Threshold Concentrations of Endothelin-1 Potentiate Contractions to Norepinephrine and Serotonin in Human Arteries

A New Mechanism of Vasospasm?

Zhihong Yang, MD, Vincent Richard, PhD, Ludwig von Segesser, MD, Erwin Bauer, MD, Peter Stulz, MD, Marko Turina, MD, and Thomas F. Lüscher, MD

Endothelin-1 is an endothelium-derived vasoconstrictor peptide. Its circulating levels are below those known to evoke direct vascular effects. To study whether low concentrations of endothelin-1 potentiate the effects of other vasoconstrictor hormones, we suspended isolated human internal mammary and left anterior descending coronary artery rings in organ chambers for isometric tension recording. In mammary artery rings, the contractions to norepinephrine (3 × 10⁻⁸ M) were potentiated by threshold (3 × 10⁻¹⁰ M) and low concentrations (10⁻⁹ M) of endothelin-1 (96 ± 35% and 149 ± 58% increase from control; p < 0.01 and 0.001; n = 6). The inhibitor of endothelial nitric oxide formation L-N⁵-monomethyl arginine did not affect the potentiating effects of the peptide. The calcium antagonist darodipine (10⁻⁷ M) prevented the potentiation of the response to norepinephrine evoked by endothelin-1. Similarly, contractions to serotonin (10⁻⁷ or 3 × 10⁻⁸ M) were amplified by endothelin-1 (3 × 10⁻¹⁰ M) in the mammary (30 ± 9%) and in the coronary arteries (59 ± 25%). Endothelin-1 (10⁻⁹ M) further potentiated the response (57 ± 23% in mammary and 87 ± 26% in coronary arteries; p < 0.05; n = 7 and 3). The sensitivity of mammary arteries to calcium chloride was markedly enhanced in the presence of endothelin-1 (3 × 10⁻¹⁰ M; concentration shift, eightfold; p < 0.01; n = 5). In contrast to endothelin-1, threshold concentrations of serotonin and norepinephrine (10⁻⁸ M) did not potentiate contractions induced by endothelin-1 (10⁻¹⁰ - 10⁻⁹ M), nor did serotonin augment the effects of norepinephrine. Thus, in human arteries threshold concentrations of endothelin-1 specifically amplify contractions induced by norepinephrine and serotonin by a calcium-dependent mechanism. This may be important in vasospastic syndromes in humans. (Circulation 1990;82:188–195)

Endothelin-1 (human and porcine) is a 21-amino acid peptide that is produced and released by endothelial cells after stimulation with thrombin, transforming growth factor β, adrenalin, phorbol ester, and the calcium ionophore A23187.¹⁻⁶ In isolated blood vessels of various animal species including humans, the peptide possesses potent vasoconstrictor properties that markedly exceed those of other cardiovascular hormones.¹,¹³⁻¹⁴ Thus, endothelin-1 has been implicated in coronary vasospasm, unstable angina, and hypertension.¹⁵⁻¹⁷ However, measurements of the circulating levels of endothelin-1 in normal subjects by a highly specific and sensitive radioimmunoassay revealed concentrations that are below those inducing significant direct pharmacological effects in isolated blood vessels.¹⁸⁻²⁰ However, higher levels of the peptide might be present at the level of the vascular smooth muscle.

Low and threshold levels of the peptide could play an important role, if endothelin-1 would sensitize the blood vessel wall to the effects of other vasoconstric-
tor substances that are found at much higher concentrations than the peptide in the circulating blood or are liberated locally from adrenergic nerves such as norepinephrine²¹ or released from activated platelets such as serotonin.²²⁻²⁶ Indeed, vasoconstrictor sub-
stances can potentiate each other’s action on vascular smooth muscle in some,27–32 but not all animal species.33 Thus, the present study was designed to investigate whether threshold concentrations of endothelin-1 can potentiate the effects of norepinephrine and serotonin in human arteries and, if so, what mechanism might be involved.

Methods

Preparation of Blood Vessels

Internal mammary arteries and veins were harvested intraoperatively from patients undergoing coronary bypass surgery for coronary artery disease.34 Human left anterior descending coronary arteries were obtained from cardiac transplant recipients undergoing transplantation for dilatative cardiomyopathy. The blood vessels were dissected free and cleaned of adherent connective tissue under a dissection microscope (Wild and Leitz AG, Zurich, Switzerland).

