Original Contributions

Endothelin Stimulated by Angiotensin II Augments Contractility of Spontaneously Hypertensive Rat Resistance Arteries

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In cultured endothelial cells, endothelin is produced after stimulation with angiotensin II. The effects of angiotensin II and endothelin-1 on vascular sensitivity to norepinephrine were studied in perfused rat mesenteric resistance arteries. Expression of endothelin messenger RNA (mRNA) was determined in endothelial cells obtained from the mesenteric circulation. Perfusion (5 hours) of the arteries with angiotensin II (10^{-7} M) potentiated contractions in arteries with endothelium induced by norepinephrine in spontaneously hypertensive rats but not Wistar-Kyoto rats. The potentiation was inhibited by phosphoramidon and an endothelin antibody. Short-term stimulation (1 hour) with angiotensin II did not cause the potentiation. Stimulation with angiotensin I (10^{-7} M; 5 hours) caused a potentiation prevented by captopril.

In endothelial cells collected from the mesenteric arterial bed of spontaneously hypertensive rats, endothelin-specific mRNA was constitutively expressed, and the level of endothelin transcripts was increased by angiotensin II (10^{-7} M). Threshold concentrations of exogenous endothelin-1 potentiated contractions induced by norepinephrine in arteries with and without endothelium of spontaneously hypertensive rats but not Wistar-Kyoto rats. Thus, angiotensin II stimulates the endothelial production of endothelin in situ and thereby potentiates contractions to norepinephrine in mesenteric resistance arteries of spontaneously hypertensive rats. This suggests that vascular endothelin production acts as an amplifier of the pressor effects of the renin-angiotensin system that may play an important role in hypertension.

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Endothelin-1 is a 21-amino acid peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells. Thrombin, arginine vasopressin, epinephrine, angiotensin II, the calcium ionophore A23187, and phorbol esters stimulate the expression of endothelin-specific messenger RNA (mRNA) and release of endothelin from cultured endothelial cells. Among the stimulators of endothelin production, angiotensin II is of particular interest in hypertension since both the circulating and vascular renin-angiotensin systems are involved importantly in the pathogenesis of high blood pressure.

Endothelin provokes potent contractions of isolated vascular tissues and therefore might play a role in the development or maintenance of hypertension, or both. However, as judged from the circulating levels of endothelin-1 in normal subjects, the vascular production of the peptide is not sufficient to induce direct pharmacological effects, although the levels are increased in certain disease states and by aging. Recently, it has been reported that threshold concentrations of exogenous endothelin-1 sensitize the vascular smooth muscle to norepinephrine and serotonin. Thus, even low concentrations of the peptide, if indeed produced in situ, might take an important part in the regulation of vascular tone.

The present study was designed to examine in isolated and perfused mesenteric resistance arteries of normotensive and spontaneously hypertensive rats 1) whether threshold concentrations of exogenous endothelin-1 potentiate contractions induced by norepinephrine and 2) whether angiotensin II stimulates contraction...
the vascular production of endothelin-1 in situ and thereby potentiates contractions to norepinephrine.

**Methods**

**Animals**

Male Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), 16–20 weeks of age, were obtained from Charles River Wiga GmbH, Sulzfeld, FRG.

**Experimental Set-up**

The entire mesenterium was removed from anesthetized rats (pentobarbital, 50 mg/kg i.p.) and was placed into cold buffer solution (4°C) of the following composition (mM): NaCl 118.6, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.1, edetate calcium disodium 0.026, and glucose 10.1. A 3-mm long segment of the third branch of the mesenteric artery (intraluminal diameter, 200 μm) was isolated under a dissection microscope and was mounted in an arteriograph chamber (Living Systems Instrumentation, Burlington, Vt.) filled with oxygenated (95% O₂-5% CO₂) buffer solution (37°C) that circulated from a reservoir (300 ml) through the chamber.

