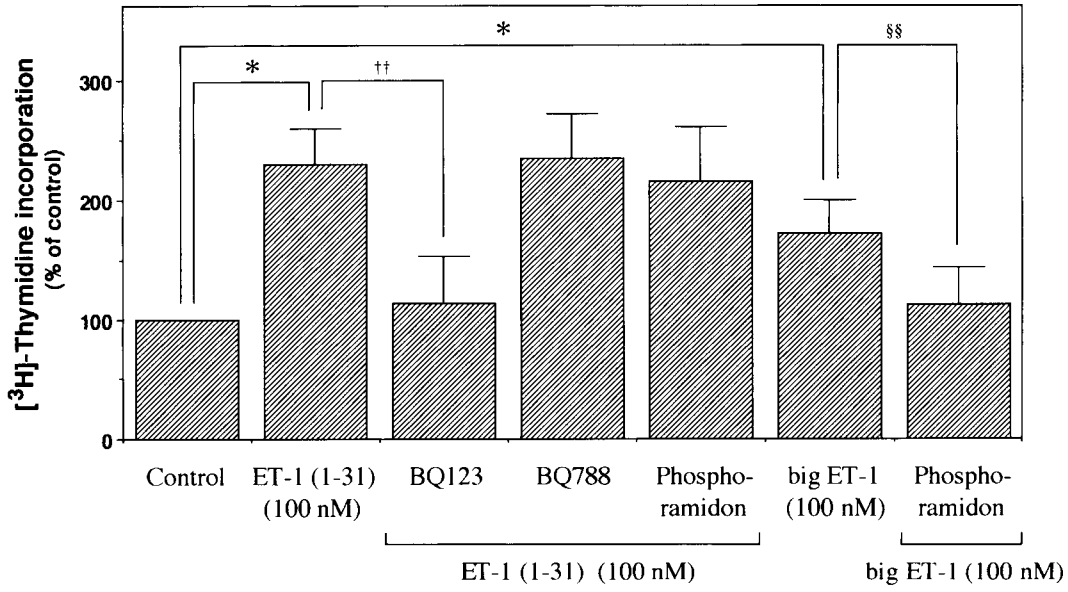

Fig. 2. Effects of phosphoramidon (10 μM), BQ123 (100 nM) and BQ788 (100 nM) on ET-1 (1 – 31) or big ET-1-induced increases in DNA synthesis in HMCs. Values are expressed as a % of the control (means ± S.D. of 5 separate experiments). The control value is the radioactivity obtained without agonists stimulation (1,050 ± 103 cpm). Each drug was added to the incubation medium throughout agonist stimulation (24 h) in each experiment. *$P<0.05$, compared to the control value. ††$P<0.05$ and §§$P<0.05$ were calculated from the values of [^{3}H]-thymidine incorporation induced by 100 nM ET-1 or big ET-1, respectively.

(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 (Fig. 2). Next, to investigate the possibility that the effect of ET-1 (1 – 31) was due to further degradation of ET-1 (1 – 31) to ET-1 by ECE in the medium or in the cells, the effect of an inhibitor of ECE, phosphoramidon (27), on the ET-1 (1 – 31)-induced increase in [^{3}H]-thymidine uptake was examined. The increase in [^{3}H]-thymidine uptake evoked by ET-1 (1 – 31) was not affected by 10 μM phosphoramidon, whereas the big ET-1-induced increase in [^{3}H]-thymidine uptake was significantly inhibited by phosphoramidon (Fig. 2).

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

As indicated by the autoradiograms in Fig. 3, ERKs in HMCs were composed of two isoforms, ERK1 (p44ERK) and ERK2 (p42ERK). After stimulation by 100 nM of ET-1 (1 – 31), ERK activities (sum of the activities of ERK1 and ERK2) rapidly increased 2.45-fold ($P<0.05$) at 5 min and peaked (3.26-fold, $P<0.05$) at 10 min (Fig. 3A). Thereafter, the activities of both ERKs rapidly declined, returning to the baseline control value 30 min after stimulation. The time course for the ERK activation induced by equimolar ET-1 was similar to that by ET-1 (1 – 31) (Fig. 3B).

