AVE 0991, a Nonpeptide Mimic of the Effects of Angiotensin-(1–7) on the Endothelium

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Abstract—Recently, we demonstrated that the heptapeptide angiotensin-(1–7) (Ang-[1–7]) exhibits a favorable kinetic of nitric oxide (NO) release accompanied by extremely low superoxide (O$_2^-$) production. In this report we describe AVE 0991, a novel nonpeptide compound that evoked effects similar to Ang-(1–7) on the endothelium. AVE 0991 and unlabeled Ang-(1–7) competed for high-affinity binding of [125I]-Ang-(1–7) to bovine aortic endothelial cell membranes with IC$_{50}$ values of 21±35 and 220±280 nmol/L, respectively. Stimulated NO and O$_2^-$ release from bovine aortic endothelial cells was directly and simultaneously measured on the cell surface by selective electrochemical nanosensors. Peak concentrations of NO and O$_2^-$ release by AVE 0991 and Ang-(1–7) (both 10 µmol/L) were not significantly different (NO: 295±20 and 270±25 nmol/L; O$_2^-$: 18±2 and 20±4 nmol/L). However, the released amount of bioactive NO was ≈5 times higher for AVE 0991 in comparison to Ang-(1–7). The selective Ang-(1–7) antagonist [D-Ala$^7$]-Ang-(1–7) inhibited the AVE 0991–induced NO and O$_2^-$ production by ≈50%. A similar inhibition level was observed for the Ang II AT$_1$ receptor antagonist EXP 3174. In contrast, the Ang II AT$_2$ receptor antagonist PD 123,177 inhibited the AVE 0991–stimulated NO production by ≈90% but without any inhibitory effect on O$_2^-$ production. Both NO and O$_2^-$ production were inhibited by NO synthase inhibition (≈70%) and by bradykinin B$_2$ receptor blockade (≈80%). AVE 0991 efficiently mimics the effects of Ang-(1–7) on the endothelium, most probably through stimulation of a specific, endothelial Ang-(1–7)-sensitive binding site causing kinin-mediated activation of endothelial NO synthase. (Hypertension. 2002;40:847-852.)

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Angiotensin-(1–7) (Ang-[1–7]), a biologically active component of the renin-angiotensin system, regulates blood pressure by counteracting the vasoconstrictor and proliferative effects of Ang II.$^1$ Ang-(1–7) was vasodilatory in many vascular beds, including canine$^2$ and porcine$^3$ coronary arteries, rat aorta,$^4$ and feline mesenteric arteries.$^5$ Chronic infusion of Ang-(1–7) in spontaneously hypertensive rats$^6$ and Dahl salt-sensitive rats$^7$ reduced mean arterial blood pressure. This antihypertensive effect could be the reason for the attenuated pressor response to Ang II and phenylephrine and the improved baroreceptor reflex function in spontaneously hypertensive rats in response to chronic infusion of Ang-(1–7).$^8$

The limited data concerning the effects of Ang-(1–7) on human vascular function are conflicting. Ang-(1–7) blocked the Ang II–induced vasoconstriction in isolated human arteries$^9$ and antagonized vasoconstriction in forearm circulation by Ang II infusion in normotensive men.$^{10,11}$ Also, a direct vasodilatation to the same extent in basal forearm circulation of both normotensive subjects and hypertensive patients caused by Ang-(1–7) was reported.$^{12}$ In contrast, Ang-(1–7) did not alter basal forearm blood flow in normotensive men$^{10,11}$ and in patients with heart failure treated with an ACE inhibitor.$^{13}$

The vasodilator bradykinin (BK) appears to be involved in the vascular and antiproliferative effects of Ang-(1–7).$^{14,15}$ There are two possibilities of interactions between BK and Ang-(1–7). First is an induction of enhanced synthesis and/or release of endothelial kinins by Ang-(1–7) and second is a potentiation of the effects of BK and/or endogenous kinins by Ang-(1–7). According to the latter, it had been shown that preincubation of isolated coronary arteries with Ang-(1–7) increased the relaxation produced by BK.$^{16–18}$ The same is true for the BK-induced blood pressure–lowering effect, which was potentiated by Ang-(1–7) in normotensive and hypertensive rats.$^{19,20}$ There are several mechanisms that appear to be responsible for the BK-potentiating effects by Ang-(1–7). It appears to involve a receptor-mediated induction of nitric oxide (NO) release$^{2,21,22}$ and/or prostaglandins$^{23,24}$ and endothelium-derived hyperpolarizing factor.$^{25}$ Although a specific Ang-(1–7) receptor has not been cloned yet, there are several sources of evidence that support its existence, based on binding studies with endothelial membrane$^{26}$ and functional studies showing the opposing and/or differential actions of Ang II and Ang-(1–7).$^{27}$ Furthermore, binding of Ang-(1–7) to ACE induces a cross-talk between...
BK receptors and ACE, leading to desensitization of BK B2 receptors,28 and binding of Ang-(1–7) to the ACE C-domain prevents to a certain extent the degradation of BK.28,29 The contribution of each of these mechanisms appears to be dependent on the vascular bed, the species, and on the investigated vessel diameter.30