The proximal end of the artery was cannulated with the afferent cannula and secured with a surgical nylon suture. The distal end was attached to the inside of the efferent cannula. A polyethylene catheter (intraluminal diameter, 30–50 μm) was placed in the afferent cannula and was connected to a pressure transducer for measurement of transmural pressure or for intraluminal application of drugs. The artery was perfused with buffer solution containing 1% bovine serum albumin (BSA) and was equilibrated under an optimal transmural pressure (30 mm Hg) for 1 hour. Then the arteries were repeatedly contracted with 10⁻⁶ M norepinephrine at 30-minute intervals until the responses remained constant. The arteriograph was placed on a stage of a microscope. Signals were derived from the video image of the vessel, which was processed by an electronic system (Living Systems Instrumentation) for continuous measurement and recording of intraluminal diameter.

In some experiments, the endothelium was removed by infusing 0.5% 3-[{(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for 30 seconds. The presence or absence of the endothelium was confirmed by acetylcholine (10⁻⁶ M).

**Protocols**

Concentration–response curves to extraluminal norepinephrine were obtained in preparations with and without endothelium (control contraction). Thereafter, the following protocols were performed: 1) After a 40-minute interval, the arteries were exposed to subthreshold concentrations of extraluminal endothelin-1 for 15 minutes, and then concentration–response curves to norepinephrine were repeated in the presence of endothelin-1. Threshold concentrations of endothelin-1 were determined from concentration–response curves to endothelin-1 obtained in pilot experiments. 2) The perfusate was exchanged for a solution containing 10⁻⁷ M angiotensin II or angiotensin I for 1 or 5 hours. Angiotensin II caused only a transient contraction lasting a few minutes. Subsequently, concentration–response curves to norepinephrine were repeated. The experiments were carried out in the presence or absence of [Sar¹, Ala³]angiotensin II (10⁻⁶ M), pepstatin A (10⁻⁷ M), phosphoramidon (10⁻⁵ M²), or captopril (10⁻⁶ M), which were applied extraluminally 30 minutes before the perfusion with angiotensin I or angiotensin II. Neither of the drugs changed the vascular diameter of the blood vessels under these conditions. In some experiments, anti-endothelin antibody was used. The antiserum had been raised in rabbits and is directed against human/porcine endothelin with a 100% cross-reactivity with rat endothelin. A 1:20,000 dilution of antiserum binds approximately 50% of radiolabeled endothelin at a concentration of 1.0 pmol/ml. The antiserum was diluted 1:100 with phosphate buffer solution (pH 7.40) and was infused intraluminally through the polyethylene catheter in the afferent cannula, which was connected to a syringe on a microinfusion pump (model 22, Harvard Apparatus, South Natick, Mass.). For the intraluminal application of the antiserum, flow rate through the vascular segment was measured repeatedly at the efferent cannula (since flow changes with changes in vascular diameter), and infusion rate of intraluminally applied drug was adjusted accordingly to assure a constant concentration of the drug in perfusate. The perfusion rate was set to get 100 times dilution of the infused solution in the perfusate. The infusion was started after 4-hour perfusion with angiotensin II and was continued until the concentration–response curves to norepinephrine were completed.

In time control experiments, the contractile response to norepinephrine remained unchanged after 5 hours (data not shown). Endothelial function was confirmed by a full relaxation to acetylcholine (10⁻⁶ M) at the very end of each experiment (i.e., after intraluminal exposure of the blood vessels to either control solution or angiotensin II and after completion of two concentration–response curves to norepinephrine).

**Northern Blotting and Densitometry**

Studies were performed in freshly obtained endothelial cells from the mesenteric circulation. In the anesthetized SHR (pentobarbital, 50 mg/kg i.p.) the superior mesenteric artery was cannulated with a polyethylene tube, flushed with 20 ml buffer solution containing 0.5% BSA, and the mesenteric arterial bed was isolated by cutting around the intestinal borders of the mesentery. The whole preparation was placed in a small chamber and was perfused with buffer solution containing 0.5% BSA (37°C, 95% O₂-5% CO₂). The perfusate was pooled in the chamber and was recirculated through the tissue at a
constant flow rate of 2 ml/min (closed circulation, total volume 10 ml). The tissue was equilibrated for 1 hour and then angiotensin II (10^{-7} M) was added to the perfusate. After 2 hours of stimulation with angiotensin II, the mesenteric vascular bed was massaged^{28} without collagenase, and endothelial cells were removed by flushing with buffer solution and collected by centrifugation at 500g for 5 minutes.

