Potentiation of Norepinephrine-Induced Contractions by Endothelin-1 in the Rabbit Aorta

Daniel Henrion, Ismail Laher

Subthreshold concentrations of endothelin-1 potentiated the norepinephrine-induced contraction in isometrically mounted rings of the rabbit aorta. Pretreatment with endothelin-1 (0.1 nM) for 10 minutes increased the sensitivity of the aortic rings to norepinephrine without affecting the maximal contraction. This amplification was unaffected by removal of the endothelium but was prevented by the protein kinase C inhibitors staurosporine (0.01 μM) and calphostin C (0.1 μM). Pretreatment of the aortic rings for 24 hours with phorbol 12-myristate 13-acetate (0.1 nM) also abolished the potentiation. Norepinephrine-induced contraction was potentiated by pretreating with phorbol 12-myristate 13-acetate (10 nM) and by increasing the concentration of K+ in the bath solution from 4.6 to 8.6 mM. The potentiation of the norepinephrine-induced contraction by endothelin-1 (0.1 nM) or by phorbol 12-myristate 13-acetate (10 nM) was not associated with an increase in norepinephrine-induced 45Ca2+ uptake or influx, whereas the potentiation due to an increase in the concentration of K+ in the bath solution from 4.6 to 8.6 mM was associated with an increase in norepinephrine-induced 45Ca2+ uptake. We conclude that endothelin-1 potentiation of the norepinephrine-induced contraction occurs in the absence of changes in stimulated Ca2+ entry and is endothelium independent. It is probable that endothelin-1 increases the sensitivity of the contractile apparatus to Ca2+ by activating protein kinase C-dependent mechanisms. (Hypertension 1993;22:78-83)

KEY WORDS • endothelins • drug synergism • aorta • norepinephrine • protein kinase C • calcium

Endothelin-1 (ET-1) is a potent vasoconstrictor produced by endothelial cells. Its direct contractile effect has been widely studied, although it is not yet completely understood. On the other hand, subthreshold concentrations of ET-1, compatible with the plasma level, potentiate the contractile effect of different agonists such as norepinephrine in human arteries and rat mesenteric beds and serotonin in rat aortic rings and pulmonary arteries. Such a phenomenon might have a pathological importance since it could be involved in hypertension and vasospastic syndrome. Indeed, the potentiation of serotonin-induced contractions by ET-1 might increase the vasospasms induced by aggregating platelets. Moreover, there is a positive correlation between ET-1 plasma level and systolic blood pressure in humans, and the vascular production of ET-1 under angiotensin II (Ang II) stimulation is higher in hypertensive rats. The amplification is nevertheless blocked by Ca2+ entry blocker in human arteries and in the rat mesenteric bed. In contrast, the direct effect of ET-1 is relatively insensitive to Ca2+ entry blockers.

Finally, low concentrations of ET-1 have been shown to activate the conversion of angiotensin I into Ang II in endothelial cells. We have previously shown in the rabbit facial artery, a muscular blood vessel, that Ang II potentiates the contractions induced by norepinephrine, histamine, and caffeine (in the absence of extracellular Ca2+). The potentiation depends on protein kinase C (PKC) activation and is not associated with changes in 45Ca2+ uptake. A similar phenomenon was found in the rabbit aorta.

In the present study, we investigated the role of PKC in the phenomenon of potentiation by ET-1 of the norepinephrine-induced contractions. In addition, we compared the increase in 45Ca2+ uptake induced by norepinephrine after potentiation by ET-1, by the PKC activator phorbol 12-myristate 13-acetate (PMA), or by an increase in K+ in the extracellular medium.

The present study suggests that ET-1 amplified the norepinephrine-induced contraction without changing the 45Ca2+ uptake. The amplification was prevented by PKC inhibition and was not affected by the removal of the endothelium. Our results suggest that vascular intracellular sensitivity to Ca2+ is enhanced during contractile amplification by ET-1 through a PKC-dependent mechanism.

