Nonpeptide AVE 0991 Is an Angiotensin-(1–7) Receptor Mas Agonist in the Mouse Kidney

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Abstract—It has been described recently that the nonpeptide AVE 0991 (AVE) mimics the effects of angiotensin-(1–7) [Ang-(1–7)] in bovine endothelial cells. In this study, we tested the possibility that AVE is an agonist of the Ang-(1–7) receptor Mas, in vitro and in vivo. In water-loaded C57BL/6 mice, AVE (0.58 nmol/g body weight) produced a significant reduction in urinary volume (0.06 ± 0.03 mL/60 min [n=9] versus 0.27 ± 0.05 [n=9]; $P < 0.01$), associated with an increase in urinary osmolality. The Ang-(1–7) antagonist A-779 completely blocked the antidiuretic effect of AVE. As observed previously for Ang-(1–7), the antidiuretic effect of AVE after water load was blunted in *Mas*-knockout mice (0.37 ± 0.10 mL/60 min [n=9] versus 0.27 ± 0.03 mL/60 min [n=11] AVE-treated mice). In vitro receptor autoradiography in C57BL/6 mice showed that the specific binding of ^{125}I-Ang-(1–7) to mouse kidney slices was displaced by AVE, whereas no effects were observed in the binding of ^{125}I-angiotensin II or ^{125}I-angiotensin IV. Furthermore, AVE displaced the binding of ^{125}I-Ang-(1–7) in *Mas*-transfected monkey kidney cells (COS) cells ($IC_{50} = 4.75 \times 10^{-8}$ mol/L) and of rhodamine–Ang-(1–7) in *Mas*-transfected Chinese hamster ovary (CHO) cells. It also produced NO release in *Mas*-transfected CHO cells blocked by A-779 but not by angiotensin II type-1 (AT_1) and AT_2 antagonists. Contrasting with these data, the antidiuretic effect of AVE was totally blocked by AT_2 antagonists and partially blocked ($\approx 60\%$) by AT_1 antagonists. The binding data, the results obtained in *Mas*-knockout mice and in *Mas*-transfected cells, show that AVE is a Mas receptor agonist. Our data also suggest the involvement of AT_2/AT_1-related mechanisms, including functional antagonism, oligomerization or cross-talk, in the renal responses to AVE. (*Hypertension*. 2004;44:490-496.)

Key Words: receptors, angiotensin ■ angiotensin ■ angiotensin II

S ince the demonstration that angiotensin-(1–7) [Ang-(1–7)] was equipotent to angiotensin II (Ang II) for releasing vasopressin (AVP) from hypothalamic-neurohypophyseal explants,[1] several studies have provided morphological and functional data, indicating a role for Ang-(1–7) in fluid homeostasis and in kidney and cardiovascular function.[2–4]

In addition to its natriuretic/diuretic action in vitro and in anesthetized animals,[4–8] Ang-(1–7) produces a potent AVP-independent antidiuretic effect in conscious water-loaded rats[9] that can be accounted for its weak glomerular effects reducing the glomerular filtration rate and by its potent tubular actions,[8,9] especially at the intramedullary collecting ducts (IMCD).[10–12] The natriuresis and diuresis observed with acute[9] or chronic[10] infusion of the Ang-(1–7) antagonist A-779 and the natriuresis observed with acute A-779 administration in anesthetized rats[11] are in agreement with these findings.

