Endothelin-3 Regulates Endothelin-1 Production in Cultured Human Endothelial Cells

Koji Yokokawa, Masakazu Kohno, Kenichi Yasunari, Koh-ichi Murakawa, and Tadanao Takeda

Effects of endothelin-3 on the secretion of endothelin-1 and other endothelium-derived substances were investigated in cultured human umbilical vein endothelial cells. The present binding study showed two distinct subpopulations of binding sites for endothelin-3 with higher and lower affinities in cultured human endothelial cells. Endothelin-3 caused an increase in intracellular Ca\(^{2+}\) and inositol 1,4,5-trisphosphate levels and activated protein kinase C in a dose-dependent manner. Endothelin-3 also caused an increase in \(^{3}H\) thymidine incorporation into cellular DNA and stimulated the production of cyclic guanosine 3',5'-monophosphate, 6-ketoprostaglandin F\(_{1\alpha}\), and immunoreactive endothelin-1 in cultured human endothelial cells. \(^{3}H\)-Monomethyl L-arginine (3\times10^{-4} \text{ mol/l}) and indomethacin (10^{-5} \text{ mol/l}) enhanced endothelin-3-induced endothelin-1 production. These results suggest that endothelin-3 bound to its specific receptors and then caused phosphoinositide breakdown, subsequently mobilizing intracellular Ca\(^{2+}\) and leading to protein kinase C activation and the initiation of DNA synthesis, resulting in the stimulation of endothelin-1 production by human endothelial cells. Furthermore, this endothelin-1 production may be suppressed by endothelium-derived relaxing factor and prostacyclin produced in response to endothelin-3 in cultured human endothelial cells. (Hypertension 1991;18:304–315)

Endothelin (ET) is a newly identified 21-residue, vasoconstrictor peptide produced by vascular endothelial cells (ECs).\(^{1}\) Recently, three distinct human ET-related genes were cloned by screening a genomic DNA library.\(^{2}\) ET-1 exhibited a more potent constrictor activity than ET-3, whereas ET-3 exerted more profound initial depressor response in vivo.\(^{2}\) The production of ET-1 in ECs can be controlled by a transcriptional gene regulation directly coupled to the intracellular signals from the phosphoinositide turnover pathway, that is, activation of protein kinase C (PKC) and increase in intracellular Ca\(^{2+}\).\(^{3}\) Emori et al\(^{4}\) have recently demonstrated that ET-3 increased inositol 1,4,5-trisphosphate (IP\(_{3}\)) and intracellular Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_{i}\)) through its specific receptors in cultured bovine ECs.

The present study was designed to examine the effect of ET-3 on the secretion of ET-1 and other endothelium-derived substances in cultured human ECs.

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Methods

Materials

Synthetic ET-1 was purchased from Peptide Institute Co., Tokyo. Rabbit anti-ET-1 serum was purchased from Peninsula Laboratories Inc., Belmont, Calif. Medium-199, penicillin, streptomycin, Versene, and fetal calf serum (FCS) were purchased from Gibco Laboratories, New York. Collagenase type II, endothelial cell growth supplement (ECGS), bradykinin, adenosine 2',5'-diphosphate (ADP), N\(^{6}\),2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (db-cAMP), forskolin, and prostaglandin I\(_{2}\) (PGI\(_{2}\)) were purchased from Sigma Chemical Co., St. Louis, Mo. Fura-2/AM was purchased from Sigma Chemical Co., St. Louis, Mo. Fura-2/AM was purchased from Wako Co., Tokyo. Ionophore A23187 and \(^{3}N\)-monomethyl L-arginine (LNMMA) were purchased from Calbiochem Co., Los Angeles. Triium-labeled IP\(_{3}\), phosphorus-32-labeled PKC, prostacyclin (iodine-125-labeled 6-ketoprostaglandin F\(_{1\alpha}\) [6-keto-PGF\(_{1\alpha}\)] assay system, iodine-125-labeled ET-1, trium-labeled thymidine, and calcium-45 were purchased from Amersham Japan Co., Tokyo. Cyclic guanosine 3',5'-monophosphate (cGMP) assay system was purchased from Yamasa Shoyu Co. Ltd., Chiba, Japan.
Measurement of Intracellular Ca\(^{2+}\) Concentration

ECs were obtained from 20-cm segments of human umbilical veins by filling the vessel lumen with collagenase type II (0.5 mg/ml), incubating them at 37°C for 10 minutes, and flushing out the loosened sheets of cells as described previously.\(^5,6\) Cells were collected, cultured in standard culture flasks, and incubated at 37°C with atmospheric air and 5% CO\(_2\) in medium-199 containing 5% (vol/vol) FCS, ECGS (50 \(\mu\)g/ml), penicillin, and streptomycin (each 50 microunits/ml). ECs were identified by typical phase contrast “cobblestone” morphology and by immunofluorescence to factor VIII antigen.\(^6\) Vascular smooth muscle cell (VSMC) contamination was less than 0.1%. The eighth to 10th passages of cultured ECs were used in the following studies.

