Angiotensin-(1-7)–Stimulated Nitric Oxide and Superoxide Release From Endothelial Cells

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Abstract—The stimulation of endothelium-dependent NO release by angiotensin-(1-7) [Ang-(1-7)] has been indirectly shown in terms of vasodilation, which was diminished by NO synthase inhibition or removal of the endothelium. However, direct measurement of endothelium-derived NO has not been analyzed. With a selective porphyrinic microsensor, NO release was directly assessed from single primary cultured bovine aortic endothelial cells. Ang-(1-7) caused a concentration-dependent release of NO of 1 to 10 μmol/L, which was attenuated by NO synthase inhibition. [D-Ala7]Ang-(1-7) (5 μmol/L), described as a selective antagonist of Ang-(1-7) receptors, inhibited Ang-(1-7)–induced NO release only by ≈50%, whereas preincubation of bovine aortic endothelial cells with the angiotensin II subtype 1 and 2 receptor antagonists EXP 3174 and PD 123,177 (both at 0.1 μmol/L) led to an inhibition of 60% and 90%, respectively. A complete blockade of the Ang-(1-7)–induced NO release was observed on preincubation of the cells with 1 μmol/L concentration of the bradykinin subtype 2 receptor antagonist icatibant (HOE 140), suggesting an important role of local kinins in the action of Ang-(1-7). Simultaneous direct measurement of superoxide (O2−) detected by an O2−-sensitive microsensor revealed that the moderately Ang-(1-7)–stimulated NO release was accompanied by a very slow concomitant O2− production with a relative low peak concentration in comparison to the O2− production of the strong NO releasers bradykinin and, especially, calcium ionophore. Thus, Ang-(1-7) might preserve the vascular system, among others, due to its low formation of cytotoxic peroxynitrite by the reaction between NO and O2−. (Hypertension. 2001;37:72-76.)

Key Words: angiotensin-(1-7) ■ nitric oxide ■ superoxide ■ endothelial cells ■ kinins

There is increasing experimental evidence that in addition to the known effector hormone angiotensin (Ang) II, the heptapeptide Ang-(1-7) is also a biologically active component of the renin-angiotensin system.1–3 Besides circulating Ang-(1-7), local production of the heptapeptide has been shown in the vasculature, where it is predominantly generated from Ang I by metalloendopeptidase 24.15.4 In endothelial cells from different sources, Ang-(1-7) is derived from carboxyl-terminal degradations of Ang I and Ang II by neutral endopeptidase (NEP 24.11) or by carboxypeptidases, respectively.5

In contrast to Ang II, Ang-(1-7) is neither dipsogen nor an aldosterone secretagogue, but similar to Ang II, it releases vasopressin,6 prostaglandins,7 and NO.8–9 Also unlike Ang II, Ang-(1-7) inhibits vascular smooth muscle cell growth.10,11 In the past years, some studies have indicated that Ang-(1-7) counterregulates the cardiovascular actions of Ang II by acting as a local modulator of the vascular tone.12 Ang-(1-7) is a vasodilatory agent in many vascular beds, including canine13 and porcine14 coronary arteries, rat aorta,15 and feline mesenteric arteries.16 Recently, it was demonstrated that Ang-(1-7) blocks the Ang II–induced vasoconstriction in human arteries.17 Long-term infusion of Ang-(1-7) in spontaneously hypertensive rats (SHR) produced a transient decrease in the mean arterial pressure, which was significant after 2 days and returned to baseline on day 7 of the 14-day treatment period.18 This antihypertensive effect could be the reason for the attenuated pressor response to Ang II and phenylephrine and the improved baroreceptor reflex function in SHR in response to long-term infusion of Ang-(1-7).19 These effects of Ang-(1-7) are probably mediated by an enhanced synthesis and/or release of endothelin kinins, which in turn stimulate the release of the vasodilatory and cardioprotective prostaglandins7,20 and NO.3,13,21 The presence of a unique endothelial receptor, which preferentially binds Ang-(1-7) and is distinct from Ang II subtype 1 (AT1) and subtype 2 (AT2) receptors, was postulated by the group of Ferrario22 from binding experiments on bovine aortic endothelial cells (BAECs). Recent studies demonstrated that Ang-(1-7) is not only a substrate but also an endogenous inhibitor of angiotensin-converting enzyme (ACE) by binding to the active site of ACE responsible for the inactivation of bradykinin (BK).23 Inhibition of the active site of ACE also induces “cross-talk” between ACE and BK subtype 2 (B2)
receptors on plasma membranes, abolishing the desensitization of the B2 receptor.\(^24\) This kind of interaction can explain the well-documented kinin-like actions of Ang-(1-7) observed in coronary vessels\(^3,9\) and in rats,\(^25–27\) without having the well-documented kinin-like actions of Ang-(1-7) observed in coronary vessels.\(^3,9\) Therefore, we directly investigated the effect of Ang-(1-7) on NO release from primary cultured BAECs via a porphyrinic microsensor placed in close proximity to the cell surface. Second, we correlated the Ang-(1-7)–induced NO release with the endothelial production of O$_2^-$.