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

ERK activation at different concentrations of ET-1 (1 – 31) were then examined using the time points of maximum response for ERKs (10 min) and compared with that of ET-1. As shown in Fig. 4A, ET-1 (1 – 31) caused an increase in ERK activity in a concentration-dependent manner (from 100 pM to 100 nM). The concentration-response curve for ERK activation induced by ET-1 was slightly more potent than that induced by ET-1 (1 – 31) (Fig. 4B). These results are similar to the results of DNA synthesis evoked by ET-1 (1 – 31) and ET-1 (Figs. 1 and 4).

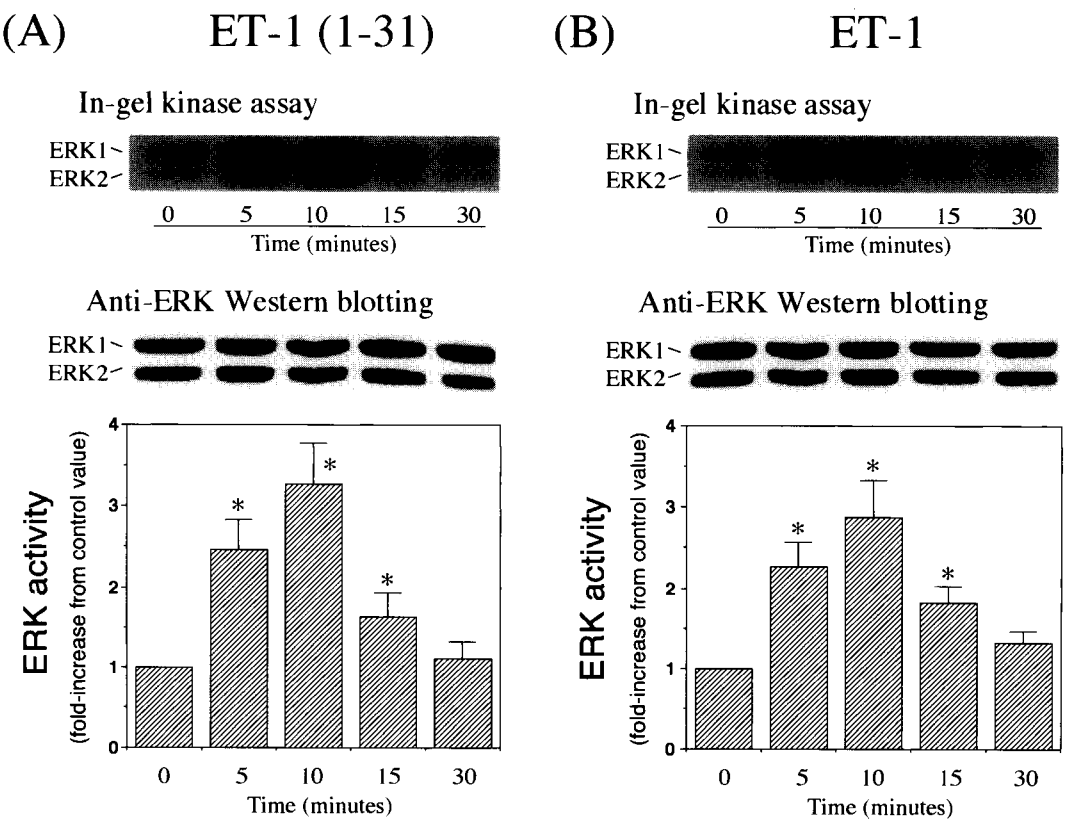

Fig. 3. Time courses of the induction of ERK1 (p44ERK) and ERK2 (p42ERK) by ET-1 (1 – 31) (A) or ET-1 (B). Cells were stimulated with 100 nM ET-1 (1 – 31) or ET-1 for the times indicated. Upper panels indicate representative autoradiograms showing the activity of ERK1/2 from the samples after stimulation by ET-1 (1 – 31) (A) or ET-1 (B), determined by in-gel kinase assays as described in Materials and Methods. Middle panels are Western blots showing the existence of ERK1/2 in HMC extracts using anti-ERK1/2 antibodies. The mean value of ERK activity (sum of the activities of ERK1 and ERK2) from non-stimulated cells (0 min) is defined as 1 in the graphs in the lower panels. Values are means ± S.D. of three separate experiments. *$P<0.05$, compared to the control value (0 min).