Iodine-125–Labeled Endothelin-3 Binding Experiments

Confluent human umbilical vein ECs grown in 6-well plates were washed with 2 ml medium A, with a composition of (mmol/l) NaCl 140, KCl 4, Na\(_2\)HPO\(_4\)1, MgCl\(_2\) 1, CaCl\(_2\) 1.25, glucose 11, \(N\)-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES) 5 (pH 7.4), and 0.2% bovine serum albumin, and were incubated in 2 ml of the same medium containing 2\(\times\)10\(^{-11}\) mol/l iodine-125–labeled ET-3 and various concentrations of unlabeled ET-3 for 60 minutes at 37°C according to previous reports.\(^7,8\) After incubation, cells were intensively washed with medium A (binding medium) at 4°C and solubilized in 1N NaOH, and the cell-bound radioactivity was determined. Specific binding was defined as total binding minus nonspecific binding determined in the presence of 2\(\times\)10\(^{-7}\) mol/l unlabeled ET-3.

The experiment was also performed to examine whether iodine-125–labeled ET-3 was internalized during the 60-minute incubation period. It has previously been shown that lowering cellular adenosine triphosphate (ATP) levels by treatment with metabolic poisons inhibits endocytosis.\(^9\) Therefore, ECs were preincubated in glucose-free media at 23°C for 30 minutes in the presence of 1 mmol/l dinitrophenol and 30 mmol/l 2-deoxyglucose to inhibit endocytosis according to previous methods,\(^9,10\) and then they were incubated with iodine-125–labeled ET-3 and were washed with ice-cold medium. The cell-bound ligand was removed by treating the cells with 0.2 mol/l acetic acid, pH 2.5, containing 0.5 mol/l NaCl for 6 minutes at 4°C, which extracts only surface-bound ligand without damaging the receptors or extracting internalized ligand,\(^10\) and the radioactivity was determined.

Measurement of Cytosolic Ca\(^{2+}\) Concentration

Cytosolic calcium levels were monitored by measuring the fluorescence of fura-2.\(^2,11\) ECs were cultured in complete medium on 36\(\times\)13.5 mm glass coverslips according to the method described previously.\(^5,12,13\) Confluent cell monolayers (2.0–2.3\(\times\)10\(^6\) cells) on the glass coverslips were washed intensively with 10 ml modified HEPES-Tyrode’s buffer (mmol/l NaCl 129, NaHCO\(_3\) 8.9, KH\(_2\)PO\(_4\) 0.8, MgCl\(_2\) 0.8, glucose 5.6, HEPES 10, and bovine serum albumin 0.035%, pH 7.4. The cell monolayer was loaded with 3 pmol/l fura-2-pentaacetoxymethyl ester in 3 ml complete medium for 60 minutes at 37°C with 95% air and 5% CO\(_2\). After incubation, the coverslips were washed with 10 ml modified HEPES-Tyrode’s buffer. The coverslips were then positioned in quartz glass cuvettes at a 45° angle to both the excitation and emission light paths with 3 ml fresh modified HEPES Tyrode’s buffer at 37°C equipped with a thermostated holder.\(^12\) Ca\(^{2+}\) fura-2 fluorescence was measured using a fluorescence spectrometer (model CAF-100, Japan Spectroscopy, Tokyo) with excitation at 340 and 380 nm and emission at 500 nm. All measurements were adjusted for autofluorescence by subtracting the values obtained in unloaded cells from those obtained in loaded cells before calculation. The free Ca\(^{2+}\) concentration was calculated from measurements of the ratio of fluorescence intensities by the following formula with the dissociation constant \((K_d)\) for intracellular fura-2 at 37°C of 224 nmol/l, as published by Grynkiewicz et al:\(^11\)

\[
[Ca^{2+}]_i = K_d (R - R_{min})/(R_{max} - R) \times F_{min380}/F_{max380}
\]

where \(R\) is ratio of fluorescence intensities, \(R_{max}\) is the maximal fluorescence ratio determined after addition of 0.2% Triton X-100 to permeabilize the cells in the presence of saturating concentrations of \([Ca^{2+}]_i\), \(R_{min}\) is the minimal fluorescence ratio determined after the subsequent addition of 20 mM ethylene glycol-bis(\(\beta\)-aminoethyl ether)-\(N,N,N',N'\)-tetraacetic acid (EGTA), \(F_{min380}\) and \(F_{max380}\) are the fluorescent values at 380 nm in the absence and in the presence of saturating \([Ca^{2+}]_i\), respectively.