We described recently that Ang-(1–7) is an endogenous ligand for the G protein–coupled receptor Mas.[13] The specific binding of the ^{125}I-Ang-(1–7) is abolished in kidneys of *Mas*-null mice, whereas no changes were observed in the specific binding of ^{125}I-Ang II and ^{125}I-Ang IV.[13] Accordingly, the antidiuresis promoted by Ang-(1–7) in water-loaded mice was absent in *Mas*-deficient mice, suggesting that the receptor Mas is essential for this renal action of Ang-(1–7).[13]

The growing body of evidence that Ang-(1–7) counteracts many of the Ang II cardiovascular effects[2,14] makes this peptide a potential target for development of orally active agonists. In this regard, a novel nonpeptide compound, AVE 0991 (AVE), has been described recently as an Ang-(1–7)

mimic.[15] In bovine endothelium, AVE produced a NO-releasing effect similar to that of Ang-(1–7).[14,16] AVE and unlabeled Ang-(1–7) competed with high affinity for the binding of ^{125}I-Ang-(1–7) to bovine aortic endothelial cell membranes,[16] suggesting that the NO-releasing activity of AVE is a receptor-mediated event. However, Ang II type 1 (AT_1) antagonist reduced and AT_2 antagonist abolished this AVE effect. In addition, the Ang-(1–7) receptor Mas antagonist A-779 produced only partial inhibition ($\approx 50\%$) of the NO release induced by AVE in these cells. These observations raise the possibility that AVE is not a Mas agonist. In this study, we tested this hypothesis in vitro and in vivo using radioligand binding and functional studies in *Mas*–wild-type (*Mas*-WT) and *Mas*-knockout (*Mas*-KO) mice and in *Mas*-transfected cells.

Materials and Methods

Animals

Swiss male mice, *Mas*-KO ($Mas^{-/-}$) male mice on the pure genetic background C57BL/6, and WT C57BL/6 control mice ($Mas^{+/+}$)[17] were used in the experiments (see data supplement, available online at http://www.hypertensionaha.org). The animal care committee from Federal University of Minas Gerais, Brazil, approved all experimental protocols.

Drugs and Reagents

The information about the drugs and reagents used in the experiments is included in the online data supplement of this journal.

Water Diuresis

Water diuresis was induced by intraperitoneal water injection (0.05 mL/g of body weight [BW]) in conscious mice as described previously.[13,18] Drugs were administered in the same injection with water load at prefixed volumes (0.01 mL/g BW). In the first set of experiments, WT mice (C57BL/6, control group) or *Mas*-KO mice were treated with: (1) 0.58 nmol/g AVE (n=9, control; n=11, *Mas*-KO mice); or (2) vehicle for AVE (10^{-2} mol/L KOH, 0.01 mL/g; n=9, control; n=9, *Mas*-KO). In the second set, Swiss mice were treated with: (1) vehicle (n=36); (2) 0.58 nmol/g AVE (n=16); (3) 46 pmol/g Ang-(1–7) antagonist A-779 (n=4); (4) 2 nmol/g losartan or valsartan (n=5); (5) 2 nmol/g AT_2 receptor antagonists PD123319 or PD123177 (n=9); (6) AVE combined with A-779; (7) AVE combined with losartan or valsartan (n=4 for each); (8) or AVE combined with PD123319 (n=5) or PD123177 (n=4). The urinary volume was measured for 60 minutes after water loading, and urine samples were obtained to determine the osmolality (Osmomether; Fiske Associates, Inc). The dose of AVE was based in preliminary experiments performed in Swiss mice.

Blood Pressure Measurement

A polyethylene catheter (PE-10 connected to PE-50) was inserted into the right carotid artery of C57BL/6 mice for blood pressure measurements. All surgical procedures were performed under ketamin and xylazine anesthesia (15 μg and 0.3 μg per g BW, respectively). One day after surgery, the basal value of mean arterial pressure (MAP) was recorded before and after water diuresis with vehicle (10 mmol/L 0.01 mL/g KOH; n=3) or AVE (0.58 nmol/L, n=3). Heart rate and MAP were recorded on a data acquisition system (MP100; Biopac Systems).

In Vitro Receptor Autoradiography of ^{125}I-Angiotensins Binding to Mice Kidneys

Kidneys of WT mice (C57BL/6) were snap-frozen in cooled isopentane ($-32°$C). Sections (16 μm) were serially cut, starting from the central area of the kidney (HM 505N; Microm), mounted on gelatin-coated slides, and dried at 4°C, before the assay.