Effects of protein kinase C inhibition, tyrosine kinase inhibition, MEK inhibition, ET receptor antagonists and phosphoramidon on ET-1 (1 – 31)-induced ERK activation

To investigate the involvement of protein kinase C (PKC) in ET-1 (1 – 31)-induced ERK activation, the effects of staurosporine, a PKC inhibitor, were examined. As shown in Fig. 5, 1 μM staurosporine strongly inhibited the ET-1 (1 – 31)-induced ERK activation. However, pretreatment with genistein at 10 μM, a chemical inhibitor of tyrosine kinase, had little effect on 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 (28), also inhibited the ERK activation induced by ET-1 (1 – 31). It was also observed that ET-1 (1 – 31)-induced ERK activation was inhibited by BQ123, but not by BQ788 and phosphoramidon (Fig. 5). These results are consistent with the results of [^{3}H]-thymidine uptake as shown in Fig. 2.

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

Figure 6 shows DNA binding activities of AP-1 from the nuclear extracts of HMCs after 2 h treatment with 100 nM ET-1 (1 – 31) or ET-1. As shown in the left panel of Fig. 6A, the incubation of consensus AP-1 oligonucleotide with HMC 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 supershifted complexes. As shown in Fig. 6B, ET-1 (1 – 31) treatment for 2 h increased AP-1 binding activity in HMCs 1.72-fold compared with the control. This increase in AP-1 DNA binding activity by ET-1 (1 – 31) was almost comparable with that by ET-1.

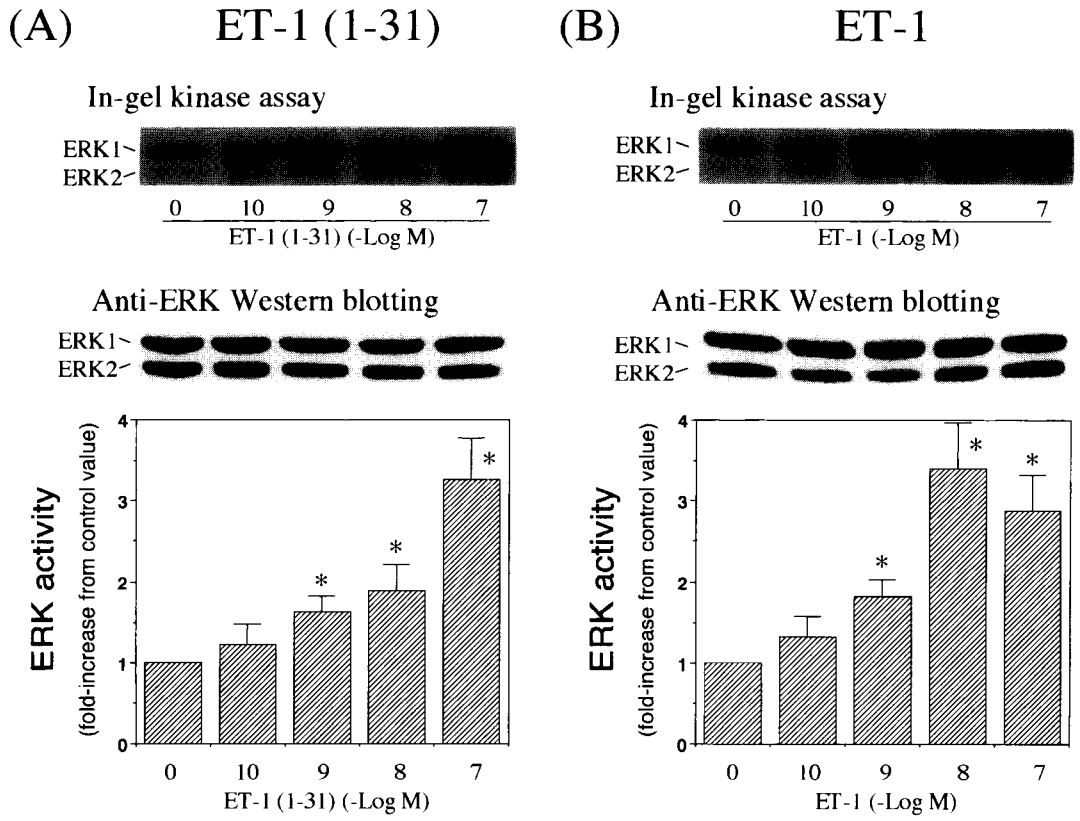

Fig. 4. Concentration-response curves for the induction of ERK1 (p44ERK) and ERK2 (p42ERK) by ET-1 (1 – 31) (A) or ET-1 (B). Cells were stimulated with ET-1 (1 – 31) or ET-1 for 10 min. Upper panels indicate representative autoradiograms showing the activity of ERK1/2 from the samples after stimulation by ET-1 (1 – 31) (A) or ET-1 (B), determined by in-gel kinase assays as described in Materials and Methods. Middle panels are Western blots showing the existence of ERK1/2 in HMC extracts using anti-ERK1/2 antibodies. The mean value of ERK activity (sum of the activities of ERK1 and ERK2) from non-stimulated cells (without agonists) is defined as 1 in the graphs in the lower panels. Values are means ± S.D. of three separate experiments. *P<0.05, compared to the control value (without agonists).