Solutions

The arteries were cut into rings (of about 5 mm in length) and placed into cold modified Krebs-Ringer bicarbonate solution (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 0.026 edetate calcium disodium, and 11.1 glucose (control solution). Calcium-free solution containing 2 mM EGTA was made by omitting Ca2+ from the control solution, and Ca2+-free K+-depolarizing solution was prepared by omitting Ca2+ from the control solution and replacing 94 mM NaCl by 94 mM KCl (total potassium concentration, 100 mM).

Experimental Setup

The rings were suspended between two stirrups in organ chambers filled with 25 ml control solution (37°C) aerated with 95% O2-5% CO2 and were connected to force transducers (Statham Universal UC2, Statham Gould, Cleveland, Ohio) and to an anchor. Changes in isometric force were recorded. The internal mammary and coronary arteries were progressively stretched until the optimal point of the length-tension relation was reached.34 The rings were then allowed to equilibrate for 45 minutes. Before the actual experiments, the presence of the endothelium was confirmed in each ring (contracted with norepinephrine 3×10−7 M in internal mammary arteries or with prostaglandin F20, 2×10−6 M in coronary arteries) by a full relaxation to acetylcholine (10−6 M) or bradykinin (10−6 M), respectively.

Protocols

Different blood vessels with endothelium were used to study the responses to norepinephrine or serotonin. Thus, mammary arteries were first exposed either to 3×10−8 M norepinephrine or 10−7 M serotonin (control response). To study the effects of endothelium-derived relaxing factor on the potentiation induced by endothelin-1, some rings were

preincubated with l-N6-monomethyl arginine (l-NMMA; 10−4 M)35 for 30 minutes. Coronary arteries were exposed to 3×10−8 M serotonin (control response). After washout, rings were consecutively incubated with different concentrations of endothelin-1 (10−10, 3×10−10, and 10−9 M) before exposure to the same concentration of norepinephrine or serotonin as under control conditions. In another series of experiments, rings were incubated with a threshold concentration of serotonin (10−8 M) or norepinephrine (10−8 M) instead of endothelin-1 and then reexposed to norepinephrine (3×10−8 M) or endothelin-1 (10−10 to 10−7 M). The time interval between each exposure to the hormones was at least 40 minutes. In certain experiments, the arteries were incubated with the dihydropyridine calcium antagonist darodipine (10−7 M) for 10 minutes.36

In another series of experiments, the rings were washed at least twice for 10 minutes with calcium-free control solution containing 2 mM EGTA. After equilibration, the vessels were exposed to calcium free solution containing 100 mM KCl (see “Preparation of Blood Vessels”). A cumulative concentration-response curve to calcium chloride (10−7 to 10−3 M) was constructed from data obtained in control rings and in preparations, studied in parallel, that were previously incubated with 3×10−10 M of endothelin-1.

Drugs

The following drugs were used: Endothelin-1 (Calbiochem, Lucerne, Switzerland); darodipine (PY 108-068; Sandoz Ltd., Basel, Switzerland); l-N6-monomethyl arginine (Calbiochem); l-norepinephrine bitartrate (Sigma Chemical Co., St. Louis, Mo.); serotonin (5-hydroxytryptamine; Serva, Heidelberg, FRG); EGTA (Sigma Chemical Co.). The concentrations of the drugs are expressed as final molar concentrations in the bath solution. All drugs were dissolved in distilled water except darodipine, which was dissolved in 100% ethanol (stock solution, 10−4 M) and diluted in 5 H2O:4 ethanol (vol:vol). The final concentration of ethanol in the organ chamber was less than 0.5%.

Calculations and Statistical Analyses

Contractions are expressed in absolute tension (g) or as a percentage of the control response. The response to calcium chloride was expressed as a percentage of the maximal response to the cation (10−3 M). The concentration exhibiting 50% of the maximal response to an agonist (EC50 value) was calculated for each ring separately and expressed as negative log M. In the potentiation experiments, the contraction induced by endothelin-1 itself was not included in the measurement of the contraction to norepinephrine or serotonin. Data are given as mean±SEM. In all experiments, n is the number of patients from whom the blood vessels were obtained. The unpaired Student’s t test or analysis of variance (ANOVA) followed by Scheffé’s test for repetitive observations were used for statistical analysis. A
two-tailed p value less than 0.05 was considered to indicate a statistical difference.