The binding assay was performed as described previously.[13] Sections were incubated with high-performance liquid chromatography–purified radioligand. For Ang-(1–7) binding: assay buffer with 5×10^{-10} mol/L ^{125}I-Ang-(1–7) and 10^{-5} mol/L of either Ang-(1–7) or A-779 (nonspecific binding [NSB]) or 10^{-5} mol/L AVE. For Ang II binding: assay buffer with 5×10^{-10} mol/L ^{125}I-Ang II and 10^{-5} mol/L Ang II (NSB) or 10^{-5} mol/L AVE. For Ang IV binding: assay buffer with 5×10^{-10} mol/L ^{125}I-Ang IV and 10^{-5} mol/L Ang IV (NSB) or with 10^{-5} mol/L AVE.

After incubation, sections were rinsed in 0.05 mol/L Tris-HCl, pH 7.4 (3×30 sec), and in distilled water (1×20 sec), dried at room temperature, and exposed to autoradiographic film (Fuji Image Plate) for 48 [Ang-(1–7)], or 24 (Ang II and Ang IV) hours. After exposure, films were scanned and quantified by densitometry (Bio-Imaging Analyzer; Fujifilm). Illustrations were made with Osiris Imaging software (version 3.12).

Fluorescent and ^{125}I-Ang-(1–7) Binding Experiments in Mas-Transfected cells

Monkey kidney (COS) cells and Chinese hamster ovary (CHO) cells were stably transfected with rat *Mas* cDNA driven by a cytomegalovirus promoter and selected by neomycin.[13] Cell lines were cultured as described previously.[13] ^{125}I-Ang-(1–7) (0.5×10^{-9} mol/L) was incubated in 24-well plates for 60 minutes at 4°C in 0.3 mL of serum-free medium (DMEM) supplemented with 0.2% BSA, 0.005% bacitracin, 0.1 mol/L PMSF, and 0.5 mol/L orthophenanthroline with *Mas*-transfected COS cells in the presence or absence of AVE (10^{-10} to 10^{-5} mol/L). After 2 washes with ice-cold serum-free DMEM, cells were disrupted with 0.1% Triton X-100. Bound radioactivity was measured in a gamma counter.

Binding of rhodamine–Ang-(1–7) in *Mas*-transfected CHO cells was performed under similar conditions using 2×10^{-9} mol/L rhodamine-labeled–Ang-(1–7) in the presence or absence of AVE (10^{-6} mol/L), CV11974 (10^{-6} mol/L), or PD123319 (10^{-6} mol/L). NSB was determined in the presence of 10^{-6} mol/L Ang-(1–7). Relative fluorescence measurements were performed on a Zeiss 510 meta laser scanning confocal microscope equipped with an oil-immersion objective lens ($\times 63$).

Imaging of Intracellular NO Production in CHO and *Mas*-Transfected CHO Cells

For fluorescence experiments, confluent cells between the second and fourth passages were platelet in 6-well plates. CHO-transfected and CHO-nontransfected cells were preincubated in freshly prepared Hanks' balanced salt solution (HBSS) containing 10^{-6} mol/L 4,5-diaminofluorescein-diacetate (DAF-2DA; Molecular Probes) for 20 minutes. After washing with HBSS, cells were incubated with 10^{-8} mol/L AVE alone or combined with 10^{-6} mol/L A-779, CV11974, or PD123319 for 30 minutes at 37°C in a humidified incubator under an atmosphere with 5% CO_2. Control cells were incubated in the same media without any drug. As negative control, cells were incubated in media lacking DAF-2DA. After the incubation time, the medium was discarded, and the cells were then washed in PBS and cover slipped using 10% glycerol (Tris, pH 7.4). Fluorescent images were obtained using a Zeiss 510 meta laser scanning confocal microscope equipped with an oil-immersion objective lens ($\times 63$).