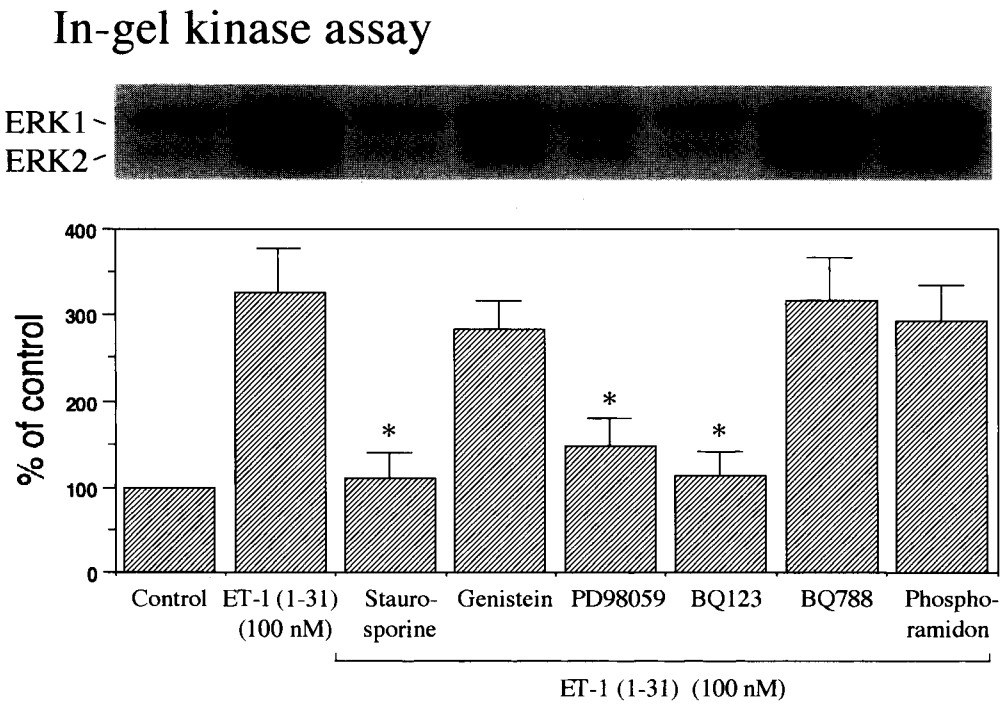

Fig. 5. Effects of PKC inhibitor, tyrosine kinase inhibitor, MAPK kinase or ERK kinase (MEK) inhibitor, ET-receptor antagonists and phosphoramidon on ERK activation by ET-1 (1 – 31). Staurosporine (1 μM), PD98059 (50 μM), genistein (10 μM), BQ123 (100 nM), BQ788 (100 nM) and phosphoramidon (10 μM) were 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 Figs. 4 and 5. Values are expressed as a % of the control (means ± S.D. of three separate experiments). The control value is the ERK activity (sum of the activities of ERK1 and ERK2) obtained without agonist stimulation. *P<0.05, compared to the value obtained by ET-1 (1 – 31) (100 nM) stimulation.