Results

Endothelin-1 and Norepinephrine

In isolated internal mammary artery rings with endothelium, norepinephrine (3×10^{-8} M) alone evoked an increase in tension of 1.2±0.3 g (Figures 1 and 2; n=6). Endothelin-1 10^{-10} M did not cause any contractions nor did it augment the contraction to norepinephrine (1.3±0.3 g). Endothelin-1 3×10^{-10} M induced only a marginal response (0.14±0.1 g), but it significantly potentiated the contractions induced by norepinephrine (1.9±0.4 g; p<0.01; 96±35% increase from control; Figures 1 and 2). Endothelin-1 10^{-9} M caused an increase in tension of 0.9±0.4 g and further augmented the contractions induced by norepinephrine (2.1±0.3 g; p<0.001; 149±58% increase from control; Figure 2).

Inhibition of the formation of endothelium-derived nitric oxide from L-arginine by L-NMMA (10^{-4} M) caused a slight increase in tension (0.2±0.1 g), demonstrating the basal formation of nitric oxide (n=7). L-NMMA, however, did not alter the potentiation of the contraction to norepinephrine evoked by endothelin-1 (3×10^{-10} M; Table 1).

Threshold concentrations of norepinephrine (10^{-8} M), which by themselves evoked only a small increase in tension (0.2±0.1 g), did not significantly affect the half-maximal (pD2 value) nor the maximal contraction induced by endothelin-1 (10^{-10} to 10^{-7} M; Table 2).

In contrast to the internal mammary artery, the contractions induced by norepinephrine (10^{-4} M) were not augmented in the presence of endothelin-1 (10^{-11} to 10^{-9} M) in the human internal mammary vein (n=6; data not shown).

Endothelin-1 and Serotonin

In isolated internal mammary arteries with endothelium, serotonin (10^{-7} M) induced an increase in tension evoked only by norepinephrine (p<0.005).

![Figure 1](image1)

**Figure 1.** Tracing of contractile responses to norepinephrine (3×10^{-8} M NE) in an isolated human internal mammary artery under control conditions (left) and after incubation with a threshold concentration of endothelin-1 (3×10^{-10} M ET) (right). The response to norepinephrine was augmented in the presence of endothelin-1 compared with the control conditions (original recording).

![Figure 2](image2)

**Figure 2.** Bar graph of contractile responses to norepinephrine in human internal mammary arteries under control conditions (open bar) and after incubation with various concentrations of endothelin-1 (10^{-10} to 10^{-9} M ET). Threshold concentrations of endothelin-1 significantly augmented the contractions induced by norepinephrine. On the other hand, the calcium antagonist darodipine (10^{-7} M PY 108-068) prevented the augmentation of the contraction to norepinephrine by endothelin-1 (shaded bar). *p<0.05, **p<0.001.

### Table 1. Effects of the Inhibitor of the Formation of Endothelium-Derived Nitric Oxide From L-Arginine L-N{sup}{sup}0-{sup}Monomethyl Arginine on the Potentiation of the Contractions Induced by Norepinephrine by Threshold Concentrations of Endothelin-1 in the Human Internal Mammary Artery With Endothelium

<table>
<thead>
<tr>
<th>Contraction to 3×10^{-8} M norepinephrine (g of tension)</th>
<th>Control</th>
<th>3×10^{-10} M Endothelin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control response</td>
<td>1.0±0.3</td>
<td>1.4±0.3*</td>
</tr>
<tr>
<td>L-N{sup}0-{sup}Monomethyl arginine (10^{-4} M)</td>
<td>1.1±0.3</td>
<td>1.5±0.2*</td>
</tr>
</tbody>
</table>

Data are mean±SEM of seven experiments.

*Statistically significant difference between the response to norepinephrine in the presence and absence of endothelin-1 (p<0.005).

### Table 2. Effects of Threshold Concentrations of Norepinephrine and Serotonin on the Contractile Responses Evoked by Endothelin-1 (10^{-10} to 10^{-7} M) in Human Internal Mammary Arteries With Endothelium

<table>
<thead>
<tr>
<th>Endothelin-1</th>
<th>pD{sub}2*</th>
<th>Maximal response†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.2±0.2</td>
<td>100±6%</td>
</tr>
<tr>
<td>Serotonin</td>
<td>8.2±0.2</td>
<td>95±4%</td>
</tr>
<tr>
<td>Control</td>
<td>7.9±0.2</td>
<td>104±10%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>8.2±0.2</td>
<td>105±10%</td>
</tr>
</tbody>
</table>

Data are mean±SEM of six or seven experiments.
pD{sub}2, negative log molar concentration of endothelin-1 causing 50% of the contraction induced by 100 mM potassium.