### Methods

#### Endothelial Cell Culture

BAECs were isolated through digestion with dispase and were cultured as previously described.\(^29\) Cells were seeded onto 6-well plates (Nunc Intermed) that were precoated with collagen R and were grown to confluence. The culture medium used for BAECs was Dulbecco’s modified Eagle’s/Ham’s F-12 medium (1:1) that contained 1 mM L-arginine, heat-inactivated FCS (20%), 50 IU/mL penicillin, 50 μg/mL streptomycin, 1 mM L-glutamine, 5 μg/mL glucuronolactone, and 5 μg/mL L-(-)-ascorbic acid. The purity of the primary cultured BAECs was characterized by uptake of fluorescent labeled LDL (DiI-Ac-LDL) and by negative staining for α-smooth muscle actin.

#### Direct NO Measurements

Primary cultures of BAECs grown to confluence in 6-well plates were used (200 to 250 μg protein or 3 × 10^5 cells per well).\(^29,30\) After removal of the culture medium through aspiration, the monolayers were washed twice with 2 mL warm (37°C) HEPES-Tyrode’s solution, pH 7.4, containing (in mM/L) KCl 2.7, NaCl 137, CaCl$_2$ 1.8, MgCl$_2$ 2, Na$_2$HPO$_4$ 0.36, glucose 5, and HEPES 10. Thereafter, the cells were preincubated for 15 minutes at 37°C with 1 mL HEPES-Tyrode’s solution containing 3-isobutyl-1-methyl-xanthine (IBMX; 100 μM/L). IBMX was added to provide identical incubation conditions as the intracellular cGMP measurements in previously reported experiments.\(^31\) Agonists, dissolved in HEPES-Tyrode’s solution, and superoxide dismutase (SOD; 0.3 μM/L) were added at the concentrations and times indicated in Results. The detection of NO with a porphyrinic microsensor and its preparation were performed as previously described.\(^31,32\) The current proportional to NO concentration was measured with the sensor, which operated in amperometric mode (EG&G PAR model 283 Potentiostats, Galvanostats) at constant potential of 0.68 V versus saturated calomel electrode.

The sensor was positioned on the surface of the cell membrane with the use of a micromanipulator, and the drugs were injected with a nanoinjector, which was positioned at close proximity (5 to 7 μm) from the cell surface. The microsensor had a response time of 0.1 ms with the use of a micromanipulator, and the drugs were injected with a nanoinjector, which was positioned at close proximity (5 to 7 μm) from the cell surface. The diameter of the sensor was 1 to 1.5 μm, the response time was 0.05 ms, and the detection limit was 1 nmol/L. The O$_2^-$ sensor also operated in amperometric mode at a constant potential of −0.23 V versus standard calomel electrode. A 3-electrode system was used for all O$_2^-$ measurements with the O$_2^-$ sensor as working electrode, SCE reference electrode, and platinum auxiliary electrode. A Princeton Applied Research PAR model 283 voltammetric analyzer interfaced with an IBM 80486 computer was used to record the amperometric (current-versus-time) signal. Calibration of the sensor was performed by constructing standard curves based on O$_2^-$ concentrations stoichiometrically generated through the reaction of xanthine and xanthine oxidase. Concentrations of O$_2^-$ were reported in nmol/L.

#### Chemicals

SOD (from bovine erythrocytes, specific activity 3300 U/mg) and collagen R were purchased from Serva. Dil-Ac-LDL was purchased from Paesel+Lorelei. Ang-(1-7), [d-Ala$_2$]Ang-(1-7), BK acetate salt, calcium ionophore (Cal) A23187, and N'-'nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. EXP 3174, PD 123,177, and icatibant (HOE 140) were synthesized at Aventis Pharma.

### Statistical Analysis

Values are expressed as mean±SEM from 5 experiments, with P<0.05 considered statistically significant. Statistical evaluation was made with ANOVA followed by unpaired Student’s t test. All analyses were made with the statistical software Microcal Origin.