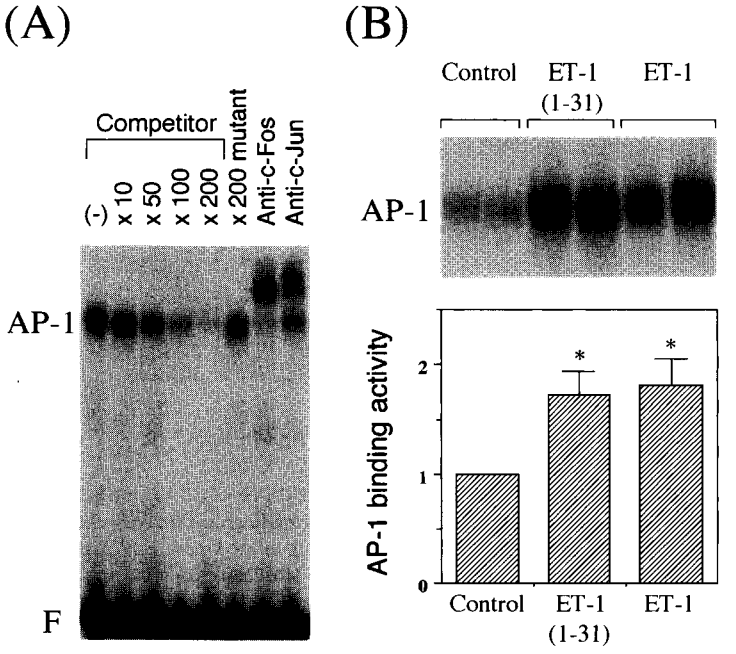

Fig. 6. AP-1 DNA binding activity from HMC nuclear extracts stimulated with 100 nM ET-1 (1 – 31) for 2 h. (A) The bracket in the left panel indicates HMC nuclear extract AP-1 DNA binding complexes induced by ET-1 (1 – 31) stimulation. F, free probe. A competition assay for AP-1 was carried out in the 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) The top panel shows the representative autoradiogram of HMC nuclear extract AP-1 DNA binding complexes induced by 100 nM ET-1 (1 – 31) and ET-1 for 2 h. The bottom panel shows their activities quantified using a bioimaging analyzer, as described in Materials and Methods. 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 means ± S.D. of three separate experiments. *P<0.05, compared to the control value.

DISCUSSION

It was previously reported that human mast cell chymase specifically converted big ETs to novel vasoactive 31-amino acid peptides, ETs (1 – 31) (12). 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 the pulmonary artery (29). It was also found that ET-1 (1 – 31) contracted rabbit afferent and efferent arterioles (13) or increased the intracellular free Ca^{2+} concentration in HMCs (14). In the present study, the presence of ET-1 (1 – 31) in healthy human plasma was confirmed. It was also found that ET-1 (1 – 31) itself stimulates HMC proliferation. These findings strongly suggest that ET-1 (1 – 31) is a novel bioactive peptide in humans.

Accumulating evidence suggests that ET-1, not only causes contraction of MCs (6, 7), but also induces their proliferation (3, 8, 9), which may result in glomerulosclerosis. It has also been reported that ET-1 is an inflammatory mediator and plays a role in the pathogenesis of glomerulonephritis through the production of inflammatory cytokines (10, 30). The cell proliferating activity of ET-1 was implicated in the activation of MAPK, especially ERK in MCs (31, 32). ERK activation was shown to lead to ribosomal S6 kinase phosphorylation (33) and c-Fos and c-Jun phosphorylation (34, 35), which are believed to initiate protein synthesis and cell proliferation. ET-1 (1 – 31), which exists in human plasma in similar quantities to ET-1, may be relevant to glomerulosclerosis or glomerulonephritis through induction of HMC growth similar to ET-1. Therefore, we investigated the effect of synthetic ET-1 (1 – 31) on HMC proliferation in comparison with the effect of ET-1.

As shown in Fig. 1, the findings revealed that the activity of ET-1 (1 – 31) to promote DNA synthesis in HMCs was comparable with that of ET-1. This suggests that ET-1 (1 – 31) has a potency to induce HMC growth similar to ET-1. Since ET-1 has been shown to cause MC proliferation at 1 to 100 nM concentrations (36, 37), ET-1 (1 – 31) may also be a potent mitogen for HMCs. However, the concentrations of ET-1 (1 – 31) used in this study were considered to be higher than those in plasma. As shown in Fig. 2, phosphoramidon, an inhibitor of metalloendopeptidases and ECE (27), at a concentration of 10 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 inhibited the proliferating activity of big ET-1 (Fig. 2). These findings are consistent with the findings of a previous study that ECE requires the C-terminal structure of big ET-1 for enzyme recognition and is not able to cleave ET-1 (1 – 31) (38). Taken together, the findings 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).