Recently, we have directly shown that Ang-(1–7) induces, through a kinin-mediated mechanism, a relatively slow release of bioactive NO from cultured endothelial cells. This NO release was associated with a concomitant, very low production of superoxide (O2−)31 as well as peroxynitrite and cytotoxic radicals. Consequently, a nonpeptide, orally active compound that mimics the favorable NO/O2− releasing profile of Ang-(1–7) might have significant therapeutic potential for the treatment of cardiovascular diseases associated with dysfunctional endothelium. This prompted us to initiate a program directed toward the synthesis and identification of a nonpeptide mimic of Ang-(1–7), which succeeded in the discovery of the compound AVE 0991, 5-formyl-4-methoxy-2-phenyl-1-[[4-[2-ethylaminocarbonylsulfonamido]-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole (Figure 1). In the vitro results obtained with this agonist of the putative Ang-(1–7) receptor and their comparison to those elicited by Ang-(1–7) are described here.

Methods

Binding of [125]I-Ang-(1–7)

Ang-(1–7) (Bachem) was radioiodinated with chloramine T and [125]I-sodium iodide in a sodium phosphate–buffered solution. The mono-labeled [125]I-Ang-(1–7) (specific activity, 2107 mCi/mg) was isolated by HPLC purification on a Hypersil C18 RP column, with an acetonitrile/TEAP solvent system.

Binding of [125]I-Ang-(1–7) was performed by the method of Tallant et al.,26 with some modifications. Briefly, 100 μg of membranes from primary cultured bovine aortic endothelial cells (BAECs, passage 1) were incubated in a total volume of 200 μL for 45 minutes at 25°C in HEPES-buffered saline (10 mmol/L HEPES, 0.1 mol/L NaCl, 5 mmol/L MgCl2) containing 0.2% BSA and protease inhibitor cocktail (Boehringer Mannheim). Saturable binding of [125]I-Ang-(1–7) was calculated by subtracting nonspecific binding (40% to 50%), determined in the presence of 10 μmol/L unlabeled Ang-(1–7) from total binding. Competition experiments with increasing concentrations of AVE 0991 and unlabeled Ang-(1–7) were performed in the presence of 10 nmol/L [125]I-Ang-(1–7). Assays were terminated by vacuum filtration (≥15 mm Hg) over Durapore filters (0.65 μm, Opak 96-well plates, Millipore) presoaked with 1% BSA. The filters were washed 3 times with each 100 μL of PBS (50 mmol/L NaHPO4 and 0.15 mol/L NaCl, pH 7.2). Radioactivity on dried filters was quantified with a gamma counter.

Specificity Measurements

AVE 0991 was evaluated in a panel of 25 radioligand binding assays at CEREP (86600 Celle L’Evescault). AVE 0991 was tested in each assay at 10 μmol/L in duplicate. The influence of AVE 0991 on Ang II AT1 receptors was tested in a radioligand binding assay with CHO cells transfected with the human recombinant AT1 receptor, using [125]I-[Sar1, Ile8]-Ang II (50 pmol/L, incubation time 60 minutes at 37°C). The influence of AVE 0991 on Ang II AT2 receptors was tested in a radioligand binding assay with Hela cells transfected with the human recombinant AT2 receptor, using [125]I-CGP42112A (50 pmol/L; incubation time, 180 minutes at 37°C).

NO and O2− Measurements

Primary cultures of BAECs grown to confluence in 6-well plates were used (200 to 250 μg protein or 3 × 105 cells per well).33,34 After removal of the culture medium by aspiration, the monolayers were washed twice with 2 mL of warm (37°C) HEPES-Tyrode’s solution, pH 7.4 (in mmol/L: KCl 2.7, NaCl 137, CaCl2 1.8, MgCl2 2, NaHPO4 0.36, glucose 5, 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 μmol/L). IBMX was added to have incubation conditions, which were identical with those used for measurement of intracellular cyclic GMP in BAECs.35 Compounds dissolved in HEPES-Tyrode’s solution and superoxide dismutase (0.3 μmol/L) were added at the concentrations and times indicated in the results.

