Evidence for Mas-Mediated Bradykinin Potentiation by the Angiotensin-(1-7) Nonpeptide Mimic AVE 0991 in Normotensive Rats

Mariana B.L. Carvalho, Fernanda V. Duarte, Raphael Faria-Silva, Beatrix Fauler, Leonor T. da Mata Machado, Renata D. de Paula, Maria J. Campagnole-Santos, Robson A.S. Santos

Abstract—We evaluated the effect of the nonpeptide mimic of angiotensin (Ang)-(1-7), AVE 0991, on the hypotensive effect of bradykinin (BK). Increasing doses of intra-arterial or intravenous BK were administered before and 30 minutes after the beginning of AVE 0991 infusion. The effect of AVE 0991 on plasma Ang-converting enzyme activity was tested using Hip-His-Leu as the substrate. The interaction of AVE 0991 with Ang-converting enzyme in vivo was tested by determining its effect on the pressor action of Ang I or Ang II. AVE 0991 produced a significant and similar potentiation of intra-arterial or intravenous bradykinin. AVE 0991 did not inhibit plasma Ang-converting enzyme activity in vitro or the pressor effect of Ang I in vivo. N\(^\text{\textsuperscript{6}}\)-nitro-L-arginine methyl ester or D-Ala\(^\text{\textsuperscript{7}}\)-Ang-(1-7) administration abolished the BK potentiation effect of AVE 0991. We further examined the BK-potentiating effect of AVE 0991, evaluating its effect on NO production in rabbit endothelial cells. The NO release was measured using the 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate. A synergistic effect of AVE 0991 and BK on NO release was observed. These results suggest that AVE 0991 potentiates bradykinin through an Ang-converting enzyme–independent, NO-dependent receptor Mas-mediated mechanism. This effect may contribute to the improvement of endothelial function by AVE 0991 in vivo. (Hypertension. 2007;50:762-767.)

Key Words: bradykinin  ●  angiotensin (1-7)  ●  AVE 0991  ●  NO  ●  endothelial function

The recent characterization of the orphan G protein–coupled receptor Mas as an angiotensin (Ang)-(1-7) receptor involved in many of its biological actions\(^\text{1,2}\) and of the Ang-converting enzyme (ACE) homologue, ACE 2, as a major Ang-(1-7)-forming enzyme\(^\text{3,4}\) gave a more strong support for the concept that Ang-(1-7) is a biologically active component of the renin- Ang system. Furthermore, there is increasing evidence for a role of Ang-(1-7) as a counterregulatory peptide of the cardiovascular effects of Ang II.\(^\text{2,5}\)

Ang-(1-7) receptor agonists are being considered as putative cardiovascular drugs for the treatment of hypertension, heart failure, atherosclerosis, and many other diseases that involve endothelial dysfunction.\(^\text{2}\) Among these compounds AVE 0991, a nonpeptide mimic of Ang-(1-7),\(^\text{5}\) has been shown to evoke effects similar to those elicited by Ang-(1-7).\(^\text{6-9}\) In endothelial cells, AVE 0991 releases NO and, to a lesser extent, superoxide.\(^\text{6}\) In keeping with the NO-releasing activity of Ang-(1-7) and AVE 0991\(^\text{7,8,10-12}\) we have shown recently that acute infusion of Ang-(1-7) or AVE 0991 could potentiate the vasodilation produced by intra-arterial acetylcholine, suggesting an improvement of endothelial function.\(^\text{13}\) This effect was blocked in the presence of the endothelial NO synthase inhibitor N\(^\text{\textsuperscript{6}}\)-nitro-L-arginine methyl ester (t-NAME) or the receptor Mas antagonist D-Ala\(^\text{\textsuperscript{7}}\)-Ang-(1-7) (A-779).

