Involvement of Endothelin in the Regulation of Human Vascular Tonus

Potent Vasoconstrictor Effect and Existence in Endothelial Cells

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Endothelin, a recently discovered endothelium-derived peptide, has been reported to produce potent vasoconstriction in various vessels of experimental animals. To study the involvement of endothelin in the regulation of vascular tonus in humans, isolated human mesenteric arteries were investigated by both pharmacological and immunohistochemical methods. The vasoconstrictor action of endothelin-1 was examined on ring segments of human mesenteric arteries. Endothelin-1 induced a slowly developing and sustained contraction, with an EC₅₀ value (half-maximal effective concentration) of 2.9×10⁻⁹ M, two orders of magnitude smaller than that of norepinephrine (EC₅₀ of 3.9×10⁻⁷ M), indicating that the vasoconstrictor action of endothelin-1 is about 100 times more potent than that of norepinephrine. The contractile effect of endothelin-1 was affected neither by adrenergic, cholinergic, histaminergic, nor serotoninergic antagonists, nor by inhibitors of arachidonic acid metabolism. The vasoconstrictor response to endothelin-1 was effectively antagonized by nicardipine, a dihydropyridine Ca²⁺ channel blocker. Endothelin-1 profoundly augmented contractile response to Ca²⁺ in partially depolarized tissues. Immunohistochemical studies revealed for the first time that endothelin-like immunoreactivity was localized in endothelial cells of human mesenteric artery. The results of the present study indicate that endothelin-1 is one of the most potent vasoconstrictors in the human mesenteric artery and that it induces vasoconstriction via an ultimately accelerating Ca²⁺ influx through voltage-dependent Ca²⁺ channels. Since endothelin-1 can be located in human endothelial cells, it may play an important physiological or pathophysiological role.

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Since the discovery of endothelium-dependent vasodilation by Furchgott and Zawadzki in 1980, it has become evident that the endothelial cells covering the luminal surface of blood vessels play a key role in the motor effects of certain vasoactive substances. In addition to mediating relaxations, endothelial cells can also facilitate contractile responses of a variety of vascular smooth muscles. This excitatory function of endothelium may be mediated by diffusible materials, that is, endothelium-derived constrictor factors (EDCFs). Endothelin (ET) is a 21–amino acid peptide exhibiting potent vasoconstrictor activity that we recently purified and sequenced from the culture medium of porcine aortic endothelial cells. It was subsequently found that the amino acid sequence of human ET is exactly identical to that of porcine ET. It was also revealed recently that many mammals, including humans, possess three distinct ET-related genes. From analysis of these genes, the three similar but distinct peptides from the “ET family” were found and named ET-1, ET-2, and ET-3, where ET-1 was formerly called human-porcine ET. Inasmuch as the mRNA for prepro–ET-1 was detected in intact endo-

See p 2022
thelial cells by Northern blot analysis. ET-1 can be considered to be expressed as one of the EDCF, that is, as an endogenous vasoconstrictor, even under physiological conditions. Recently, we reported that plasma concentrations of ET-1 and big ET-1 were elevated in patients with acute myocardial infarction. ET-1 produces potent vasoconstriction in various vessels of experimental animals.

It is generally known that the splanchnic vascular bed receives as much as 25% of cardiac output. The resistance of the peripheral vascular bed is important for the maintenance of blood pressure and, therefore, the mesenteric resistance vessels appear to be particularly important for the control of systemic blood pressure. It is also known that various functional components of the mesenteric vascular bed are highly sensitive to circulatory disturbances under a wide range of conditions, including hypovolemic shock and increased blood concentrations of various vasoactive substances. Therefore, it is of interest to clarify the responsiveness of the human mesenteric vasculature to various vasoactive substances.

In the present study, to investigate the importance of ET in the regulation of vascular tonus in humans, the vasoconstrictor effects of ET-1 on human mesenteric resistance vessels were characterized quantitatively and compared with those produced by norepinephrine, an endogenous potent vasoconstrictor. Furthermore, to exemplify the actual expression of ET in human endothelial cells, the tissue was subjected to an immunohistochemical investigation with an antibody raised against ET-1.