Total cytoplasmic RNA was separated on 1.2% agarose gels, blotted and hybridized to an endothelin specific complementary DNA (cDNA) probe.^{27,28} For slot blotting, cells were washed in phosphate buffered saline and lysed in Tris-EDTA buffer (10 mM Tris-Cl, pH 7.0, 10 mM EDTA, 0.5% NP-40) by three cycles of freeze-thawing in the presence of placental RNase inhibitor (RNasin). After each cycle of freezing and thawing, fresh RNasin was added to a concentration of 0.5 units/ul. Subsequently, cell lysates were centrifuged in an Eppendorf centrifuge at highest speed for 15 minutes at 4°C to remove cell debris and nuclei. The resulting supernatants were purified further by means of Sephadex G-50 spin columns as described.^{28} Purified, RNA containing lysates were slot-blotted directly onto Hybond N membrane (Amersham, UK), air-dried, and irradiated with ultraviolet light for 3 minutes at 302 nm to cross-link the RNA to the membrane. Hybridization of membranes to random-primed, radioactive probes (endothelin,^{27} major histocompatibility complex [MHC])^{29} occurred overnight at 65°C.^{27} The endothelin specific probe used was a 1.1 kb, partial cDNA probe, cloned in the EcoRI sites of the vector pUC 18. Using this enzyme, the insert was recovered and isolated. The isolated insert was random-primed for radioactive labeling. The MHC class I probe is a full-length cDNA probe (1.8 kb) cloned in the Pst I site of pUC 9. Exposure of hybridized blots to Kodak X-OMAT AR film was overnight at -70°C for MHC controls; slot blots for preproendothelin mRNA were exposed for 3 days to visualize hybridization signals. Densitometric analyses of hybridization signals were performed by scanning autoradiographs at 525 nm, the given arbitrary optical density (OD) units were obtained by normalization of signals with respect to the OD values obtained for the MHC internal control (highest OD value within a given series was arbitrarily taken as 100%).

**Drugs**

The following drugs were used (unless otherwise stated from Sigma Chemical Co., St. Louis, Mo.); acetylcholine hydrochloride, angiotensin I, angiotensin II, captopril (Squibb Institute, Princeton, N.J.), CHAPS, endothelin-1 (Calbiochem, Lucerne, Switzerland), endothelin antiserum (Peptide International, Inc., Louisville, Ky.), l-norepinephrine, pepstatin A, N-(α-harnnopyranosylhydroxyphosphinyl)-Leu-Trp (phosphoramidon), RNasin (Boehringer, Mannheim, FRG), [Sar^1, Ala^8]angiotensin II. Endothelin-1 was dissolved in distilled water containing 0.05% BSA, endothelin antisemur in phosphate buffer, and all other drugs in distilled water. Drugs were applied cumulatively and the concentrations are expressed as final molar concentrations.

**Statistics**

Data are given as mean±SEM. In each set of experiments, n equals the number of animals studied. Statistical evaluation was done by paired and unpaired Student’s t test. Means were considered significantly different when p<0.05.