Bradykinin (BK) is an endogenous peptide that causes vasodilation depending on endothelial factors, such as NO, the endothelium-derived hyperpolarizing factor, or prostaglandins.\(^\text{14}\) Ang-(1-7) enhances the effects of BK in a variety of models.\(^\text{15-20}\) However, Ang-(1-7) does not directly activate the BK B\(_1\) receptor,\(^\text{12}\) but it does amplify the effects of BK via ACE\(^\text{19}\) and through receptor-mediated release of prostaglandins and NO.\(^\text{15,17,18,20}\) It should be emphasized that ACE inhibition is not the major mechanism for the BK-potentiating activity in normotensive Wistar rats.\(^\text{17,18}\) In fact, Ang-(1-7) seems to increase the BK hypotensive effect by modulating a possible cross-talk among receptors or intracellular phosphorylating cascades rather than by preventing the BK degradation by ACE.\(^\text{2,16}\) This BK-potentiating activity, at least in spontaneously hypertensive and Wistar rats, seems to be a receptor Mas-mediated event, given that it is blocked in the presence of A-779,\(^\text{15,17,18,20}\) a selective antagonist for the Ang-(1-7) receptor.\(^\text{1}\) In Sprague-Dawley rats, however, other mechanisms are apparently involved.\(^\text{21-23}\)
Considering that other Ang peptides produced by enzymatic hydrolysis of Ang-(1-7) can potentiate BK, it would be important to determine the effect of the nonpeptide AVE 0991 on the BK effect and whether this potentiating activity can be blocked by the Ang-(1-7) receptor antagonist A-779. In this study, we addressed this question by determining the effect of AVE 0991 on the hypotensive effect of BK and by evaluating the role of Mas and NO on this effect.

**Methods**

**Animals**

Experiments were performed in 77 conscious male Wistar rats, weighing 282±4.5 g, bred at the animal facility of the Biological Sciences Institute (CEBIO, Federal University of Minas Gerais). All of the animal procedures were performed in accordance with institutional guidelines (Federal University of Minas Gerais).

**General Surgical Procedures**

Twenty-four hours before the experiments, under anesthesia with 2.5% tribromoethanol (1.0 mL/100 g), a polyethylene catheter (polyethylene-10 connected to polyethylene-50) was introduced into the descending aorta, through the left carotid artery, for intra-arterial injections. The correct position of the catheter was verified in postmortem examination. Other catheters were implanted into the abdominal aorta (through the femoral artery) for mean arterial pressure (MAP) and heart rate measurements and the femoral vein for intravenous infusions and injections. After recovery from anesthesia, the animals were kept in individual cages with free access to water and chow.

**Arterial Pressure Measurements**

The arterial pressure was monitored by a solid-state strain gauge transducer connected to a computer through a data acquisition system (MP 150, BIOPAC Systems, Inc). MAP and heart rate were calculated from the pulsatile pressure with the AcqKnowledge software. All of the parameters were continuously collected. The experiments were conducted in conscious rats.

**Drugs**

Ang I, Ang II, BK, L-NAME, A-779, and AVE 0991 were dissolved in isotonic saline (0.9% NaCl) immediately before use. Ang I, Ang II, and A-779 were from Bachem. BK and L-NAME were purchased from Sigma Chemical Co. AVE 0991 was a generous gift from Dr Juergen Puenter from Aventis Pharma.

**Experimental Protocols**

**Protocol 1: Effect of AVE 0991 on the Hypotensive Action of Intra-Arterial BK in Wistar Rats**

Intra-arterial bolus injections of BK (6.2, 12.5, 25.0, and 50.0 ng) were made before and within 30 minutes of intravenous infusion of AVE 0991 (46 pmol/min for 60 minutes; n=7; and 460 pmol/min for 60 minutes, n=6) or vehicle (6 μL/min for 60 minutes; n=6). An interval of 3 minutes was allowed between BK injections. After the first series of BK injections, the catheter was washed with isotonic saline. The doses of the drugs and the time points for determining their effects were chosen based on preliminary experiments.

**Protocol 2: Effect of AVE 0991 on the Hypotensive Action of BK in Wistar Rats Treated Previously With L-NAME**

After the first series of intra-arterial BK injections (6.2, 12.5, 25.0, and 50.0 ng), rats were treated with L-NAME (30 mg/kg, IV) just before AVE 0991 (230 pmol/min for 60 minutes; n=7) or isotonic saline infusion (6 μL/min for 60 minutes; n=5).