Methods

Small segments of mesenteric arteries were obtained from 11 male patients, aged 36–65 years (54.5±2.9 years; mean±SEM), who were undergoing segmental resection of the intestine for intestinal malignancy. None of the patients had signs of vascular disease, metabolic disease, or connective tissue disorders. The patients received diazepam, hydroxyzine hydrochloride, and/or atropine for premedication, and anesthesia was induced with sodium thiopental and maintained with nitrous oxide and ethane. Supplementary doses of pancuronium bromide were given as needed. Arterial segments were 1 mm or less in outside diameter and had a macroscopically normal appearance. Immediately after removal, the vessel segments were transferred to an ice-cold Krebs-Ringer solution of the following composition (mM): NaCl 113, KCl 4.8, CaCl₂ 2.2, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, and glucose 5.5.

Pharmacological Analysis

After the arterial segment was freed from surrounding connective tissues, a ring segment 4-mm long piece of the tissue was mounted in a silicon-coated 20-ml organ bath with two metal holders, one being anchored, and the other being connected to a force-displacement transducer (model TB-611T, Nihon-Kohden, Tokyo, Japan) for measurement of isometric contractions. The solution was maintained at 37°C and was aerated with a mixture of 95% O₂ and 5% CO₂. A resting tension of 1 g was applied to the tissue, and an equilibration period of 2 hours was allowed. During this period, the tissue was washed with fresh solution every 15 minutes. After equilibration, the maximum response to KCl (50 mM) was measured repeatedly at 30-minute intervals until a steady response was obtained (usually, three to four times). Subsequently, the dose-response relation to ET-1 or norepinephrine (NE) was determined by means of a cumulative application. When the effects of the various blocking agents were examined, they were applied 10 minutes before starting the cumulative application of ET-1. For normalization, the responses to ET-1 and NE were expressed as the percent of the maximum response to KCl.

For measurement of the dose-response relation to Ca²⁺ for the contractile effect, the arterial strips were first equilibrated in a nominally Ca²⁺-free Krebs-Ringer solution containing 25 mM KCl (NaCl substituted with KCl) to produce partial depolarization for at least 1 hour, and CaCl₂ was added cumulatively, so that the designated concentration of Ca²⁺ was attained. ET-1 was applied 20 minutes before the first addition of CaCl₂.

Immunohistochemistry

For the immunohistochemical studies, small segments of the mesenteric arteries were fixed with 0.01 M picric acid and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), rinsed serially with 7.5%, 15%, and 30% sucrose in 0.1 M PB, and embedded in Tissue-Tek (Miles Scientific Inc., Naperville, Illinois). After the 10 μm-thick frozen sections were made with a cryostat (Reichert-Jung, Vienna, Austria), the avidin-biotin-peroxidase complex (ABC) method of immunohistochemistry was performed according to the method of Hsu et al. Briefly, the sections were incubated with 0.3% H₂O₂ in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. They were subsequently incubated serially with the solutions as follows: 1) 2% normal goat serum (Vector Laboratories, Inc., Burlingame, California) for 20 minutes at room temperature to reduce nonspecific background staining; 2) rabbit anti-ET-1 serum (Peptide Inst., Osaka, Japan) at a dilution of 1:1,000 for 24 hours at 4°C; 3) biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc.) at a dilution of 1:100 for 1 hour at 37°C; and 4) the ABC reagent (avidin plus biotinylated horseradish peroxidase, Vector Laboratories, Inc.) at a dilution of 1:100 for 1 hour at 37°C. Each solution was diluted with 0.1% Triton X-100 in phosphate-buffered saline (pH 7.2), and between each step, the sections were rinsed with a wash solution (0.5% NaCl and 0.1% Triton X-100 in 0.01 M PB, pH 7.2) three times for 5 minutes. Then, the sections were rinsed with 0.05 M Tris-HCl buffer (pH...
7.6) and immersed in 0.02% 3,3′-diaminobenzidine and 0.005% H$_2$O$_2$ in 0.05 M Tris-HCl buffer for 7–10 minutes to visualize localization of peroxidase. The sections were dehydrated, mounted, and observed with a Zeiss microscope. The anti–ET-1 serum did not crossreact with the following peptides at high doses (1–10 µM): peptide YY, calcitonin gene-related peptide (human), secretin (human), somatostatin, and β-endorphin (human). The specificity of immunostaining was assessed by performing the following experiments: 1) incubation of the sections with a nonimmune rabbit serum diluted 1:1,000 instead of the anti–ET-1 serum, 2) incubation of the sections with the anti–ET-1 serum subjected to solid-phase absorption by passage through a column loaded with activated CH-Sepharose 4B (Pharmacia Inc., Tokyo, Japan) to which ET-1 conjugated. The anti–ET-1 serum was passed through the resin, which underwent no conjugation, and which served as the control for this phase of the experiment.