### Results

Figure 1a shows the result of simultaneous assessment of the changes in NO and O$_2^-$ concentrations with time after the addition of 10 μmol/L Ang-(1-7). The peptide stimulated NO release with a peak concentration of 280±30 nmol/L (n=5), which was accompanied after a delay of 3.0±0.3 s by a very low peak O$_2^-$ production of 18±2 nmol/L (n=5). The rates of NO and O$_2^-$ increases were 120±10 and 16±3 nmol · L$^{-1}$ · s$^{-1}$ (each n=5), respectively. In comparison with Ang-(1-7), the receptor-independent Cal A23187 and BK showed different kinetics of NO and O$_2^-$ release (Figures 1b and 1c). A23187 (1 μmol/L) and BK (0.1 μmol/L) evoked significantly higher peak NO concentrations (560±25 and 490±20 nmol/L, respectively) associated with faster rates of NO increases (650±20 and 280±30 nmol · L$^{-1}$ · s$^{-1}$, respectively) than did Ang-(1-7). Cal A23187- and BK-induced peak concentrations of O$_2^-$ were 80±6 and 32±4 nmol/L, respectively, which were significantly higher than the peak O$_2^-$ concentrations induced by Ang-(1-7).

The rates of increases in O$_2^-$ concentrations (120±12 and 26±3 nmol · L$^{-1}$ · s$^{-1}$, respectively) for Cal A23187 and BK were much faster than that observed for Ang-(1-7). The peak NO concentration increased with increasing concentrations of Ang-(1-7) (Figure 2). The observed release of NO from BAECs was in exactly the same concentration range of Ang-(1-7) as determined for the release of cGMP (unpublished data).

As shown in Figure 2, the Ang-(1-7)–stimulated NO release from BAECs was significantly (and competitively) reduced by ~60% when the cells were preincubated for 20 minutes with a 200 μmol/L concentration of the NOS inhibitor L-NAME. Complete inhibition was observed with preincubation (5 minutes) of the cells with a 1 μmol/L concentration of the B2 receptor antagonist icatibant (HOE 140).

The specificity of the Ang-(1-7)–stimulated NO release from BAECs was determined through preincubation of the cells for 20 minutes with either the Ang-(1-7) receptor.
antagonist [d-Ala7]Ang-(1-7), the AT1 antagonist EXP 3174 (active metabolite of losartan), or the AT2 antagonist PD 123,177 (Figure 3). Preincubation of the cells with both [d-Ala7]Ang-(1-7) (5 μmol/L) and EXP 3174 (0.1 μmol/L) attenuated the Ang-(1-7)–induced NO release by \( \approx 50\% \) to \( \approx 60\% \), whereas preincubation with PD 123,177 (0.1 μmol/L) caused an inhibition of \( \approx 90\% \). The selected concentrations of the applied antagonists were maximally effective on the Ang-(1-7)–induced NO release.

**Discussion**

The present study demonstrated for the first time through direct measurements of NO via a porphyrinic microsensor that exogenously added Ang-(1-7) stimulated the release of the vasodilator NO from primary cultured BAECs. The Ang-(1-7)–induced NO release was concentration dependent (ED50 \( \approx 3 \) μmol/L) and inhibited to 60% by L-NAME. This inhibition rate is in the range (60% to 70%) observed for NO release stimulated by other NOS agonists (CaI, BK, etc). Ang-(1-7) is able to activate endothelial NOS, which is in line with the reported vasorelaxant effect of Ang-(1-7) on precon-
trated canine or porcine coronary arteries, which could be blocked by pretreatment with NOS inhibitors.3,13,14

The presence of a unique Ang-(1-7) receptor distinct from AT1 and AT2 receptors was postulated from binding studies with membranes from cultured BAECs and supported by the ability of the Ang-(1-7) analog [d-Ala7]Ang-(1-7) to compete the binding of [125I]Ang-(1-7) and to block some of the biological effects of Ang-(1-7).22,36 However, up to now, there has been no direct evidence of the existence of a unique Ang-(1-7) receptor, and in our study on cultured BAECs, [d-Ala7]Ang-(1-7) failed to completely block (50% blockade only) the NO release induced by Ang-(1-7). Moreover, the reduction of the Ang-(1-7)–stimulated NO release by the AT1 antagonist EXP 3174 and, even more pronounced, by the AT2 antagonist PD 123,177 of 60% and 90%, respectively, observed in our experiments supports the view that AT2, but also AT1, receptors might be involved in the reported actions of Ang-(1-7).