**Results**

**Angiotensin II**

In SHR mesenteric resistance arteries with endothelium, the contractions to low and medium concentrations of norepinephrine were potentiated after a 5-hour perfusion with angiotensin II (10^{-7} M), while the maximal response was not changed (Figure 1, left panel). In contrast, in WKY mesenteric resistance arteries with endothelium, angiotensin II did not alter the response to norepinephrine (Figure 2). In the SHR, after stimulation with angiotensin II, subsequent contractions to norepinephrine were increased 1.3-fold for 10^{-7} M (p<0.01), 4.7-fold for 3×10^{-7} M (p<0.005), 2.4-fold for 10^{-6} M (p<0.005), and 1.4-fold for 3×10^{-6} M (p<0.005), respectively. In SHR preparations without endothelium, contractions to norepinephrine were not changed by angiotensin II (Figure 1, right panel). Stimulation with angiotensin II for only 1 hour did not alter the response to norepinephrine in SHR arteries with endothelium (data not shown). The potentiating effect of angiotensin II observed in arteries of SHR with endothelium was inhibited in the presence of [Sar^1, Ala^8]angiotensin II (10^{-6} M, n=5, data not shown), pepstatin A (10^{-7} M, n=5, data not shown), phosphoramidon (10^{-3} M), or endothelin antibody (Figure 3).
Angiotensin I

In arteries of SHR with endothelium, the contractions induced by medium concentrations of norepinephrine (3 × 10^{-7}, 10^{-6} M) were potentiated after 5 hours of stimulation with angiotensin I, whereas the maximal response was not changed (Figure 4). After stimulation with angiotensin I, the contractions were increased 2.4-fold for 3 × 10^{-7} M (p < 0.05) and 1.6-fold for 10^{-6} M norepinephrine (p < 0.005). The potentiation was inhibited in the presence of captopril (10^{-6} M, Figure 4).

Expression of Endothelin Specific Messenger RNA

Total cytoplasmic RNA (equivalent of 10^6 cells/slot) obtained from mesenteric endothelial cells of the SHR ex vivo demonstrated a low basal level of preproendothelin mRNA (Figure 5). However, on stimulation of the mesenteric arterial bed with angiotensin II for 2 hours, endothelin transcript levels were increased 3.4-fold as compared with time control (Figure 5, n = 4, p < 0.0005). Hybridization of the same blot for MHC class I antigens confirmed essentially identical amounts of total mRNA analyzed.

Exogenous Endothelin-1

Threshold concentration of extraluminal endothelin-1 (3 × 10^{-10} M) potentiated the contraction induced by 3 × 10^{-7} and 10^{-6} M of extraluminal norepinephrine in arteries of SHR with endothelium (Figure 6, right panel), whereas the maximal response to norepinephrine was unaffected. The increase in contractility obtained in the presence of endothelin-1 was 3.5-fold for 3 × 10^{-7} M (p < 0.01) and 2.3-fold for 10^{-6} M (p < 0.01) of norepinephrine, respectively. Lower concentration of endothelin-1 (3 × 10^{-11} M) also potentiated the contraction induced by norepinephrine (10^{-6} M, increase 1.7-fold [NS] and 4.8-fold [p < 0.05], respectively; at 3 × 10^{-7} M 1.5-fold [NS] and 2.1-fold [p < 0.05], respectively; and at 10^{-6} M 1.2-fold [p < 0.05] and 1.3-fold [p < 0.05], respectively) in SHR but not in WKY arteries.

In contrast, in WKY, the contractions to norepinephrine were not potentiated by threshold concentrations of the peptide (3 × 10^{-12}, 3 × 10^{-11} M) (Figure 6, left panel). Similarly, in arteries without endothelium, threshold concentrations of endothelin-1 (10^{-12}, 10^{-11} M) potentiated contractions induced by norepinephrine (at 10^{-7} M 1.5-fold [NS] and 4.8-fold [p < 0.05], respectively; at 3 × 10^{-7} M 1.5-fold [NS] and 2.1-fold [p < 0.05], respectively; and at 10^{-6} M 1.2-fold [p < 0.05] and 1.3-fold [p < 0.05], respectively) in SHR but not in WKY arteries.

Discussion

The present study demonstrates that angiotensin II stimulates the vascular production of endothelin in...
perfused mesenteric resistance arteries of SHR in situ and thereby induces an endothelium-dependent potentiation of vascular sensitivity to norepinephrine.