**Drugs and Statistics**

Drugs used were ET-1 (porcine, Peptide Inst., Osaka, Japan); NE (Wako Pure Chemicals, Osaka, Japan); bunazosin (Eisai, Tokyo, Japan); atropine sulfate (Tanabe, Osaka, Japan); diphenhydramine hydrochloride (Tokyo Kasei, Tokyo, Japan); methysergide hydrogen maleinate (Sandoz Ltd., Basel, Switzerland); and bovine serum albumin (fraction V), indomethacin, nicardipine, nordihydroguaiaretic acid (Sigma Chemical, St Louis, Missouri). ET-1 was dissolved in phosphate-buffered saline (pH 7.4) containing 0.05% bovine serum albumin.

Values are expressed as mean±SEM. Statistical analyses were performed with the one-way analysis of variance (ANOVA), followed by the Bonferroni method or the Student’s $t$ test for paired values.

**Results**

ET-1 produced a dose-dependent contraction of the human mesenteric artery. ET-1–induced vasoconstriction was quite slow to develop and long lasting: 15–20 minutes were required to attain a steady-state tension at each dose, and it took longer than 2 hours to attain the full dose-response relation (Figure 1B). The maximally developed tension did not return to the initial level even after repeated washings with a fresh solution for as long as 3 hours (data not shown). NE also caused a dose-dependent contractile response, which appeared to be rapidly developing and reversible (Figure 1A).

The dose-response curves to ET-1 and NE are illustrated in Figure 2. The maximum responses to ET-1 and NE were identical to and slightly larger, respectively, than that to KCl (50 mM). However, the EC$_{50}$ value (the concentration eliciting half-maximal response) of ET-1 was $2.9\times10^{-9}$ M, which was more than two orders of magnitude smaller than that of NE ($3.9\times10^{-7}$ M; Table 1). The dose-response curve for ET-1 in the artery with intact endothelial cells was identical to that in endothelium-denuded tissue, which was produced by rubbing the intima with a

**Figure 1.** Typical traces for the dose-response relation for the vasocontractile effects (increase in tension, g) of norepinephrine (A) and endothelin-1 (B) on human mesenteric arteries. Numbers in the figure indicate –log molar concentration of the drug. Transverse bars indicate time courses (□, 30 minutes; ■, 10 minutes). Note that time course of the dose-response curve for norepinephrine was markedly shorter than that for endothelin-1.

**Figure 2.** Dose-response curves for norepinephrine (□, n=8) and endothelin-1 in the absence (● [control], n=8) or presence of antagonists (○, n=6) or $3\times10^{-6}$ M nicardipine (△, n=5) for vasoconstrictive effects on human mesenteric arteries. Antagonists included bunazosin ($10^{-7}$ M), atropine sulfate ($10^{-7}$ M), diphenhydramine hydrochloride ($10^{-7}$ M), and methysergide hydrogen maleinate ($10^{-7}$ M). Responses are expressed as percents of maximum contraction to 50 mM K$^+$. Points and bars represent means and SEM, respectively.
TABLE 1. E_{C50} Values and Maxima of Endothelin and Norepinephrine Vasocontractile Effects on Human Mesenteric Arteries

<table>
<thead>
<tr>
<th></th>
<th>E_{C50} M (CI)</th>
<th>Maximal response (%)</th>
<th>n</th>
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<tr>
<td><strong>Endothelin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.9x10^{-9}</td>
<td>98.5±4.8</td>
<td>8</td>
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<tr>
<td></td>
<td>(1.2x10^{-9} to 7.3x10^{-9})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Nicardipine</td>
<td>1.7x10^{-8s}</td>
<td>98.5±1.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(3x10^{-8} M)</td>
<td>(2.3x10^{-9} to 1.2x10^{-7})</td>
<td></td>
</tr>
<tr>
<td>+ Antagonists</td>
<td>2.9x10^{-9}</td>
<td>96.6±4.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(3.8x10^{-10} to 2.2x10^{-9})</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Norepinephrine</strong></td>
<td>3.9x10^{-7}</td>
<td>115.1±4.6*</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(1.9x10^{-7} to 7.9x10^{-7})</td>
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Maximal response indicates percent of 50 mM K^{+}-induced contraction (mean±SEM).