Measurement of \(\text{"Ca}^{2+}\) Uptake

Confluent ECs were incubated in modified HEPES Tyrode’s buffer with 100 \(\mu\)l of \(\text{"CaCl}_2\) and agents to be tested for 5 minutes. ECs were then incubated with ET-3 for next 5 minutes. After incubation the medium was aspirated, and the cells were quickly washed with ice-cold buffer and denatured with 0.5 ml HCl (0.1N). The radioactivity of each sample was determined in a liquid scintillation counter.\(^14\)

Measurement of IP\(_3\) Levels, Protein Kinase C Enzyme Activity, and Cellular Cyclic GMP Content

For the measurement of IP\(_3\) levels,\(^15\) cells were cultured on 6-well plates. When cells were confluent, the medium was then removed and replaced with 1 ml modified HEPES Tyrode’s buffer. Stimulation was initiated by adding ET-3, and the reaction was terminated by adding 1 ml of 5% trichloroacetic acid (TCA). Samples were extracted with diethyl ether, and then titrated to pH 7.5 with NaHCO\(_3\). Amsed’s [\(^3\)H]IP\(_3\) assay system was used for the assay of IP\(_3\) as described in “Materials.”
For the measurement of PKC activity, confluent cells were incubated at 37°C in freshly replaced serum-free medium without addition or with various concentrations of ET-3. Activations were terminated by rapid washing of cells with 3 x 2 ml aliquots of cold extraction buffer (mmol/l) (HEPES 20, MgCl₂ 15, EGTA 20, ethylenediaminetetraacetic acid [EDTA] 1, and dithiothreitol 1, pH 7.4), followed by scraping cell layers into 300 μl extraction buffer containing 0.2% (vol/vol) Triton X-100 and 5 mmol/l phenylmethylsulfonylfluoride according to the method of Resink et al. Cell lysates were collected after centrifugation for 30 minutes at 100,000 g. PKC enzyme activity was measured by an assay kit and was detected by phosphorylation of the epidermal growth factor (EGF) receptor-derived peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH), which contains a major PKC phosphorylation site within the EGF receptor.

For the measurement of cGMP levels, confluent ECs cultured in 6-well plates were preincubated for 15 minutes at 37°C in 1.4 ml incubation buffer (mmol/l) (NaCl 137, KCl 2.7, Na₂HPO₄, 8, KH₂PO₄ 1.5, CaCl₂ 0.5, MgCl₂ 0.5, isobutylmethylxanthine [IBMX] 1, and indomethacin 0.01). The incubation was started by adding 100 μl of various concentrations of ET-3, and was stopped by adding 25 μl HC1 (IN) according to the method previously published. Intracellular cGMP was released into the buffer within 1 hour. The buffer was then removed and neutralized with 25 μl NaOH (IN), and cGMP was measured by radioimmunoassay using cGMP assay kit as described above.

### Measurement of Prostacyclin

Confluent ECs cultured in 6-well plates were incubated with various concentrations of ET-3 in 1 ml of serum- and ECGS-free medium at 37°C. The reactions were terminated with 150 μl of 1N HC1 according to the method previously reported. The reaction mixture was then extracted with 5 ml methyl formate through a Sep-Pak C18 cartridge by the method described previously. In ECs to ET-3, and forskolin (10⁻⁶ mol/l), db-cAMP (10⁻⁶ mol/l), and PGF₁₂ (10⁻⁶ mol/l) on ET-1 production were also examined. ECs were incubated with various agonists for 6 hours in the presence or absence of IBMX (0.1 mmol/l), and then immunoreactive ET-1 was determined by the radioimmunoassay as above.

This antibody cross-reacted 5% with ET-3 and 84% with ET-2. The cross-reacted ET-3 concentration in the medium were under 1.5 pg/ml at most in this study.

### Calculations and Statistical Analysis

The statistical significance of the results was evaluated by the analysis of variance, and probability values were determined by Student's t test.