Reports that describe the effects of AT1 and AT2 antagonists on the Ang-(1-7)–induced functional responses are not consistent, possibly due to organ and species differences in Ang II receptor subtype specificity. Attenuation only by an AT2 antagonist in porcine aortic smooth muscle cells37 or only by an AT1 antagonist in canine coronary arteries8 or a lack of inhibition in canine coronary arteries13 by both AT1 and AT2 antagonists was reported. Hence, it might be speculated that Ang-(1-7) interacts with multiple binding site on endothelial cells, all of which promote the activity of the endothelial kinin system. This hypothesis is supported by the observed complete inhibition of the Ang-(1-7)–induced NO release on BAECs by the B2 receptor antagonist icatibant and clearly indicates that the observed NO release is correlated with an enhanced synthesis and release of endogenous kinins followed by subsequent stimulation of B2 receptors.38 At the present, the mechanisms by which Ang-(1-7) exerts its kinin-mediated NO release are still a matter of speculation (Figure 4). From experiments in canine coronary arteries that show an inhibition of the Ang-(1-7)–induced nitrite production by protease inhibitors, which block the local formation of kinins, it was suggested that the kallikrein-kinin system becomes activated via stimulation of a specific receptor for Ang-(1-7).9 Similarly, prior findings of our group revealed that the NO release from BAECs induced by Ang II is associated with an enhanced synthesis and/or release of endogenous kinins.8 More recent investigations demonstrated the ability of Ang-(1-7) to inhibit ACE activity with an IC50 value of 3 and 0.65 μmol/L in human plasma17 and canine lung,23,28 respectively. Thus, the applied Ang-(1-7) concentrations of 1 to 10 μmol/L, at which we observed an increased NO synthesis on BAECs, could have partially inhibited endothelial ACE activity. Furthermore, Ang-(1-7) has been shown to indirectly sensitize B2 receptors via induction of a cross-talk between the B2 receptors and ACE on plasma membranes without having a direct effect on the B2 receptors and the BK hydrolyses.23 Activation of the unique non-AT1, non-AT2 receptor deduced from binding studies on membranes from cultured BAECs22 is also a possible explanation for the observed stimulated synthesis and release of NO by Ang-(1-7). However, the signal transduction pathway coupled to this putative Ang-(1-7) receptor is unknown. We found that Ang-(1-7) (10 and 100 μmol/L) did not change intracellular free Ca2+ (fluorimetrically assessed with the Ca2+ indicator dye Fura-2) in BAECs in comparison with a 5- to 6-fold rise in intracellular free Ca2+ induced by 0.1 μmol/L BK. This finding is in line with the reported failure of Ang-(1-7) to stimulate Ca2+ in human astrocytes.1 These findings together with the demonstrated kinin-mediated action of Ang-(1-7) are difficult to explain because the stimulation of endothelial B2 receptors is correlated with an increase in intracellular cytosolic Ca2+.39 However, very recent data indicate that there are other intracellular mechanisms that are able under certain conditions, such as, fluid shear stress and insulin, to activate endothelial NOS through phosphorylation of the enzyme at basal levels of intracellular Ca2+.40

We could show through the simultaneous measurement of NO and O2− that the moderate NO release by Ang-(1-7) is associated with very low concomitant production of O2−. The kinetics of the Ang-(1-7)–induced NO and O2− release are much different than those particularly observed for Cal and BK. In comparison, the stronger NO releasers BK and Cal caused faster O2− production with significantly higher peak O2− concentrations. Consequently, our data imply that rapid NO generation is correlated to high O2− production. Because it has been suggested that the generation of O2− and subsequent fast reaction between O2− and NO to form peroxynitrite and cytotoxic radicals may play a role in endothelial dysfunction and vascular injury, Ang-(1-7) with its NO/O2− releasing profile might be able to preserve a functional vascular system.

A rapid consumption of local l-arginine, the substrate of endothelial NOS, due to rapid NO production may be the reason for a disarrangement of NOS, which starts to produce

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**Figure 4. Proposed mechanisms of action responsible for the Ang-(1-7)–mediated NO synthesis in endothelial cells (EC). Stimulation of “Ang-(1-7) receptors” (non-AT1, non-AT2 receptors) most likely causes an enhanced concentration of endogenous kinins with subsequent stimulation of B2 receptors. Similarly, AT2 receptor stimulation by Ang II activates the kinin/NO system. NEP indicates neutral endopeptidase; PEP, prolyl endopeptidase.**
O$_2^-$ It has been demonstrated that isolated neuronal NOS may also produce O$_2^-$ when tetrahydrobiopterin and L-arginine are not present in sufficient amounts. 35 Moreover, dysfunctional NOS seems to be present in the main source of Cal-stimulated O$_2^-$ production in aortas of prehypertensive and old SHR. Whether the Ang-(1-7)- and BK-mediated O$_2^-$ production is formed via a dysfunctional NOS and/or other enzymes such as xanthine oxidases and NAD(P)H-dependent oxidases remains to be elucidated.

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References
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