The above findings suggest that ET-1 (1 – 31) binds to its receptor(s) without further proteolytic degradation and induces a proliferation of HMCs. As shown in Fig. 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 no evidence shows that the receptor of ET-1 (1 – 31) is identical to that of ET-1, the findings 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 (3, 39), to mobilize Ca^{2+} from intracellular and extracellular sources (40), and to activate PKC (41) in MCs. In mitogenesis, accumulating evidence shows that the activation of ERKs, belonging to the MAPK family, play a critical role in MC proliferation induced by ET-1 (31, 32, 42). Therefore, the effect of ET-1 (1 – 31) on ERK activity in HMCs was investigated and compared with that of ET-1. As shown in Fig. 3A, application of ET-1 (1 – 31) to HMCs resulted in a rapid and significant activation of ERK1 and ERK2. The potency of ET-1 (1 – 31) in activating ERKs was slightly less than that of ET-1 (Fig. 4). However, in a previous study, ET-1 (1 – 31) was almost equipotent in proliferation of human coronary artery smooth muscle cells (15). One explanation for the discrepancy in the potencies of ET-1 (1 – 31) and ET-1 between previous and present studies, is the difference of receptor distribution. In any way, the potency of ET-1 (1 – 31) as a mitogen for HMCs would be significant.

Next, the signaling cascade from the extracellular stimuli to intracellular response induced by ET-1 (1 – 31) in HMCs was examined. Much 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 (37). The present findings, shown in Fig. 5, also revealed that ET-1 (1 – 31)-induced ERK activation was dependent on PKC because pharmacological PKC inhibition by 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 tyrosine kinase inhibitor genistein (10 μM) inhibited ERK activation by ET-1 (1 – 31) less than staurosporine (Fig. 5). However, since it was reported that the inhibition of tyrosine kinase causes reduction of [^{3}H]-thymidine uptake in MCs (37), involvement of tyrosine kinase cannot be completely excluded. Another problem of genistein is the specificity for tyrosine

kinase because it also inhibits cAMP-dependent protein kinase and protein kinase C at higher concentration (43). It has been suggested that ERKs are activated by MEK, which exist just upstream of the signal transduction cascade of ERK activation (28). Therefore, the effect of the selective MEK inhibitor PD98059 on ET-1 (1 – 31)-induced ERK activation was examined. As shown in Fig. 5, pretreatment with 50 μM PD98059 significantly inhibited the activation. These findings are consistent with the theory that MEK activity directly relates to ERK activation. However, since the inhibitors for protein kinases used have a specificity problem, mechanisms other than PKC and MEK for ERK activation cannot be ruled out. Further studies are required to clarify the intracellular signaling pathway leading to cell proliferation induced by ET-1 (1 – 31). It was also observed that ET-1 (1 – 31)-induced ERK activation was inhibited by BQ123, but not by BQ788 (Fig. 5). These results are consistent with the results of [^{3}H]-thymidine incorporation suggesting that ET-1 (1 – 31) stimulation, which is transduced to intracellular ERK activation, is mediated through ET_A or ET_A-like receptors of HMCs.

It has been suggested that ERKs play a central role in the formation of transcription factor AP-1 complex (44). ERKs are known to induce c-fos mRNA expression by phosphorylating Elk-1/TCF transcription factors (44). Previously, ET-1 treatment has been shown to increase c-fos or c-jun mRNA in MCs (36, 45). Therefore, the effect of ET-1 (1 – 31) on AP-1 DNA binding activity in HMCs as well as ET-1 was examined. The present study, using gel shift analysis, provided the first evidence that AP-1 DNA binding activity, which contains c-Fos and c-Jun proteins, was significantly increased by the addition of ET-1 (1 – 31) as well as ET-1 (Fig. 6). The present finding is consistent with a report that ET-1 stimulates AP-1 DNA binding activity in rat mesangial cells (36). Thus, it is likely that the activation of AP-1 is implicated in the stimulation of HMC growth by ET-1 (1 – 31).

In conclusion, immunoreactive ET-1 (1 – 31) was detectable in plasma but the level was slightly lower than that of ET-1 in healthy young humans. ET-1 (1 – 31) is a novel vasoactive peptide of the ET family that may be deeply involved in chymase-related pathophysiological processes in humans. This is the first reported evidence that ET-1 (1 – 31) is comparable to ET-1 in HMC 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 the transcription factor AP-1 complex. Potency of ET-1 (1 – 31) for ERK activation is comparable to that of ET-1 and angiotensin II, known mitogens for HMCs. Since the existence of chymase in human kidney has been reported, the pathogenesis of glomerulosclerosis or glomerulonephritis 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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