### Results

**Iodine-125–Labeled Endothelin Binding**

To characterize the specific binding site for ET-3 in human umbilical vein ECs, a radioligand binding assay was performed by using iodine-125–labeled ET-3 (Figure 1). Specific binding, which was more than 85% of total binding at any concentration of iodine-125–labeled ET-3 and less than 5% of the total radioactivity added in the incubation medium, was saturable (Figure 1A) and time dependent at 37°C, reaching an apparent equilibrium after 60 minutes (binding of iodine-125–labeled ET-3 at 5, 10, 30, 45, 60, and 90 minutes was 27%, 44%, 83%, 95%, 100%, and 100%, respectively). The ET-3 binding analysis was then performed with 60-minute incubation. Scatchard analysis of the data revealed the presence of two distinct subclasses of binding sites for ET-3: one with high affinity (apparent Kₐ, 1.5 x 10⁻¹² mol/l) and low capacity (Bmax, 66 sites/cell), and the other with low affinity (apparent Kₐ, 3.9 x 10⁻¹⁰ mol/l) and high capacity (Bmax, 1,380 sites/cell) (Figure 1B).

The experiments were also performed by acid extraction to examine whether iodine-125–labeled ET-3 was internalized during the 60-minute incuba-
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0.22

0.02

0.01

Figure 1. Plots show binding of iodine-125-labeled endothelin-3 to cultured human endothelial cells. Panel A: Total minus nonspecific binding of 125I-endothelin-3. Panel B: Scatchard plot for specific binding of 125I-endothelin-3. Data are mean of three determinations.

Figure 2. Line graph shows competitive displacement experiments of iodine-125-labeled endothelin-3 binding to cultured human endothelial cells with unlabeled endothelins and big endothelin-1. Cells were incubated with 125I-endothelin-3 in the presence of indicated concentrations of unlabeled endothelin-1 (△), endothelin-2 (○), endothelin-3 (●), and big endothelin-1 (○). Data are mean of three determinations.

...tion period. The acid treatment removed 10% (3,150±311 cpm, n=4) of the cell-bound hormone (30,275±457 cpm, n=4) in control cultures incubated for 60 minutes with iodine-125-labeled ET-3. No significant difference was observed in the acid-extracted radioactivity between the culture treated with metabolic poisons to inhibit internalization (3,275±525 cpm, n=4) and control. However, after the 24-hour incubation period with iodine-125-labeled ET-3, the acid-extracted radioactivity decreased to 50% (1,600±374 cpm, n=4) of cultures incubated with iodine-125-labeled ET-3 for 60 minutes. Total cell-bound radioactivity was not affected by the treatment with metabolic poisons (30,318±308 cpm, n=4) or by the 24-hour incubation (30,203±371 cpm, n=4).

In competitive experiments, binding of iodine-125-labeled ET-3 was dose-dependently displaced by unlabeled ET-3 (IC50 value was 1.9×10^-9 mol/l), whereas the displacement was not complete with ET-1, ET-2, and big ET-1 (IC50 value was 10^-9, 10^-9, and 3×10^-9 mol/l, respectively) (Figure 2).

Effect of Endothelin-3 on [Ca2+]i

The ET-3-induced Ca2+ mobilization was studied by measurement of changes in [Ca2+]i, using fura-2 as a Ca2+ indicator (Figure 3). Addition of ET-3 (100 nmol/l) resulted in a rapid increase in [Ca2+]i that peaked within 60 seconds and then declined gradually to a steady level in the presence of extracellular Ca2+ (Figure 3A). This also occurred after addition of thrombin (0.03 units/ml), known to increase [Ca2+]i in cultured ECs (Figure 3B). The [Ca2+]i increased in response to ET-3 in a dose-dependent manner (maximum Ca2+ elevation was 150±13 nmol/l, 50% maximal concentration [EC50] was 1.3×10^-9 mol/l) (Figure 3D). To determine the role for extracellular Ca2+, the experiment was performed in the presence of 1 mmol/l EGTA to exclude extracellular Ca2+. The later sustained phase of [Ca2+]i (at 5 minutes) was significantly abolished by EGTA treatment, but the initial transient rise in [Ca2+]i was not (Figures 3C and 3D). Calcium antagonists nifedipine (10^-7 mol/l) and diltiazem (10^-9 mol/l) did not significantly affect the ET-3-induced increase in [Ca2+]i (data not shown).