Methods

Aorta Preparation

The thoracic aorta was isolated from adult New Zealand male rabbits (2-4 kg) that were exsanguinated...
and decapitated under pentobarbital anesthesia (50 mg/kg IV combined with heparin 1000 IU/kg). Ring segments 3 mm in length were cleaned of fat and connective tissues and were mounted between two stainless steel wires in a 30-mL organ bath containing physiological salt solution (PSS) of the following composition (in mM): NaCl 160, KCl 4.6, CaCl₂ 1.5, MgSO₄ 1.2, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) 5.0, and glucose 11.0. The pH of the PSS was adjusted to 7.4 with NaOH (1 M), and the solution was bubbled with 100% oxygen. The PSS contained propanolol (1 µM) to inhibit β-adrenergic receptors and desmethyllumipramine (0.1 µM) and deoxycortico-
steroide (10 µM) to reduce neuronal and extraneuronal norepinephrine uptakes. A PSS containing 8.6 mM K⁺ was prepared by increasing the amount of KCl and by proportionally decreasing the amount of NaCl in the PSS. One wire was attached to a fixed support, and the second wire was connected to a moveable holder supporting a tension transducer (model FT 03, Grass Instruments, Inc., Quincy, Mass) so that isometric force measurements could be recorded on a physiograph (model SD90, Grass). The artery segments were allowed to recover for 60 minutes, and the PSS was replaced at 15-minute intervals during this time. After this recovery period, a 1 g preload, resulting in optimal stretch, was applied to the aortic segments, which were then allowed to equilibrate for an additional 90 minutes.

**Contractile Response to Norepinephrine**

A concentration-response curve to norepinephrine was made by cumulative additions of norepinephrine to the PSS. After the maximal response was obtained, the PSS was replaced several times until tissues returned to baseline tension. The PSS was replaced at 15-minute intervals throughout the experiment. Several dose-response curves to norepinephrine were performed at 90-minute intervals until successive concentration-response curves no longer changed in sensitivity. A final concentration-response curve to norepinephrine was then made after pretreatment of the ring segments with ET-1 (0.1 nM) or PMA (10 nM) for 10 minutes or after changing the PSS for a PSS containing K⁺ (8.6 mM) for 10 minutes. Control segments did not receive ET-1 (0.1 nM), PMA (10 nM), or K⁺ (8.6 mM). Some tissues were pretreated with staurosporine (0.01 µM) for 20 minutes or calphostin C (0.1 µM) for 50 minutes before the addition to ET-1 (0.1 nM) or PMA (10 nM) for 10 minutes or before changing the PSS for one containing K⁺ (8.6 mM) for 10 minutes. In some other ring segments, the endothelium was removed by gently rubbing the inner surface of the artery wall. The absence of endothelial function was confirmed by the absence of relaxation to acetylcholine (10 µM) after precontraction with norepinephrine (1 µM). In control segments, acetylcholine (10 µM) caused a maximum relaxation. In all experiments, time-dependent changes in tissue sensitivity were determined by carrying out parallel experiments with dimethylsulfoxide, the solvent vehicle for staurosporine and calphostin C, or ethanol, the solvent for PMA.

**Pretreatment of Aorta With PMA**

Tissues were incubated for 24 hours at 4°C in a PSS containing the phorbol ester PMA (0.1 µM). This protocol has been described previously. Control segments were incubated 24 hours at 4°C in normal PSS. In some experiments, PMA was replaced by the inactive phorbol ester 4a-PMA (0.1 µM).