**Protocol 3: Effect of AVE 0991 on the BK-Potentiating Activity of AVE 0991 in Wistar Rats**

Intra-arterial bolus injections of BK (6.2, 12.5, 25.0, and 50.0 ng) were made before and within 30 minutes of intravenous infusion of AVE 0991 (230 pmol/min for 60 minutes) combined with A-779 (180 pmol/min for 60 minutes; n=5).

**Protocol 4: Effect of AVE 0991 on the ACE Activity in Wistar Rats**

The effect of AVE 0991 on ACE was tested in vivo using the following procedures: intravenous bolus injections of Ang I (2.5, 5.0, 10.0, and 20.0 ng) or Ang II (1.2, 2.5, 5.0, and 10.0 ng) were made before and within 30 minutes of intravenous infusion of AVE 0991 (230 pmol/min for 60 minutes; n=6; 460 pmol/min for 60 minutes; n=6) or vehicle (6 μL/min for 60 minutes; n=7 for Ang I and n=8 for Ang II); and intravenous bolus injections of BK (0.25, 0.5, 1.0, and 2.0 μg) were made before and within 30 minutes of intravenous infusion of AVE 0991 (230 pmol/min for 60 minutes; n=11).

**Protocol 5: Effect of AVE 0991 on the ACE Activity In Vitro**

To evaluate the effect of AVE 0991 on the ACE activity, different concentrations of this compound (10⁻³ to 10⁻⁸ mol/L) were tested, using Hip-His-Leu as substrate and a pool of rat plasma sample as the source of enzyme. The plasma was obtained from rats not used for the in vivo experiments. The product His-Leu released from the substrate was quantified by fluorometry, as described by Santos et al.

**Intracellular NO Measurement**

Confluent rabbit endothelial cells (RECs) between the fourth and ninth passages were plated in 6-well plates and used to evaluate NO release. RECs were preincubated in freshly prepared Krebs-Ringer-Hepes salt solution containing 2.5×10⁻⁷ mol/L of 4-amino-5-methylamino-2′-7′-difluorofluorescein diacetate (Molecular Probes) for 20 minutes, as described previously by Kojima et al. After washing with Krebs-Ringer-Hepes salt solution, cells were incubated with 10⁻⁶ mol/L of AVE 0991, 10⁻⁷ mol/L of BK, or AVE 0991 combined with BK for 15 minutes at 37°C in a humidified incubator under an atmosphere with 5% CO₂. Control cells were incubated in the same media without any drug. After the incubation period, the medium was discarded, and cells were then washed in Krebs-Ringer-Hepes salt solution and coverslipped using hydromount. Fluorescent images were obtained using a Zeiss 510 metalaser scanning confocal microscope equipped with an oil-immersion objective lens (×63).

**Statistical Analysis**

Numerical values are given as mean±SEM. Comparisons were made by 2-way ANOVA with Bonferroni posttest or Student’s paired t test when appropriate using GraphPad Prism version 4.0 for Windows (GraphPad Software). For in vitro studies, comparisons were made by 1-way ANOVA followed by Bonferroni posttest. The criterion for statistical significance was set at P<0.05.

**Results**

**Effect of AVE 0991 Infusion in Normotensive Wistar Rats**

Figure 1 shows the effect of different doses of AVE 0991 on the hypotensive action of BK. Infusion of AVE 0991 at 46 pmol/min for 60 minutes significantly increased the BK-evoked hypotension in normotensive Wistar rats (Figure 1A), although no difference was observed with the higher dose of BK. A more consistent potentiation was seen with a 5-fold increase in AVE 0991 (25 ng: −23±2 mm Hg versus −18±2 mm Hg, before; and 50 ng: −30±2 mm Hg versus −24±2 mm Hg, before; P<0.05; Figure 1B). A similar result was observed with 460 pmol/min per 60 minutes of AVE (Figure 1C). Isotonic saline infusion did not change the
The hypotensive effect of BK (Figure 1D). AVE 0991 infusion did not change baseline MAP or heart rate (Table). It should be noted that the baseline MAP values were slightly higher than expected because of the occlusion of the left carotid artery necessary for the BK injections.