Detection of NO by a porphyrinic nanosensor and its preparation were performed as previously described.33,34 The current, which is proportional to NO concentration, was measured by the porphyrinic sensor. The sensor operated in an amperometric (current proportional to NO concentration versus time) mode at a constant potential of 0.68 V versus a saturated calomel electrode (SCE). An amount of NO detected by the sensor was calculated on the basis of colorimetric measurements (integrated area under current-time curve). A microsensor capable of almost instantaneous indirect detection of O2− was prepared according to the general procedure described previously33 and subsequently modified in our laboratory for single-cell measurements.36 The O2− sensor consists of two electrodes: The first electrode is for detection of the total concentration of H2O2 generated stoichiometrically by the fast dismutation of O2− by superoxide dismutase, and the second electrode is for the measurement of basal H2O2 concentration. The difference between the currents generated by these two electrodes was used as an analytical signal for indirect O2− determination. Both microsensors were operated at a potential of ~0.23 V versus SCE. The O2− sensor was combined with the NO sensor in one unit (tandem sensor) of total diameter of ~2 to 3 μm. Two separate instruments (Gamry FAS1 femtostats) were used for the recording of NO and O2−.

Statistical Analysis

Values are expressed as mean±SEM from 3 to 5 experiments, with a value of P<0.05 considered statistically significant. Statistical evaluation was done by ANOVA followed by an unpaired Student t test. All analyses were made with the statistical software, Microcal Origin.

Results

[125]I-Ang-(1–7) Binding Experiments

Saturation isotherms of the binding of [125]I-Ang-(1–7) to BAEC membranes revealed a Kd value of 2.9±0.1 nmol/L and a Bmax value of 32.4±2.5 fmol/mg protein (n=4). AVE 0991 and unlabeled Ang-(1–7) competed the specific binding of [125]I-Ang-(1–7), with IC50 values of 21±35 and 220±280 nmol/L, respectively (each n=3). The Ang-(1–7) analogue [D-Ala7]-Ang-(1–7) totally competed the specific binding of [125]I-Ang-(1–7), with an IC50 value of 0.41±3.0 μmol/L (n=3).
The rate of Ang-(1–7)–stimulated NO was 270 \pm 110 \text{ nmol/L}. The rate of Ang-(1–7)–evoked an almost identical peak concentration of NO release (20 mmol/L) (n=5) (Figure 2a). Also, the rate of O$_2^-$ release (16±3.0 nmol/L/s) was faster than that observed for AVE 0991, but the peak concentration was not significantly different. However, the duration of NO release was much shorter than that observed for AVE 0991. Therefore, the amount of NO released (by colorimetric measurement) after stimulation with Ang-(1–7) was ~5 times lower than that measured for AVE 0991 (Figure 3).

NO release stimulated by different concentrations of AVE 0991 was significantly inhibited when the cells were preincubated for 5 minutes with 200 mmol/L of the NO synthase inhibitor N$	ext{^6}$-monomethyl-L-arginine (L-NMMA). A strong inhibition of NO release was also observed when the cells were preincubated for 5 minutes with 1 mmol/L of the BK B$_3$ receptor antagonist icatibant (HOE 140) (Figure 4a). The inhibitory effects of L-NMMA and icatibant on O$_2^-$ production were similar (Figure 4b).

To assess the specificity of AVE 0991–stimulated NO release, cells were preincubated for 20 minutes with either the Ang-(1–7) receptor antagonist [D-Ala$^7$]-Ang-(1–7), the Ang II subtype 1 (AT$_1$) antagonist EXP 3174 (active metabolite of losartan), or the Ang II subtype 2 (AT$_2$) antagonist PD 123,177 (Figure 5a). Both [D-Ala$^7$]-Ang-(1–7) (5 mmol/L) and EXP 3174 (0.1 mmol/L) significantly (and noncompetitively) reduced the NO release by ~43% to 55% at 10 mmol/L of AVE 0991, whereas preincubation with PD 123,177 (0.1 mmol/L) caused a competitive inhibition of ~90%. In contrast, the attenuation of the AVE 0991–stimulated O$_2^-$ production by [D-Ala$^7$]-Ang-(1–7) and PD 123,177 was much weaker. EXP 3174 had no inhibitory effect on AVE 0991–induced O$_2^-$ production (Figure 5b). The selected concentrations of the applied antagonists were maximally effective on AVE 0991–stimulated NO release.

AVE 0991–induced NO release from BAECs was exactly in the same concentration range (EC$_{50}$=2.1±3.0 mmol/L, n=4) as assessed for intracellular cyclic GMP production in BAECs.

### Discussion

The present study demonstrated that the new nonpeptide compound AVE 0991 is able to evoke effects on endothelial cells similar to that observed for heptapeptide Ang-(1–7).