**Calcium-45 Uptake**

Measurements of the uptake of ⁴⁵Ca²⁺ were made at 37°C using the methods described by Meisheri et al. and modified so that ⁴⁵Ca²⁺ uptake and wall force could be measured simultaneously. After stabilization of the contractile response to norepinephrine (0.1 µM), ⁴⁵CaCl₂ (0.67 µCi/mL) was added to the PSS for 90 minutes. Tissues were then exposed to norepinephrine (0.1 µM) for 3 minutes, either in the absence or presence of ET-1 (0.1 nM), PMA (10 nM), or K⁺ (8.6 mM). Resting values of ⁴⁵Ca²⁺ uptake were determined by adding ⁴⁵CaCl₂ (0.67 µCi/mL) to the PSS for 93 minutes and by omitting the addition of norepinephrine (0.1 µM). In some experiments, arteries were incubated with ⁴⁵CaCl₂ for 93 minutes and ET-1 (0.1 nM), PMA (10 nM), or K⁺ (8.6 mM). After exposure to ⁴⁵CaCl₂, either with or without norepinephrine and/or ET-1 (0.1 nM), PMA (10 nM), (0.1 µM), PMA (10 nM), or K⁺ (8.6 mM), the ⁴⁵Ca²⁺ content was determined as described below.

**Calcium-45 Influx**

Unidirectional influx measurements were made according to the method of Meisheri et al. After the response to norepinephrine (1 µM) reached a maintained level, in either the absence or the presence of endothelin (0.1 nM, 10 minutes), the tissues were exposed to a PSS containing ⁴⁵CaCl₂ (0.67 µCi/mL) but otherwise identical to the experimental solution. After exposure to ⁴⁵Ca₂⁺ for 90 seconds, ⁴⁵Ca²⁺ content was determined as described below.

**Calcium-45 Content**

The PSS containing ⁴⁵CaCl₂ (0.67 µCi/mL) was changed for an ice-cold Ca²⁺-free PSS containing EGTA (0.1 mM). Simultaneously, the organ bath temperature was decreased to 0°C. Tissues were then removed and placed in 100 mL of ice-cold bubbled PSS for 45 minutes to remove the extracellularly free bound ⁴⁵Ca²⁺. Artery segments were then blotted dry, weighed, and incubated overnight in EDTA (5 mM) before counting in a liquid scintillation counter (model LS 7800, Beckman Instruments, Carlsbad, Calif). Values of net uptake of ⁴⁵Ca²⁺ are expressed as micromoles per kilogram and as micromoles per kilogram per minute for ⁴⁵Ca²⁺ influx measurement.

**Statistical Analysis**

Results are expressed as mean±SEM. EC₅₀ was calculated for each individual concentration-response curve to norepinephrine after log/logit transformation. Comparisons between groups were made using a one-way analysis of variance followed by a Scheffé’s F test when significant. A value of P<.05 was considered significant.

**Drugs**

ET-1, norepinephrine, captopril, PMA, and 4a-PMA were purchased from Sigma Chemical Co., St. Louis, Mo. Staurosporine was purchased from Kyowa Hakko USA Inc., New York, NY. Calphostin C was purchased.
from Kamiya Biomedical Co., Thousand Oaks, Calif. All other reagents were of analytical grade. Stock solutions of norepinephrine contained hydrochloric acid (0.01 M). Staurosporine and calphostin C were dissolved in dimethylsulfoxide. PMA and 4α-PMA were dissolved in pure ethanol. 45CaCl2 (23.1 mCi/mg) was purchased from New England Nuclear, Boston, Mass.

Results

Effect of Endothelin-1 on Norepinephrine-Induced Contractions

Norepinephrine contracted the aortic ring segments (Fig 1) with an EC50 of 0.48±0.05 μM (n=11) and a maximal response of 7.3±0.28 g (n=11). ET-1 (0.1 nM) pretreatment did not modify the maximum force obtained (7.53±0.15 g, n=8) but increased the sensitivity of norepinephrine (Table 1). Pretreatment of the aorta with staurosporine (0.01 μM) or calphostin C (0.1 μM) affected neither the maximum response to norepinephrine nor the EC50 but did abolish the potentiation effect of ET-1 (0.1 nM) (Fig 1). The norepinephrine EC50 in the presence of ET-1 (0.1 nM) after staurosporine (0.01 μM) or calphostin C (0.1 μM) pretreatment was not significantly different from the control values (Table 1).