Effect of Endothelin-3 on 45Ca Influx Into Human Umbilical Vein Endothelial Cells

We also investigated a role for extracellular Ca2+ in response to ET-3 with the use of 45Ca. The 45Ca uptake into ECs increased in a time-dependent manner (Table 1). In our experiments, the changes in 45Ca uptake were
FIGURE 3. Effects of endothelin-3 on intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) in human endothelial cells. Endothelin-3 (100 nmol/l) caused rapid increase in [Ca\(^{2+}\)\(_i\)] levels in the presence (panel A) or absence (panel C) of extracellular Ca\(^{2+}\). Thrombin (0.03 units/ml) also raised intracellular Ca\(^{2+}\) levels (panel B). Panel D: Dose-dependent response of the initial transient peak of [Ca\(^{2+}\)\(_i\)] in response to endothelin-3 in the presence (•) or absence (○) of extracellular Ca\(^{2+}\) and the later sustained phase (at time 5 minutes) of endothelin-3 in the presence (•) or absence (○) of extracellular Ca\(^{2+}\) on [Ca\(^{2+}\)\(_i\)] in human endothelial cells. Data are mean±SEM of four experiments. ***p<0.001 compared with values in presence of extracellular Ca\(^{2+}\).

large and variable within the first 5 minutes but were comparatively small and sustained after 5 minutes. Therefore, the experiments were performed after a 5-minute incubation with \(^{45}\)Ca. Incubation of the ECs with ET-3 (100 nmol/l) induced an increased uptake of extracellular Ca\(^{2+}\) (Table 1). Ni\(^{2+}\) significantly suppressed ET-3-induced \(^{45}\)Ca influx through the cell membrane in a dose-dependent manner, but nifedipine did not (Table 2).

Effect of Endothelin-3 on IP\(_3\) Production in Human Umbilical Vein Endothelial Cells

As shown in Figure 4A, ET-3 (100 nmol/l) induced a marked increase in IP\(_3\) levels within 15 seconds, reached peak levels within 30 seconds, and then gradually fell to steady-state levels at 5 minutes. ET-3 increased IP\(_3\) levels in a dose-dependent fashion (maximum IP\(_3\) elevation, 150±11.7% increase; EC\(_{50}\), 10\(^{-8}\) mol/l) (Figure 4B).

Effect of Endothelin-3 on Protein Kinase C-Dependent Phosphorylation of Peptide

Stimulation of cells with ET-3 (100 nmol/l) induced a rapid increase in the incorporation of \(^{32}\)P into the EGF receptor-derived peptide, which was evident within 5 minutes and was sustained for at least 30 minutes (Figure 5A). Basal activity of PKC (expressed as 0% increase in Figure 5) was 16 pmol phosphate transferred per minute. ET-3 dose-dependently induced increase in phosphorylation (maximum response, 183±25%; EC\(_{50}\), 10\(^{-8}\) mol/l) (Figure 5B).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>ET-3 (100 nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>5.0±0.16</td>
<td>...</td>
</tr>
<tr>
<td>-2</td>
<td>6.1±0.17</td>
<td>...</td>
</tr>
<tr>
<td>0</td>
<td>6.6±0.08</td>
<td>...</td>
</tr>
<tr>
<td>1</td>
<td>6.8±0.08</td>
<td>7.2±0.18</td>
</tr>
<tr>
<td>3</td>
<td>7.1±0.08</td>
<td>9.0±0.19*</td>
</tr>
<tr>
<td>5</td>
<td>7.4±0.09</td>
<td>9.1±0.20\†</td>
</tr>
<tr>
<td>10</td>
<td>7.7±0.09</td>
<td>9.5±0.22\†</td>
</tr>
</tbody>
</table>

After incubation of the endothelial cells for 5 minutes with 100 \(\mu\)l \(^{45}\)CaCl\(_2\), the cells were incubated with endothelin-3 (100 nmol/l) and \(^{45}\)Ca uptake was measured. Data are mean of four experiments±SEM (×10,000 counts/min/10\(^5\) cells). \*p<0.05, \†p<0.01 compared with respective control values.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Endothelin-3 (100 nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>7.4±0.09</td>
<td>9.1±0.20*</td>
</tr>
<tr>
<td>Ni(^{2+}) (50 (\mu)mol/l)</td>
<td>7.4±0.14</td>
<td>8.5±0.23\†</td>
</tr>
<tr>
<td>Ni(^{2+}) (100 (\mu)mol/l)</td>
<td>7.5±0.16</td>
<td>8.2±0.22\§</td>
</tr>
<tr>
<td>Ni(^{2+}) (500 (\mu)mol/l)</td>
<td>7.4±0.12</td>
<td>7.9±0.19\§</td>
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<td>Ni(^{2+}) (1 (\mu)mol/l)</td>
<td>7.5±0.12</td>
<td>7.8±0.22\§</td>
</tr>
<tr>
<td>Ni(^{2+}) (5 (\mu)mol/l)</td>
<td>7.5±0.14</td>
<td>7.8±0.24\§</td>
</tr>
<tr>
<td>Nifedipine (10 (\mu)mol/l)</td>
<td>7.4±0.10</td>
<td>8.8±0.18*</td>
</tr>
</tbody>
</table>