Evaluating the Mechanism of Potentiation of BK-Evoked Hypotension by AVE 0991

Treatment with L-NAME (30 mg/kg IV) before intravenous infusion of isotonic saline did not abolish the hypotensive effect of BK in normotensive rats (see the data supplement available at http://hyper.ahajournals.org). However, the potentiation of BK-evoked hypotension by AVE 0991 was completely blocked in rats treated previously with L-NAME (Figure 2A). Baseline MAP was significantly increased after L-NAME injection (152±7 mm Hg at 30 minutes versus 118±5 mm Hg, for the group treated with AVE 0991; P<0.05 and 157±4 mm Hg at 30 minutes versus 125±8 mm Hg, in the control group; P<0.05). Therefore, although the absolute changes in MAP produced by BK after L-NAME treatment in the control group were actually higher than those observed in baseline conditions, the percent changes in blood pressure were not different.

Table. Effect of the Nonpeptide AVE 0991 Infusion on MAP and HR in Nonanesthetized Wistar Rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>131±7</td>
<td>129±6</td>
</tr>
<tr>
<td>AVE 0991 (46 pmol)</td>
<td>131±3</td>
<td>129±5</td>
</tr>
<tr>
<td>AVE 0991 (230 pmol)</td>
<td>137±4</td>
<td>129±4</td>
</tr>
<tr>
<td>AVE 0991 (460 pmol)</td>
<td>128±5</td>
<td>131±4</td>
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Values are expressed as mean±SEM. HR indicates heart rate.

Figure 1. Linear regression showing the dose-response hypotensive effect of intra-arterial BK in conscious Wistar rats, before and within 30 minutes of the intravenous infusion of the following: (A) AVE 0991 (46 pmol/min; n=8); (B) AVE 0991 (230 pmol/min; n=7); (C) AVE 0991 (460 pmol/min; n=8); and (D) isotonic saline (6 μL/min; n=6). Values are expressed as mean±SEM. *P<0.05, 2-way ANOVA followed by Bonferroni vs before infusion.

Figure 2. Linear regression showing the dose-response hypotensive effect of intra-arterial BK in conscious Wistar rats before and within 30 minutes of intravenous infusion of the following: (A) AVE 0991 (230 pmol/min) in animals treated previously with L-NAME (30 mg/kg, n=7) or (B) AVE 0991 (230 pmol/min) associated with A-779 (180 pmol/min; n=5). Values are expressed as mean±SEM.
To evaluate whether the BK-potentiating activity of AVE 0991 was a receptor-mediated response, we determined the effect of AVE 0991 combined with the Ang-(1-7) receptor Mas antagonist A-779. Infusion of A-779 combined with the nonpeptide AVE 0991 abolished the BK-potentiating activity of the Ang-(1-7) receptor agonist (Figure 2B).

Evaluating the Effect of AVE 0991 on ACE Activity In Vivo

Infusion of AVE 0991 at 230 pmol/min for 60 minutes did not change the pressor effect of intravenous Ang I at any dose (data not shown). A similar result was observed with a 2-fold increase in AVE 0991 (460 pmol/min; 2.5 ng: 6±1 mm Hg versus 9±2 mm Hg, before; and 20.0 ng: 33±5 mm Hg versus 27±4 mm Hg before; Figure 3A). Isotonic saline infusion did not change the hypertensive effect of Ang I (Figure 3B). Likewise, infusion of AVE 0991 at 230 pmol/min for 60 minutes did not change the pressor effect of intravenous Ang II (data not shown). A higher dose of AVE 0991 (460 pmol/min) also did not alter the increase in blood pressure induced by Ang II (1.25 ng: 10±2 mm Hg versus 11±1 mm Hg, before; and 10.0 ng: 31±5 mm Hg versus 31±4 mm Hg before; Figure 3C). Vehicle infusion did not change the hypertensive effect of Ang II at any dose (Figure 3D).