The expression of the concentration-response curve to norepinephrine after ET-1 (0.1 nM) as a percentage of the control concentration-response curve (Fig 1) allowed the quantification of the potentiating effect of ET-1. It was the highest when the concentration of norepinephrine was low. Potentiation by ET-1 (0.1 nM) expressed as a percentage of the control values was 314±53%, 219±28%, 206±28%, and 131±10% at norepinephrine concentrations of 0.01, 0.03, 0.1, and 0.3 μM, respectively (n=8 per group, P<.05) when the data were expressed as a percentage of the control.

Removal of the endothelium did not affect the maximum response to norepinephrine (7.35±0.40 g, n=5 versus 7.30±0.28 g, n=11), but it decreased the EC50 (Table 1) and suppressed the ability of acetylcholine (10 μM) to relax the norepinephrine (1 μM)-induced contraction (−5±5% versus 85±12% relaxation before removal of the endothelium, n=5, P<.05). However, the removal of the endothelium did not affect the potentiating effect of ET-1 (0.1 nM): after endothelium remov-
at 4°C in PSS containing PMA (0.1 μM) did not change the maximal response (7.35±0.25 g, n=5), it significantly increased the EC50 (Table 1). This procedure abolished the contractile response to PMA (1 μM), which was 2.20±0.42 g in the control (n=6) and 0.15±0.10 g (n=5, P<.05) after preincubation. The inactive phorbol ester 4α-PMA (0.1 μM) did not affect either the concentration-response curve to norepinephrine or the potentiation effect of ET-1 (0.1 nM) (not shown).

The potentiation of the norepinephrine-induced contraction by ET-1 (0.1 nM) was still present after 24 hours at 4°C (Fig 1) as well as after preincubation with 4α-PMA (expressed as a percentage of the control, it was 285±80%, 244±70%, and 145±25% at norepinephrine concentrations of 0.03, 0.1, and 0.3 μM, respectively, n=4 per group, P<.05). By contrast, preincubation of the aorta segments with PMA (0.1 μM) for 24 hours at 4°C greatly attenuated the amplification of the norepinephrine-induced contraction by ET-1 (0.1 nM) (Fig 1).

\[ 4Ca^{2+} Uptake \]

\[ 4Ca^{2+} \] uptake was determined in rabbit aortic ring segments at rest or after norepinephrine (0.1 μM). Exposure to norepinephrine (0.1 μM) for 3 minutes significantly increased \[ 4Ca^{2+} \] uptake (Table 2). This increase was not influenced by prior exposure to ET-1 (0.1 nM) despite an increase in force due to potentiation effect of ET-1 (0.1 nM). Exposure of the segments to ET-1 (0.1 nM) alone had no effect on \[ 4Ca^{2+} \] uptake (Table 2). The expression of the increase in \[ 4Ca^{2+} \] uptake due to norepinephrine per unit of the corresponding force developed (micromoles per kilogram of weight per gram of force developed) showed that the Ca^{2+} requirement to develop 1 g of wall force was 2.1-fold less after pretreatment of the aorta with ET-1 (0.1 nM) (18.3±2.9 μmol/kg per gram, n=17, to 8.7±1.6 μmol/kg per gram, n=13, P<.05; Fig 2).

The increase in \[ 4Ca^{2+} \] uptake due to norepinephrine (0.1 μM) was not influenced by a prior exposure to PMA (10 nM for 10 minutes) despite an increase in force due to potentiation effect of PMA (10 nM, Table 2). Exposure of the segments to only PMA (10 nM) did not change the \[ 4Ca^{2+} \] uptake (Table 2). As for ET-1, the expression of the \[ 4Ca^{2+} \] uptake per unit force developed showed that the Ca^{2+} requirement for 1 g of wall force was 1.6-fold less after pretreatment of the aorta with PMA (10 nM) (18.3±2.9 μmol/kg per gram, n=17, to 11.4±12.0 μmol/kg per gram, n=8, P<.05; Fig 2).