Data are mean of four experiments±SEM (×10,000 counts/min/10\(^5\) cells). \*p<0.001, \†p<0.01, \§p<0.05 compared with the respective control values.

TABLE 2. Effects of Ni\(^{2+}\) and Nifedipine on Endothelin-3-Induced \(^{45}\)Ca Uptake

\(\mu\)l \(^{45}\)CaCl\(_2\), the cells were incubated with endothelin-3 (100 nmol/l) and \(^{45}\)Ca uptake was measured. Data are mean of four experiments±SEM (×10,000 counts/min/10\(^5\) cells). \*p<0.001, \†p<0.01, \§p<0.05 compared with the respective control values.
Effect of Endothelin-3 on DNA Synthesis in Human Umbilical Vein Endothelial Cells

Shown in Figure 6 are the effects of ET-3 on [3H]thymidine incorporation into DNA in human umbilical vein ECs. When ECs were incubated with ET-3 alone in a serum- and ECGS-free medium for 24 hours, DNA synthesis of ECs was stimulated in a dose-dependent manner. This effect of ET-3 was synergistically enhanced in the presence of ECGS (50 μg/ml), which itself also had the potency to increase the thymidine incorporation (0.886±0.1536×10^5 dpm/10^5 cells) compared with the control values (0.636±0.1335×10^5 dpm/10^5 cells, p<0.05).

Effect of Endothelin-3 on Cyclic GMP Levels

ET-3 induced an increase in cGMP levels in a dose- and time-dependent manner (Table 3). LNMMA (3×10^{-4} mol/l) significantly attenuated the ET-3–induced increase in cGMP levels but not the basal release of cGMP from human umbilical vein ECs (Table 4).

Effect of Endothelin-3 on PGI2 Production by Human Umbilical Vein Endothelial Cells

The cells were grown to confluence under the conditions described above, and then we measured the amount of 6-keto-PGF_1α, a stable metabolite of PGI2, which was secreted into the culture medium from ECs in the presence of ET-3 at various concentrations. ET-3–induced PGI2 production was in a time- and dose-dependent manner (maximum concentration, 0.3±0.1 ng/10^5 cells; EC50, 2×10^{-8} mol/l) (Figure 7). ET-3 did not enhance the production of PGI2 in the presence of 10^{-5} mol/l indomethacin (Figure 7B).
We also examined the effect of ET-1 on PGI₂, cGMP, and thymidine incorporation. ET-1 even at high doses (10⁻⁷ mol/l) did not change PGI₂, cGMP level, and thymidine incorporation compared with basal levels in the ordinary culture condition (data not shown).

**Effect of Endothelin-3 on Production of Endothelin-1 by Human Umbilical Vein Endothelial Cells**

Cultured human umbilical vein ECs produced immunoreactive ET-1 in the medium in a time-dependent manner. This release was enhanced in the presence of ET-3 in a dose-dependent fashion (Fig. 8). As shown in Table 4, ET-3–induced increase in the production of ET-1 was more augmented in the presence of LNMMMA at 3×10⁻⁴ mol/l or indomethacin at 10⁻⁵ mol/l.

**Effect of Bradykinin, Ionophore A23187, and ADP on Production of Endothelin-1**

ECs were incubated for 6 hours with bradykinin (10⁻⁷ mol/l), ionophore A23187 (10⁻⁶ mol/l), or ADP (3×10⁻⁵ mol/l) (Table 5). Bradykinin (10⁻⁷ mol/l) and ADP (3×10⁻⁵ mol/l) were found to suppress the ET-1 production by ECs in the presence of IBMX (0.1 mmol/l). Ionophore A23187 markedly enhanced the ET-1 release, and this enhancement was attenuated by the presence of IBMX.