AVE 0991 at 230 pmol/min was also infused intravenously in animals receiving intravenous bolus injections of BK (0.25 µg: −9±1 mm Hg before, −14±2 mm Hg after; 0.5 µg: −12±1 mm Hg before, −19±2 mm Hg after; 1.0 µg: −17±2 mm Hg before, −21±2 mm Hg after; and 2.0 µg: −22±2 mm Hg before, −25±1 mm Hg after). The BK potentiation observed in this condition was in sharp contrast to what would be expected if AVE 0991 was blocking ACE. Indeed, the potentiation observed was even smaller than the one observed with the animals receiving intra-arterial BK.

Evaluating the Effect of AVE 0991 on the ACE Activity In Vitro

Using a fluorescence-based assay to determine ACE activity, we observed that AVE 0991 did not inhibit the conversion of Hip-His-Leu into its metabolite His-Leu in the presence of ACE in concentrations ≤10 µmol/L (data not shown).

Effect of AVE 0991 on the NO Release Induced by BK in RECs

Figure 4 shows NO measurements using 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate expressed as the percentage of NO production in relation to control cells. The fluorescence in the cells increased in an NO concentration-dependent manner. In the presence of BK (10⁻⁷ mol/L), AVE 0991 (10⁻⁸ mol/L) or AVE 0991 (10⁻⁸ mol/L) combined with BK (10⁻⁷ mol/L), fluorescent images were obtained using a Zeiss 510 metalaser scanning confocal microscope equipped with an oil-immersion objective lens (×63). *P<0.01 vs BK 10⁻⁷ mol/L; #P<0.01 vs AVE 0991, 10⁻⁸ mol/L. Values are expressed as the percentage of NO production in relation to control cells.
mol/L), a slight increase in NO release in RECs was observed (Figure 4B). The NO release by RECs was 4-fold higher in the presence of AVE 0991 alone (10^{-8} \text{ mol/L}; P<0.01; Figure 4C), whereas the combination of AVE 0991 (10^{-8} \text{ mol/L}) and BK (10^{-7} \text{ mol/L}) produced a 9-fold increase in NO release by RECs (P<0.001; Figure 4D).

**Discussion**

The major finding of this study was that short-term AVE 0991 infusion significantly increased the hypertensive effect of intra-arterial BK administration in normotensive rats. In addition, the potentiation of BK-induced hypotension by AVE 0991 was completely blocked by pretreatment with l-NAME or A-779 infusion. In vitro studies in RECs supported the evidence obtained with l-NAME that this effect involves NO release. These results are in accordance with previous observations in normotensive Wistar rats showing that Ang-(1-7) given in bolus or by infusion potentiated the hypertensive effect of BK.\(^{17,18}\)

The endothelial cells are one of the sites where Ang-(1-7) and its analogs may exert their effects, as evidenced by the lack of relaxation induced by AVE 0991 or Ang-(1-7) in endothelium-denuded vessels from dogs,\(^{11}\) rats,\(^{22,27}\) pigs,\(^{10}\) and mice.\(^{8}\) In keeping with this evidence, Mas is expressed in endothelial cells,\(^{2}\) which also seems to be the primary site for the vascular effects of BK.\(^{28}\) Moreover, the vasodilatation produced by Ang-(1-7) and AVE 0991 in mice aorta rings is blocked by A-779\(^{28}\) and absent in vessels taken from Mas-deficient mice.\(^{18}\)

It has been described that in vitro AVE 0991 is more potent than Ang-(1-7) in promoting NO release.\(^{6}\) However, in the present study, the dose of AVE 0991 necessary to produce an effect similar to the one described for Ang-(1-7) for BK potentiation\(^{18}\) was \(~750-1500\)-fold higher. These contrasting results, which have also been obtained for acetylcholine potentiation,\(^{13}\) may be related to the fact that Ang-(1-7) vascular actions may be mediated by multiple mechanisms. In addition to Mas-binding, Ang-(1-7) may generate other fragments with BK-potentiating activity\(^{24}\) and can bind to ACE.\(^{19,29}\) Furthermore, the extent of binding of AVE 0991 to plasma proteins, which may impact its biodisponibility and distribution, is not available in the literature, making difficult a more precise pharmacokinetic and pharmacodynamic comparison of this compound with Ang-(1-7).