Antagonists include bunazosin 10^{-7} M, atropine sulfate 10^{-7} M, diphenhydramine hydrochloride 10^{-7} M, methysergide hydrogen maleinate 10^{-7} M.

CI, 95% confidence interval.

*p<0.05, tp<0.001 from control of endothelin.

stainless steel rod (data not shown). The vasoconstrictor effect of ET-1 was not affected by the presence of a mixture of the following antagonists: bunazosin 10^{-7} M (a1-adrenergic), atropine sulfate 10^{-7} M (muscarinic), diphenhydramine hydrochloride 10^{-7} M (H_{1}-histaminergic), and methysergide hydrogen maleinate 10^{-7} M (serotonergic; Figure 2 and Table 1). In the human mesenteric artery, this mixture of antagonists almost abolished the individual vasoconstrictor effects of NE (10^{-7} M) and serotonin (10^{-7} M) (data not shown). Furthermore, the effect of ET-1 was unaffected by the inhibitors of cyclooxygenase (indomethacin 10^{-5} M) and lipoxygenase (nordihydroguaiaretic acid 10^{-4} M) (data not shown).

The dose-response curve for the contractile effect of ET-1 was significantly shifted to the right by nicardipine (3x10^{-9} M), a dihydropyridine Ca^{2+} channel blocker (Figure 2 and Table 1). However, this dose of nicardipine did not affect the dose-response relation for NE (data not shown). In addition, as shown in Figure 3, pretreatment with a low concentration of ET-1 (10^{-9} M) greatly potentiated the dose-response relation of Ca^{2+} for the contractile effect and reduced the E_{C50} value of Ca^{2+} (2.9x10^{-4} M at control vs. 5.2x10^{-5} M in the presence of ET-1; p<0.01, n=5) in the preparations partially depolarized by 25 mM K^{+}.

In the immunohistochemical studies, ET-like immunoreactivity was demonstrated to be localized in the luminal surface of the human mesenteric artery (Figure 4A). The control study with nonimmune rabbit serum gave negative results (data not shown). Furthermore, once the anti-ET-1 serum was passed through the column loaded with activated CH-Sepharose 4B to which ET-1 was conjugated, the immunoreactivity was significantly reduced (Figure 4B). In contrast, the sections were well stained by the anti-ET-1 serum passed through the resin that underwent no conjugation. These results indicate that the immunoreactivity was specific to ET-1. In the section from adjacent tissue, we confirmed that the same surface layer was specifically stained by the antisera against von Willebrand factor (factor VIII-associated antigen, BioGenex Lab., Dublin, California), which has been considered as one of the best markers for endothelial cells (data not shown), indicating that the luminal surface that exhibits ET-like immunoreactivity consists of endothelial cells. Furthermore, ET-like immunoreactivity in the luminal surface disappeared after deendothelialization, which was performed by rubbing the intima with a stainless steel rod (data not shown).

**Discussion**

ET has been shown to cause vasocontraction in various vessels of experimental animals.7,11-14 The present study shows that ET-1 also induces a dose-dependent contractile response in the human artery. The dose range of ET-1 needed to produce contraction of the human mesenteric artery was low, with an E_{C50} value of 2.9x10^{-9} M. This value is smaller than those of other vasoconstrictors, for example, NE (3.9x10^{-7} M, the present study; 1.2x10^{-6} M20), serotonin (2.0x10^{-7} M21), or neuropeptide Y (3.0x10^{-8} M22). The contractile effect of vasopressin (2.0x10^{-9} M23) is comparable to that of ET-1 in the mesenteric artery. However, ET-1 and vasopressin exert distinct actions, depending on the vascular tissues. For example, while vasopressin causes vasoconstriction in various peripheral arteries, it induces an endothelium-dependent relaxation of the canine cerebral artery.24 In contrast, ET-1 produced only potent contractions in every kind of blood vessel thus far examined in vitro in our laboratory (cerebral arteries of cats, dogs,
rabbits, and pigs; coronary arteries of pigs and dogs; mesenteric arteries of dogs and humans; and aortae and renal arteries of rats). The response to ET-1 was affected neither by the antagonists for NE, acetylcholine, serotonin, and histamine nor by the inhibitors of cyclooxygenase and lipoxygenase. Furthermore, the effects of ET-1 were also independent of the presence or absence of endothelial cells. These results suggest that ET-1 is the most potent vasoconstrictor directly acting on the vascular smooth muscle of the human mesenteric artery.