**Effect of Forskolin, db-cAMP, and PGI₂ on Production of Endothelin-1**

The exogenous forskolin (10⁻⁶ mol/l), db-cAMP (10⁻⁶ mol/l), or PGI₂ (10⁻⁶ mol/l) significantly suppressed the basal release of ET-1 from ECs, and these suppressive effects were enhanced in the presence of IBMX (0.1 mmol/l) (Table 5).

**Discussion**

First, this study demonstrated that ET-3, bound to its specific receptor, stimulated phosphoinositide turnover, mobilized Ca²⁺ from intracellular and extracellular pools, activated PKC, and enhanced DNA synthesis in human umbilical vein ECs. The present binding study showed two distinct subpopulations of binding sites for ET-3 with higher and lower affinities in cultured human umbilical vein ECs. Our results are consistent with recent studies reported by Emori et al. indicating that cultured bovine ECs express ET-3 receptor subtype. Other studies have reported that at least two subtypes of binding sites for ET isopeptides exist in the membranes of chick heart and rat lung; one subtype with preferential binding affinity to ET-1 and ET-2 and the other to ET-3, suggesting that the population of ET receptor subtypes may differ from one tissue to another. The measurements of [Ca²⁺], with fura-2...
demonstrated a biphasic pattern of ET-3–induced Ca2+ mobilization: an initial transient increase in [Ca2+]i because of intracellular mobilization followed by a sustained plateau phase because of Ca2+ influx across the plasma membrane. The second plateau phase of the Ca2+ response to ET-3 was significantly inhibited by EGTA treatment but was not by nifedipine, one of the dihydropyridine Ca2+ channel antagonists. Ni2+, an inhibitor of the voltage-dependent Ca2+ channels of T type, was found to suppress the ET-3–induced active 45Ca transport from the extracellular space, whereas nifedipine did not, indicating that Ca2+ influx in response to ET-3 is partially involved in the voltage-dependent Ca2+ channels of T type more than those of L type. Moreover, we have found that ET-3 stimulated IP3 formation in human umbilical vein ECs, indicating that one of the initial membrane events in the action of ET-3 is to induce phospholipase C–mediated phosphatidylinositol 4,5-bisphosphate hydrolysis, which liberates IP3 and subsequently mobilizes Ca2+ from portions of the endoplasmic reticulum in human umbilical vein ECs. Recent reports have shown that ET-1 causes phosphoinositide hydrolysis in cultured vascular smooth muscle cells, glomerular mesangial cells, mouse Swiss 3T3 fibroblasts, osteoblastic cells, and human umbilical vein ECs. Sarafotoxin, a snake venom that has a high degree of structural similarity to ET, has been reported to stimulate phosphoinositide hydrolysis in heart and brain. Very recently it has been shown that ET-3 induces phosphoinositide breakdown in cultured bovine ECs. ET-3 was also found to activate PKC and stimulate DNA synthesis in this study. Thus, ET-3 may use the inositol lipid pathway as a signal transduction mechanism, resulting in activation of PKC in the Ca2+ messenger system and initiation of DNA synthesis in human umbilical vein ECs. DNA synthesis in response to ET-3 was found to be enhanced in the presence of ECGS, which was usually used in endothelial cell culture. Interaction between ECGS and ET-3 on ECs remains to be confirmed.

Second, the present study showed that ET-3 caused an increase in ET-1 production in human umbilical vein ECs. Yanagisawa et al have reported that the messenger RNA for preproET-1 was rapidly upregulated by the active phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) and the Ca2+...
ET-3 induces the release of PGI₂ or increase in systemic arterial blood pressure in anesthetized rats. ET-3 stimulates PGI₂ production by ET-3 is through the phosphoinositide turnover pathway. These results suggest that ET-3 directly stimulates the release of EDRF from human umbilical vein ECs, and this was attenuated in the presence of LNMMA, which was a false substrate for the enzyme synthesizing nitric oxide, indicating that ET-3 stimulates ET-1 production because of its intracellular Ca²⁺ mobilization and PKC activation in human umbilical vein ECs.

Third, endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide, the amino acid L-arginine is the precursor of nitric oxide in ECs. Nitric oxide activates soluble guanylate cyclase and in turn increases cGMP levels in ECs and VSMCs. The present study showed that ET-3 caused the increase in cGMP levels in cultured human umbilical vein ECs, and this was attenuated in the presence of LNMMA, which was a false substrate for the enzyme synthesizing nitric oxide, indicating that ET-3 stimulates the production of EDRF in cultured human umbilical vein ECs. This study also showed that ET-3 induced an increase in the levels of intracellular cyclic GMP in cultured ECs to in vivo conditions should be carefully performed because cell cultures represent at best "activated cells."