Interestingly, AVE 0991 had no effect on blood pressure in the conscious rats. However, at least in anesthetized rats, Ang-(1-7) increases cardiac output and produces significant vasodilatation in many vascular territories, decreasing total peripheral resistance. These changes have been observed after acute\(^{30}\) or chronic\(^{31}\) increases in plasma Ang-(1-7). The opposite changes in cardiac output and total peripheral resistance could explain the absence of important changes in blood pressure after Ang-(1-7) administration. Although similar studies have not been performed with AVE 0991, our hypothesis is that similar changes in cardiac output and total peripheral resistance might occur in response to this compound.

We have found that AVE 0991 at concentrations \(\leq 10 \mu\text{mol/L}\) had no noticeable effect on rat ACE activity, as evaluated by the hydrolysis of Hip-His-Leu. Similar results were obtained with human plasma ACE (M.B.L.C., R.D.P., and R.A.S.S., unpublished data, 2007). We also observed that in vivo intravenous infusion of AVE 0991 did not alter the hypertensive action of Ang I, nor did it produce any significant change in the pressor effect of Ang II. More importantly, because ACE contributes to pulmonary BK inactivation, potentiation of BK given intravenously by ACE inhibition is much more evident than the one observed with the intra-arterial administration of this nonapeptide.\(^{32}\) This was not the case with AVE 0991. Indeed, AVE 0991 potentiation of the intravenous BK effect was even smaller than that observed with the intra-arterial route. Taken altogether, these findings indicate that nonenzymatic endothelial mechanisms are the primary factors involved in BK potentiation by AVE 0991. In fact, the potentiation of BK by AVE 0991 can be considered as a further evidence that AVE 0991 improves endothelial function in rats.\(^{13}\)

l-NAME treatment abolished the augmented hypertensive effect of BK elicited by AVE 0991, suggesting that BK potentiation is involved in the facilitation of NO release from the endothelium. Accordingly, we have observed that AVE 0991 potentiates the release of NO induced by BK in RECs. Our results, however, do not rule out the involvement of arachidonic acid derivatives\(^{33}\) or the endothelium-derived hyperpolarizing factor\(^{16,20,34}\) in this effect.

The BK-potentiating activity of AVE 0991 was completely abolished by the Ang-(1-7) receptor Mas antagonist A-779. This effect cannot be attributed to an influence of A-779 alone, because in a previous study from our laboratory, we observed no consistent changes in BK-evoked hypotension when A-779 was infused alone.\(^{18}\) We and others have shown that A-779 antagonizes the effects of Ang-(1-7) and AVE 0991 in several preparations,\(^{16}\) including the effect of AVE 0991 in Mas-transfected Chinese hamster ovary cells,\(^{7}\) mouse kidney,\(^{7}\) mouse aorta,\(^{8}\) and the potentiation of acetylcholine vasodilatation, in vivo,\(^{13}\) indicating that at least some of the AVE 0991 effects are receptor Mas-mediated. In summary, in this study we have obtained evidence that AVE 0991 potentiates BK by a Mas-mediated mechanism involving facilitation of NO release.

**Perspectives**

The results of the present study show that the nonpeptide Ang-(1-7) analog, AVE 0991, induces BK potentiation without altering the Ang I or Ang II pressor effect. This observation is in keeping with the absence of a noticeable effect of AVE on ACE activity in vitro. The evidence obtained with l-NAME and A-779 in vivo and in endothelial cells in vitro indicates the involvement of a Mas-mediated mechanism involving facilitation of NO release in the potentiation of BK by AVE 0991. Furthermore, these data, which are in line with previous observations,\(^{13}\) suggest that AVE 0991 can be used to improve endothelial function in vivo.

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Disclosures

None.

References

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Figure S1: Linear regression showing the dose-response hypotensive effect of Intra-arterial BK in conscious Wistar rats, before and within 30 minutes of intravenous infusion of isotonic saline (6 µL/ min) in animals previously treated with L-NAME (30mg/Kg, n=5). (A) Values are expressed as mean ± SEM and (B) values are expressed as percentage of baseline blood pressure.