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

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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 vasoconstrictor effect 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 than in normotensive 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.

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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+. 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, Na2HPO4 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 propranolol (1 μM) to inhibit β-adrenergic receptors and desmethylimipramine (0.1 μM) and deoxycorticosterone (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 and desmehtylimipramine (0.1 μM) and deoxycorticosterone (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) for 45 minutes 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 Scheffe’s F test when significant. A value of P<.05 was considered significant.

Drugs

ET-1, norepinephrine, captopril, PMA, and 4α-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...
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 to 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 potentiating 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 removal, the potentiating effect of ET-1 (0.1 nM) was 300±58%, 325±75%, 238±38%, and 131±10% at norepinephrine concentrations of 0.01, 0.03, 0.1, and 0.3 μM, respectively (n=5 per group, P<.05).

Preincubation of the Aorta With PMA

After 24 hours at 4°C in a PSS, the concentration-response curves were modified. The maximal response was 6.80±0.39 g (n=6), and the EC50 was 0.46±0.07 μM (n=6). However, although incubation of the segments for 24 hours
ET-1, endothelin-1; PMA, phorbol myristate acetate. The increase in wall force due to norepinephrine (0.1 μM) 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). 45Ca2+ uptake was determined after the pretreatment in response to norepinephrine (third column, in which the 45Ca2+ uptake corresponds to the increase in 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.

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) (not shown).

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45Ca2+ Uptake

45Ca2+ 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 45Ca2+ 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) (Fig 1). Exposure of the segments to only PMA (10 nM) did not change the 45Ca2+ uptake (Table 2). As for ET-1, the expression of the 45Ca2+ uptake per unit force developed showed that the Ca2+ 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 4.6 to 8.6 mM, amplifying the norepinephrine (0.1 μM)-induced contraction (Table 2), the corresponding 45Ca2+ uptake also increased (Table 2). Moreover, the 45Ca2+ 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).

45Ca2+ Influx

The resting level of unidirectional 45Ca2+ influx was 26.61±0.96 μmol/kg per minute (n=30) in ring segments of aorta exposed to 45Ca2+ for 90 seconds. Pretreatment of arteries with ET-1 (0.1 nM) for 10 minutes did not alter the resting influx of 45Ca2+, which was 30.08±1.78 μmol/kg per minute (n=6). Norepinephrine (1 μM) caused a significant increase in unidirectional 45Ca2+ influx (55.58±3.62 μmol/kg per minute, n=21) that was not altered (50.89±1.73 μmol/kg per minute after pretreatment with ET-1 (0.1 nM) for 10 minutes). The corresponding increase in 45Ca2+ uptake also 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).

**Fig 2.** Bar graph shows changes in the 45Ca2+ 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.
The ability of small increases in extracellular K+ to release of norepinephrine from the rat mesenteric artery produced contractions together with a decrease in the open state probability of voltage-gated Ca2+ channels, and might not depend on alterations in release and possibly amplification of vascular responses by Ang II and ET-1 due to the release of Ca2+ from internal stores.

The transient contractile response to caffeine is as demonstrated by Nelson et al. The present study provides evidence that the amplification of vascular tone due to norepinephrine by a subthreshold concentration of ET-1 depends on a voltage-gated intracellular Ca2+ influx, and is independent of factors released by the endothelium. Moreover, the modulation of blood vessel tone by ET-1 might be of physiological and pathological importance since it takes place at sub-threshold concentrations compatible with circulating ET-1 levels and since this phenomenon might be involved in hypertension and vasospastic syndromes.

The possibility that ET-1 amplifies the norepinephrine-induced contraction by depolarizing vascular smooth muscle cells and consequently by increasing the influx of Ca2+ into these cells during the contraction elicited by norepinephrine is unlikely since ET-1 (0.1 nM) and PMA (10 nM) both potentiated the norepinephrine-induced contraction without increasing the associated uptake or influx of 45Ca2+. A small increase in the amount of K+ in the extracellular medium potentiated both the norepinephrine-induced contraction and uptake of 45Ca2+ from the extracellular medium. This is consistent with our previous finding that another vasoactive peptide, Ang II, amplifies the vasoconstriction due to serotonin, thus providing a nonspecific mechanism for agonist amplification. The present study demonstrates that the exogenous acute activation of PKC by PMA amplifies 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, adrenergic receptor stimulation, and to K+ depolarization.
In porcine coronary arteries, both ET-1 and a phorbol ester amplify serotonin-induced contraction without a significant increase in free cytosolic Ca\(^{2+}\). 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.\(^2\) 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., Consigny, and Dohi et al., 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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