†Constrictions are expressed as percent of the increase in tension evoked by 100 mM KCl.
tension of 1.4±0.3 g (Figure 3; n=7). Subthreshold concentrations of endothelin-1 10⁻¹⁰ M augmented the contractions to serotonin (1.7±0.3 g) but not significantly. Threshold (3×10⁻¹⁰ M) and low concentrations of endothelin-1 (10⁻⁹ M) significantly augmented the contractions induced by serotonin to 1.8±0.3 and 1.9±0.3 g, respectively (30±9% and 57±23% increase from the control; Figure 3; p<0.05).

Similar results were obtained in human left anterior descending coronary arteries (Figure 4). The contractions to serotonin (3×10⁻⁸ M) increased from 2.3±0.4 to 3.8±1.1 g in the presence of 3×10⁻¹⁰ M endothelin-1 (59±25% increase from control) and to 4.4±1.2 g with 10⁻⁹ M of the peptide (87±26% increase; p<0.05 compared with the control response; n=3).

In internal mammary arteries, threshold concentrations of serotonin (10⁻⁸ M), which by themselves induced a small increase in tension (0.3±0.1 g), did not augment the contractions elicited by endothelin-1 (10⁻¹⁰ to 10⁻⁷ M; Table 2).

**Norepinephrine and Serotonin**

In certain experiments, a possible interaction between norepinephrine and serotonin was studied.

In isolated mammary artery rings with endothelium, the contraction induced by norepinephrine (3×10⁻⁸ M) did not differ in the presence of serotonin (3×10⁻⁸ M). Although the monoamine by itself evoked a small increase in tension (0.3±0.1 g), the contraction induced by norepinephrine in the presence of the monoamine (0.8±0.1 g; n=5) was identical to that obtained under control conditions (0.8±0.2 g; n=5).

**Endothelin-1 and Calcium**

**Calcium sensitivity.** In arteries with endothelium suspended in Ca²⁺-free control solution containing 100 mM KCl, calcium chloride (10⁻⁷ to 10⁻⁵ M) evoked concentration-dependent contractions (Figure 5, n=5). In the presence of endothelin-1 (3×10⁻¹⁰ M), the sensitivity of the blood vessels to calcium chloride was significantly enhanced. The half-maximal concentration of calcium chloride (EC₅₀) in the absence of endothelin-1 was 4.1±0.2, but it was 5.0±0.2 in the presence of the peptide (Figure 5; concentration shift at EC₅₀, eightfold; p<0.01).

**Calcium channel blocker.** The dihydropyridine calcium antagonist darodipine (10⁻⁷ M) did not affect
the contractions induced by endothelin-1 $10^{-9}$ M (0.8±0.4 g) nor those induced by a higher concentration of the peptide ($10^{-7}$ M; 77±5% and 71±18% of the contraction to 100 mM KCl in the presence and absence of darodipine; $n=3$–10). However, the augmentation of the contraction to norepinephrine induced by endothelin-1 ($10^{-7}$ M) was prevented by darodipine ($10^{-7}$ M; Figure 2; 1.4±0.2 g; $n=6$; $p<0.05$ vs. the response to norepinephrine in the presence of endothelin-1 $10^{-9}$ M, but not significant vs. the control response to norepinephrine).

**Discussion**

The present study demonstrates that in the human internal mammary artery threshold concentrations of endothelin-1 potentiate the vasoconstrictor effects of norepinephrine and serotonin. Because endothelin-1 sensitized the vascular smooth muscle of the human arteries to extracellular calcium and because the calcium antagonist darodipine prevented the potentiating effects of the peptide, a calcium-dependent mechanism must be involved.

An amplification of the effects of vasoconstrictor substances by low concentrations of other hormones and autacoids has been described for serotonin in the caudal artery and in the perfused kidney of the rat, and in the ear and femoral artery of the rabbit. In the canine femoral artery, however, no significant interaction between α-adrenergic and serotoninergic receptors has been noted, indicating that amplification responses depend on the species and blood vessels studied and the agonists used. This study demonstrates that endothelin-1 potentiates contractions induced by norepinephrine and serotonin in human arteries. Unlike the femoral artery of the rabbit where most agonists can augment each other’s action, the potentiating effects of endothelin-1 in the human internal mammary artery were specific for the peptide, because low concentrations of serotonin did not augment contractions induced by either norepinephrine or endothelin-1 and because threshold concentrations of norepinephrine did not potentiate the contractions evoked by the peptide.