The maximally developed tension induced by ET-1 did not return to the initial level even after repeated washings with fresh solution, indicating that the effect of ET-1 is almost irreversible. However, this phenomenon was not due to irreversible impairment of the tissue, inasmuch as the artery immediately relaxed after application of 5 x 10^-7 M isoproterenol (β-adrenergic agonist) (data not shown). In the specific binding studies in which 125I-labeled ET-1 was used in the porcine coronary artery and rat aorta, 26 it has been shown that dissociation of ET-1 from the membrane is extremely slow. This may partly account for the reasons why ET-1 produces apparently irreversible contraction of arteries in vitro and why it causes a sustained increase in blood pressure when injected intravenously as a bolus in anesthetized rats. 7,12

It has been shown that ET-1–induced vasoconstrictions are extremely sensitive to extracellular Ca2+ and are antagonized by Ca2+ channel blockers on the various blood vessels. 7,13,27–30 In the present study, a small dose of nicardipine, which did not affect the NE-induced vasocontraction, efficiently suppressed the effect of ET-1. Furthermore, ET-1 profoundly augmented Ca2+-induced contraction and reduced the EC50 value of Ca2+ in partially depolarized vascular smooth muscle. These effects of ET-1 are apparently similar to those of K+ depolarization and dihydropyridine Ca2+ channel agonists, such as Bay K 8644. The current studies have indicated that ET-1 markedly augments dihydropyridine-sensitive, voltage-dependent Ca2+ channel currents in freshly dispersed smooth muscle cells from the porcine coronary artery by the whole-cell mode 28 and the cell-attached mode 31 of the patch-clamp technique. It has been reported, however, that the ET receptor is distinct from the dihydropyridine receptor. 26,29,32 Taken together, these data suggest that the contractile action of ET-1 on the human mesenteric artery appears to be closely but not directly associated with activation of voltage-dependent Ca2+ channels. In rat aorta, ET-1 has been shown to elicit vasoconstriction almost independently of the activation of the voltage-dependent Ca2+ channels. 33 In contrast, the present results clearly showed that ET-1–induced vasocontraction on the human blood vessel was sensitive to the dihydropyridine Ca2+ channel blockers. Dihydropyridine Ca2+ channel blockers are considered to be important therapeutic drugs for treatment of cardiovascular diseases, for example, essential hypertension 34–36 and coronary vasospasm. 37–40 The present findings imply that the potent in vivo vasorelaxant actions of dihydropyridine Ca2+ channel blockers may be partly attributed to the antagonism of the action of endogenous ET.

In the present study, the actual presence of ET-1–like immunoreactivity in human mesenteric endothelial cells was clearly exemplified by the immunohistochemical procedure. An autoradiographic study has demonstrated that the high-affinity binding sites for ET-1 localize on the media of human arteries. 41 These findings suggest that ET-1 plays a physiological or pathophysiological role as an endogenous bioactive substance. The splanchnic vascular bed seems to play a central role in the maintenance of systemic blood pressure because of its large capacity for blood distribution. Therefore, if the regional mesenteric vascular bed encounters some disorders, for example, arteriosclerosis, local injury, hypoxia, and the like,
then the endothelial cells would be assumed to be occasionally put in abnormal circumstances that may be somehow analogous to the culture state. In such a situation, the production and release of ET from endothelial cells would be expected to be accelerated since endothelial cells have been shown to produce and release a large amount of ET-1 into the medium when they are cultured. Inasmuch as the effects of ET-1 have been shown to be long lasting in vivo, and also in vitro, ET-1 of mesenteric endothelial origin would produce either prolonged vasoconstriction of the local mesenteric vasculature or a sustained increase in the systemic blood pressure if released. However, actual release of ET-1 in vivo has yet to be demonstrated. Taken together, it can be speculated that ET-1 may be concerned with the pathogenesis of some cardiovascular diseases by a mechanism ultimately involving activation of voltage-dependent Ca\(^{2+}\) channels in human vascular smooth muscle.

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KEY WORDS • endothelin-1 • mesenteric artery • norepinephrine • calcium channel blocker
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