In contrast, as the amount of K⁺ in the PSS increased from 4.0 to 8.6 mM, amplifying the norepinephrine (0.1 μM)-induced contraction (Table 2), the corresponding \[ 4Ca^{2+} \] uptake also increased (Table 2). Moreover, the \[ 4Ca^{2+} \] uptake per unit force developed was significantly increased (18.3±2.9 μmol/kg per gram, n=17, to 32.9±6.5 μmol/kg per gram, n=5, P<.05; Fig 2).

\[ 4Ca^{2+} \] Influx

The resting level of unidirectional \[ 4Ca^{2+} \] influx was 26.61±0.96 μmol/kg per minute (n=30) in ring segments of aorta exposed to \[ 4Ca^{2+} \] for 90 seconds. Pretreatment of arteries with ET-1 (0.1 nM) for 10 minutes did not alter the resting influx of \[ 4Ca^{2+} \], which was 30.08±1.78 μmol/kg per minute (n=6). Norepinephrine (1 μM) caused a significant increase in unidirectional \[ 4Ca^{2+} \] influx (55.38±3.62 μmol/kg per minute, n=21) that was not altered (50.89±1.73 μmol/kg per minute).

**Table 2. \[ 4Ca^{2+} \] Uptake in Rabbit Aorta Rings and Effect of Endothelin-1, Phorbol Myristate Acetate, and K⁺**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Force (g) before treatment</th>
<th>Force (g) after treatment</th>
<th>[ 4Ca^{2+} ] uptake (μmol/kg) after treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>1.38±0.13</td>
<td>1.49±0.14</td>
<td>127.9±7.6 (17)*</td>
<td>100.7±5.4 (9)</td>
</tr>
<tr>
<td>ET-1 (0.1 nM)</td>
<td>1.23±0.14</td>
<td>1.65±0.15*</td>
<td>110.3±5.6 (13)*</td>
<td>95.9±6.6 (9)</td>
</tr>
<tr>
<td>PMA (10 nM)</td>
<td>1.33±0.08</td>
<td>1.91±0.15*†</td>
<td>125.0±10.0 (8)*</td>
<td>103.3±6.3 (6)</td>
</tr>
<tr>
<td>K⁺ (8.6 mM)</td>
<td>0.90±0.35</td>
<td>1.58±0.14*</td>
<td>150.0±19.5 (5)*</td>
<td>98.0±5.4 (8)</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; PMA, phorbol myristate acetate. The increase in \[ 4Ca^{2+} \] uptake was determined before (first column) and after pretreatment with either ET-1 (0.1 nM), PMA (10 nM), or K⁺ (8.6 mM) (second column). \[ 4Ca^{2+} \] uptake was determined after the pretreatment in response to norepinephrine (third column, in which the \[ 4Ca^{2+} \] uptake corresponds to the increase wall force given in second column) or after the pretreatment in the absence of norepinephrine (fourth column). Values represent mean±SEM. Numbers in parentheses are number of rings.

*P<.05, one-way analysis of variance (ANOVA) compared with the corresponding control (column 2 vs column 1 and column 3 vs column 4).

†P<.05, one-way ANOVA compared with solvent.

![Fig 2. Bar graph shows changes in the \[ 4Ca^{2+} \] uptake per unit force developed by ring segments of aorta in response to norepinephrine (0.1 μM) alone (Control, n=17) or after endothelin-1 (ET-1) (0.1 nM, n=13), phorbol myristate acetate (PMA) (10 nM, n=8), or K⁺ (8.6 mM, n=5). Values are mean±SEM. *P<.05, one-way analysis of variance, compared with the control.](http://hyper.ahajournals.org/)