Fourth, very recently it has been reported that EDRF inhibits the release of ET-1 from porcine aorta and that both LNMMA and methylene blue augmented the thrombin-stimulated but not the basal release of ET-1 from porcine aorta with endothelium. Our present study also showed that LNMMA enhanced the ET-3-stimulated release of ET-1 from cultured human umbilical vein ECs, suggesting that ET-3-induced EDRF suppressed the release of ET-1 from human umbilical vein ECs mediated by a cGMP-dependent pathway. Moreover, the present study showed that ET-3-induced ET-1 production significantly increased in the presence of indomethacin. PGI₂ is known to increase cellular cAMP, and this elevation may interact with the G-protein–phospholipase C system leading to the presumable inhibition of the generation of the second messengers—IP₃, diglyceride, and the protein kinase C-dependent events. The present study also showed that the exogenous PGI₂ (10⁻⁸ mol/l), db-cAMP (10⁻⁶ mol/l), and forskolin (10⁻⁶ mol/l) significantly suppressed the production of ET-1, and these suppressive effects were enhanced in the presence of IBMX (0.1 mmol/l). These results indicate that ET-3–induced PGI₂ inhibits the release of ET-1 from human umbilical vein ECs mediated by a cAMP-dependent inhibitory pathway. The present study showed that LNMMA and indomethacin each enhanced ET-1 production by cultured human umbilical vein ECs, but LNMMA plus indomethacin did not cause more enhancement of ET-1 production than LNMMA alone, indicating that there are strong interactions among EDRF, PGI₂, and ET-1 production. Recently, nitroglycerin has been reported to stimulate synthesis of PGI₂ in cultured human umbilical vein ECs, suggesting that ET-3–induced EDRF may enhance the production of PGI₂.
reports have shown that ECs produce not only PGI₂ but also thromboxane A₂ in a ratio of 5:1 to 10:1.63 Cyclooxygenase inhibitors inhibited the production of both metabolites equally. The role for thromboxane A₂ in response to ET-3 remains to be clarified.64

Finally, we tried to examine the effects of bradykinin, ionophore A23187, and ADP, which could initiate a series of events similar to ET-3 in ECs. Bradykinin (10⁻⁷ mol/l) and ADP (3×10⁻⁵ mol/l) were found to suppress the ET-1 production by ECs in the presence of IBMX (0.1 mmol/l). Ionophore A23187 markedly enhanced the ET-1 release. Bradykinin, ADP, and calcium ionophore A23187 are reported to stimulate cGMP production in cultured ECs,68 suggesting that the production of ET-1 may be regulated by the balance between cGMP production and Ca²⁺ mobilization stimulated by these agonists. Ionophore A23187 is known to induce the remarkable increase in intracellular Ca²⁺ levels, indicating that under our experimental conditions, Ca²⁺ mobilization or PKC-dependent pathways may be predominant over cGMP production in the case of A23187. ET-3 may have the potency to induce ET-1 production under the balance among cAMP, cGMP-mediated, and Ca²⁺-mobilizing, or PKC-dependent mechanisms.

EC₅₀ values for [Ca²⁺]i, IP₃, and phosphorylation by PKC induced by ET-3 (nearly 10⁻⁸ mol/l) were higher than the apparent Kᵣ values for receptor binding (10⁻¹⁰ or 4×10⁻¹⁰ mol/l). These data suggest the existence of large numbers of spare receptors for ET-3 in cultured human umbilical vein ECs. ET-3 at 10⁻¹² mol/l also significantly enhanced the release of ET-1 from human umbilical vein ECs, suggesting that stimulation of ET-1 release from ECs may be a consequence of the activation of the high affinity ET-3 receptors, although EC₅₀ values for phosphatidylinositol breakdown were higher. This discrepancy may be partly accounted for by the different incubation periods used for the study: one examined chronic response and others acute.

In summary, our observations showed that ET-3 bound to its specific receptors and then induced phosphoinositide breakdown, Ca²⁺ mobilization from intracellular and extracellular spaces, activation of PKC, and initiation of DNA synthesis in human umbilical vein ECs. ET-3, one of the ET isopeptides, then stimulated ET-1 production by ECs. However, ECs also produced EDRF and PGI₂ in response to ET-3, which would prevent the production of an excessive amount of ET-1 by human umbilical vein ECs.

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