Stimulation of arteries with angiotensin II potentiated the contraction induced by low and medium concentrations of norepinephrine in SHR but not in WKY rat. The potentiation cannot be attributed to time-dependent changes of vascular sensitivity because in time control experiments the contractility of the preparations was not changed. The potentiation was endothelium-dependent and mediated by angiotensin II receptors since the response was prevented in the presence of the angiotensin II receptor antagonist [Sar\textsuperscript{1},Ala\textsuperscript{8}]angiotensin II. This suggests that angiotensin II stimulates the production of an endothelium-derived substance that potentiates the contraction to norepinephrine.

In line with this interpretation, angiotensin II stimulated the induction of endothelin specific mRNA in endothelial cells collected from the perfused mesenteric arterial bed of SHR after stimulation with angiotensin II. The expression of endothelin specific mRNA was increased about 3.4-fold after stimulation, indicating that endothelin-1 is produced in situ in mesenteric resistance arteries of the SHR in response to angiotensin II.

Endothelin-1 is generated from big endothelin-1 by proteolytic processing.\textsuperscript{30} The production of endothelin from stimulated endothelial cells requires de novo protein synthesis and thus is very slow, reaching the maximum after 4–5 hours of incubation.\textsuperscript{1,4} In line with this observation, in intact mesenteric resistance arteries the endothelium-dependent potentiation was observed only after 5 hours but not after 1 hour of stimulation with angiotensin II. Although big endothelin exhibits vasoconstrictor activity, it is a much weaker agonist than endothelin-1.\textsuperscript{30} The aspartic protease inhibitor pepstatin A and the metalloproteinase inhibitor phosphoramidon inhibit the conversion of big endothelin-1 to endothelin-1.\textsuperscript{23,24} Administration of phosphoramidon to rats suppresses the hypertensive effect of big endothelin-1 without affecting the pressor response induced by endothelin-1,\textsuperscript{24} and this intervention also prevented the potentiation of norepinephrine-induced contractions evoked by angiotensin II in mesenteric resistance arteries with endothelium. Similarly, an endothelin antibody completely prevented the potentiation response as well. Thus, endothelin produced in situ in the mesenteric resistance circulation of the SHR mediates the endothelium-dependent potentiation of the response to norepinephrine induced by angiotensin II.
In line with this interpretation, low concentrations of exogenous endothelin-1 potentiated contractions induced by norepinephrine in SHR but not in WKY and thus mimicked the potentiation observed with angiotensin II. Although the concentration of exogenous endothelin-1 used was lower in WKY than SHR (because of the higher sensitivity to the peptide in WKY), the contractions evoked by threshold concentrations of endothelin-1 did not differ in the two strains. Thus, it is unlikely that the potentiation observed in SHR is due to the slightly higher concentration of the peptide. Indeed, $3 \times 10^{-11}$ M of endothelin-1 potentiated the contraction in SHR but not in WKY. Threshold concentrations of endothelin-1 must directly sensitize vascular smooth muscle since removal of the endothelium did not affect the potentiation. As in a previous study with normotensive rats where the potentiating effects of endothelin-1 were observed only in very old animals, the present study suggests premature aging of resistance arteries of SHR.

Angiotensin II may be derived either from the circulating renin-angiotensin system or from the vascular wall itself. Indeed, locally generated angiotensin II is released from perfused rat mesenteric arteries after stimulation of $\beta$-adrenergic receptors. Angiotensin I also potentiated the contraction to norepinephrine in SHR. Since the potentiating effect of angiotensin I was blocked by the angiotensin converting enzyme inhibitor captopril, the peptide must be converted in situ to angiotensin II by the angiotensin converting enzyme on endothelial cells. This indicates that the converting enzyme is operative in mesenteric resistance arteries of SHR and is interconnected with the vascular production of endothelin.

The fact that the potentiation was observed only in SHR suggests a pathophysiological role of endogenous endothelin in hypertension. As judged from the circulating levels of endothelin-1, the vascular production of the peptide is low. Therefore, the potentiating effects of threshold concentrations of the peptide may be particularly important in hypertension and contribute to the increased peripheral vascular resistance.

References


**KEY WORDS** • endothelium • angiotensin II • vascular resistance • captopril • norepinephrine • endothelin • spontaneously hypertensive rats • Wistar-Kyoto rats
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