AVE 0991–stimulated NO release from cultured BAECs is due to the activation of endothelial NO synthase (eNOS). This is supported by the fact that the production of NO was significantly inhibited by the L-arginine analogue L-NMMA. The amount of AVE 0991–stimulated NO production was ~5
times higher than that stimulated by Ang-(1–7), but the respective EC_{50} values of AVE 0991 and Ang-(1–7) for NO release were in a similar range (2.1±3.0 and ~3 μmol/L, respectively). The release of NO from eNOS of endothelial cells is always concomitantly accompanied by the release of superoxide.37 The O_{2}\textsuperscript{−} production, in turn, is related to the kinetics of NO release.38,39 A favorable kinetic is characterized by a slower rate of NO release. The rate of NO release by AVE 0991 was relatively slow, which led to a very small generation of O_{2}\textsuperscript{−}. In comparison, a fast rate of NO release stimulated by calcium ionophore A23187, bradykinin, or acetylcholine generates an O_{2}\textsuperscript{−} concentration several times higher than that observed for AVE 0991. The main source of O_{2}\textsuperscript{−} production was inhibited by L-NMMA, similar to that observed for NO. Moreover, the similar EC_{50} values for both NO and O_{2}\textsuperscript{−} generation by AVE 0991 are due to an Ang-(1–7)–mediated stimulation of eNOS. The rate of O_{2}\textsuperscript{−} production by eNOS is triggered by a rapid consumption of local L-arginine (substrate of eNOS).

A specific receptor for Ang-(1–7) has not been cloned yet. However, several functional and receptor binding studies provide evidence for the existence of a unique Ang-(1–7) receptor, distinct from AT\textsubscript{1} and AT\textsubscript{2} receptors.27 We could confirm the data of Tallant et al26 that showed a specific binding of [^{125}\text{I}]-Ang-(1–7) on membranes from cultured BAECs. AVE 0991 competed this binding with an ~10-fold higher affinity in comparison to Ang-(1–7). The concentrations up to 10 μmol/L the AT\textsubscript{1} receptor antagonist EXP 3174 and the AT\textsubscript{2} receptor antagonist PD 123,177 did not change specific binding of [^{125}\text{I}]-Ang-(1–7).

The Ang-(1–7) analogue [D-Ala\textsuperscript{7}]-Ang-(1–7), which is able to block some of the biological effects of Ang-(1–7),27 totally competed with the specific binding of [^{125}\text{I}]-Ang-(1–7) on BAECs, as also shown by Tallant et al.26 However, [D-Ala\textsuperscript{7}]-Ang-(1–7) failed to completely block (~50% inhibition only) NO release stimulated by AVE 0991 or Ang-(1–7).21

According to the specificity of AVE 0991 versus Ang II receptors, we observed a significant reduction of AVE 0991–stimulated NO release by EXP 3174 and even more pronounced by PD 123,177 (~50% and 90%, respectively). This significant but not completely inhibitory effect of EXP 3174 and PD 123,177 in the AVE 0991–stimulated NO release are in line with our findings on the Ang-(1–7)–stimulated NO release21 but in contrast to previous results from Brosnihan,23 which revealed any inhibitory efficacy of AT\textsubscript{1} and AT\textsubscript{2} receptor antagonists on the Ang-(1–7)–mediated NO release. However, our observed incomplete inhibition of the AVE 0991–mediated NO release on BAECs, together with the results of our binding experiments, are in favor of the previous suggestion that the Ang-(1–7) as well as the AVE
0991 effects could be mediated by non--AT$_2$ PD 123,177–sensitive and non--AT$_1$ EXP 3174–sensitive Ang II binding sites.

Although eNOS activity appears to be the source of both NO and O$_2$– production, the influence of the Ang II antagonists on the AVE 0991–induced O$_2$– productions was quite different from their effects on the respective NO production. PD 123,177 had no significant effect on O$_2$– production, whereas EXP 3174 was void of any inhibitory efficacy. In addition, the inhibitory effect of [D-Ala$^7$]-Ang-(1–7) on O$_2$– production was weaker (~35% inhibition) when compared with the respective inhibition of NO production. At present, these different influences of the Ang II antagonists on NO and O$_2$– production cannot be explained.

The moderate NO release by AVE 0991 was associated by a low concomitant production of O$_2$–. Since it is suggested that generation of O$_2$– and subsequent fast reaction between O$_2$– and NO to form peroxynitrite and cytotoxic radicals play a role in endothelial dysfunction and vascular injury, the nonpeptide AVE 0991 has a similar NO/O$_2$– releasing profile to Ang-(1-7) and might be a valuable compound for the preservation of the functional vascular system.

**Perspectives**

After the recent clinical findings described by Zeiher et al. and Heitzer et al., endothelial dysfunction is confirmed as a prognostic marker and will probably be recognized in the near future as a unique cardiovascular disease. The discovery of the nonpeptide and orally active Ang-(1–7) agonist AVE 0991 as a potent mimic of the unique NO/O$_2$– releasing profile of Ang-(1-7) opens the venue toward a novel treatment of patients with endothelial dysfunction.

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**References**