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The ability of small increases in extracellular K+ to release of norepinephrine from the rat mesenteric artery have shown that ET-1 potentiated norepinephrine-induced contraction while decreasing the sensitivity of intracellular contractile mechanisms associated with Ca2+-dependent vasoconstriction, providing a nonspecific mechanism for agonist amplification. The present study showed that staurosporine and calphostin C, two mechanistically distinct inhibitors of PKC, suppress the amplifying effect of ET-1. We have also previously shown a similar effect of these inhibitors on the Ang II potentiating property in the rabbit facial artery and aorta. Thus, Ang II and ET-1 might share a common mechanism to potentiate the norepinephrine-induced contraction. The concentrations and incubation times of staurosporine and calphostin C used in the present study were similar to those used in the latter studies. In all cases, they did not affect the norepinephrine-induced contraction. Moreover, in such conditions, they presumably do not inhibit myosin light chain kinase activity since they do not influence the response to readmission of Ca2+ in the depolarized rabbit femoral artery, basilar artery, and aorta, as well as in rat midcerebral artery and mesenteric arteries. The immunohistochemical and functional studies have shown that calphostin C is more specific for PKC than staurosporine.

Our results indicate that after pretreatment with PMA for 24 hours at 4°C, the potentiating effect of ET-1 in the rabbit aorta was abolished. A similar protocol decreases the contractility to serotonin, norepinephrine, K+, and phorbol esters in dog carotid artery rings without decreasing the PKC activity. By contrast, in our present study, as well as in a previous one, the contractile response to PMA (1 μM) and the potentiation phenomenon were abolished after this procedure. In isolated cells, chronic exposure to phorbol esters has been shown to downregulate PKC as a consequence of an increased rate of degradation of the enzyme. Such a loss of PKC activity was not observed by Merckel et al in carotid segments, suggesting either a difference due to the technique (isolated cells versus whole artery segment) or a difference in the PKC subtypes involved. Hunwiler et al have shown a different recovery of PKC isoforms after a 24-hour treatment with a phorbol ester, and in rabbit aortic cells, the subtype III of PKC disappears completely after such a treatment. The potentiation phenomenon we observed could depend on a PKC subtype sensitive to such a procedure since no more contraction to PMA was detected in the present study after incubation (24 hours at 4°C) with PMA (0.1 μM). Such an issue should be examined by measuring the PKC isoforms involved in the potentiation phenomenon. The specificity of action of PMA was controlled by the lack of downregulation effect (contraction to PMA and potentiation phenomenon still present) after incubation with the inactive phorbol ester 4α-PMA.

Additional support for a role of PKC activation in the potentiation effect of ET-1 is provided by the demonstration that the exogenous acute activation of PKC by PMA amplified the norepinephrine-induced contraction while decreasing the ratio of Ca2+ uptake per unit of force (present study). This reproduced the amplification due to ET-1. Particularly, in both cases, less Ca2+ was necessary to obtain a contraction on addition of norepinephrine. Phorbol esters have also been shown to amplify the vasoconstriction due to serotonin, by adrenergic receptor stimulation, and to K+ depolariza-
In porcine coronary arteries, both ET-1 and a phorbol ester amplify serotonin-induced contraction without any significant increase in free cytosolic Ca$^{2+}$,18 a finding that is in keeping with our observation that ET-1 and PMA potentiate norepinephrine-induced contraction without increasing the uptake of $[^{45}]$Ca$^{2+}$.

In a recent study made in the rat, Yoshida et al.22 demonstrated a pressor response when Ang II and ET-1 were infused in combination and not separately. However, combined infusion of ET-1 and norepinephrine failed to increase blood pressure. This contrasts with our findings, since our results and those of others, eg, Yang et al.,4 Consigny,7 and Dohi et al.,8 indicate a nonspecific increase in vascular constriction after exposure to ET-1.

In conclusion, we provided evidence that ET-1-induced potentiation of the norepinephrine-induced contraction depends on the activation of PKC without involving an increase in Ca$^{2+}$ uptake in the vascular smooth muscle cells.

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