The endothelial layer not only produces endothelin-1,6–8 but also endothelium-derived nitric oxide, which is a potent vasodilator. The production of endothelium-derived nitric oxide from L-arginine can be inhibited by a methylated form of the amino acid L-NMMA. In human arteries, endothelium-derived nitric oxide must be continuously formed, because L-NMMA evoked endothelium-dependent contractions as it has been reported in arteries of experimental animals. Inhibition of the endothelial L-arginine pathway with L-NMMA, however, did not augment the potentiating effects of endothelin-1 on the contractions induced by norepinephrine. In line with this observation in the human internal mammary artery, endothelial removal only minimally enhances the direct contractile effects of endothelin-1 at high concentrations of the peptide.

Endothelin-1 has been proposed as an endogenous activator of voltage-operated calcium channels. More recent studies, however, have demonstrated that the peptide does not interfere with the binding of dihydropyridine calcium antagonists to vascular smooth muscle membranes. In addition, the sustained contractions induced by the peptide differ from those caused by the calcium channel activator Bay K 8644. Thus, endothelin-1 most likely binds to a specific receptor on vascular smooth muscle cells. In line with that interpretation, the dihydropyridine calcium antagonist darodipine did not prevent the direct contractions induced by low or medium concentrations of endothelin-1 in the human internal mammary artery. Similar observations have been made in most, but not all arteries obtained from experimental animals with different calcium antagonists.

However, darodipine prevented the potentiation of the response to norepinephrine by endothelin-1, indicating that influx of extracellular calcium through voltage-operated calcium channels importantly contributes to the potentiating effects of the peptide. Indeed, endothelin-1 markedly augmented the sensitivity of depolarized vascular smooth muscle to extracellular calcium. Thus, the indirect (potentiating) vascular effects of endothelin-1 may involve activation of voltage-operated calcium channels, as it has been proposed in the porcine coronary artery. In addition, the peptide releases calcium from intracellular stores and in turn may reduce the buffering effects of the superficial sarcoplasmic reticulum during influx of extracellular calcium.

Activation of voltage-operated calcium channels may occur either directly (after activation of the endothelin-1 receptor, most likely involving a G protein linked to the channels) or indirectly through changes in membrane potential evoked by an agonist (involving nonspecific ion channels). In canine veins, the depolarization induced by endothelin-1 occurs at lower concentrations than in arteries. Because membrane potential of the human arteries was not measured in this study, a contribution of this vascular action of endothelin-1 to its potentiating effects cannot be excluded. However, the fact that changes in membrane potential have been reported at much higher concentrations than those required to potentiate the contractions induced by norepinephrine or serotonin in human arteries argues against this hypothesis. Indeed, if changes in membrane potential would mediate these effects, one would expect a more pronounced response in veins than in arteries. However, in contrast to human arteries, the potentiating effects of endothelin-1 could not be demonstrated in human veins (i.e., human internal mammary vein). Thus, the potentiating effects of endothelin-1 in human arteries most likely involve direct activation of voltage-operated calcium channels.

The amplifying effects of endothelin-1 may have important clinical implications. Thus, even slight
elevations of circulating (as demonstrated in the coronary sinus of patients with coronary artery disease) and particularly local endothelin-1 concentrations at the level of the smooth muscle may profoundly affect vascular tone and in turn local blood flow. In cultured endothelial cells and the intact porcine aorta, the formation and secretion of the peptide can be stimulated by thrombin, and in the former, formation and secretion can be stimulated by tumor-transforming factor β and epinephrine. In unstable angina and myocardial infarction, local platelet activation occurs. Thus, at sites where platelets are activated, thrombin, which is formed after activation of the coagulation cascade, and tumor transforming factor β, which is released from platelets, are abundantly present and would stimulate the formation of the peptide. Serotonin is also released from platelets under these conditions, and its vasoconstrictor action is amplified by endothelin-1. This may be particularly important, because, in contrast to dog and pig arteries, the monoamine does not induce endothelium-dependent relaxation in human coronary and internal mammary arteries. Thus, endothelin-1 is also released from platelets. The human coronary artery and the internal mammary artery can undergo spasm, which is a clinically relevant problem immediately after coronary bypass surgery. In the pig coronary artery, endothelial damage and regeneration are associated with endothelium dysfunction and vasospasm. Similar changes may occur in certain arterial bypass grafts after surgical handling. If endothelin-1 were produced by functionally altered endothelial cells, it can contribute to the development of vasospasm, particularly if the sympathetic nervous system or platelets are activated.

Indeed, in the human coronary circulation, ergonovine, which activates serotoninergic receptors, is the most reliable substance to provoke vasospasm in patients with variant angina.

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