Statistics

The results are expressed as the mean$\pm$SEM. Statistical analyses for radiolabeled ligand binding were performed using unpaired t test. Statistical analyses for other data were performed using unpaired t test or ANOVA followed by Student Newman–Keuls test for multiple comparisons when appropriate (GraphPad Prism 4.0). The level of significance was set at $P < 0.05$.

Results

Water Diuresis in *Mas*-WT and *Mas*-KO Mice

In the first set of experiments, we evaluated the effect of AVE in water-loaded WT mice (C57BL/6) and *Mas*-KO mice. The
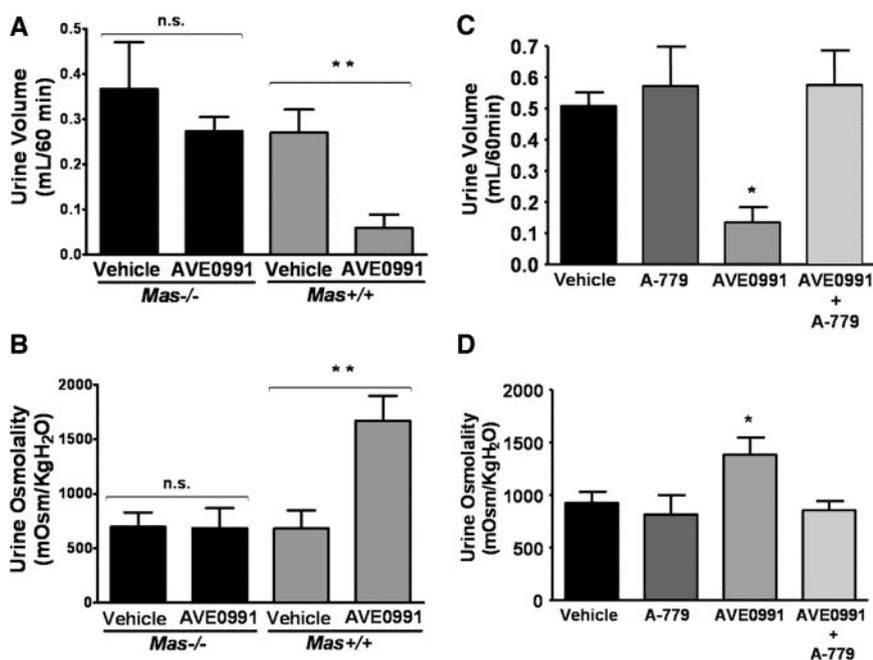
Figure 1. Effect of intraperitoneal AVE (0.58 nmol/g) on the urinary volume (A) and urinary osmolality (B) of water-loaded *Mas*-KO (*Mas*$^{-/-}$; n=18) and *Mas*-WT (*Mas*$^{+/+}$; n=20) mice. Effect of AVE (n=16), A-779 (0.46 pmol/g; n=7), vehicle (0.01 mL/g; n=40), and AVE plus A-779 (n=4) on the urinary volume (C) and urinary osmolality (D) of water-loaded Swiss mice. **$P<0.01$ compared with vehicle-treated *Mas*$^{+/+}$ mice. n.s. indicates nonsignificant. *$P<0.05$ compared with vehicle-treated Swiss mice.

urine volume measured after water load in WT and *Mas*-KO was similar. As shown in Figure 1A, AVE (0.58 nmol/g) produced a significant decrease of water diuresis in WT mice compared with vehicle-treated animals (0.06±0.03 mL versus 0.27±0.05; n=9 for each group; $P<0.01$). The antidiuretic effect of AVE was associated with an increase in urine osmolality (1669±231.0 mOsm/KgH$_2$O versus 681.1±165.8 mOsm/KgH$_2$O in vehicle-treated mice; $P<0.01$). The genetic deletion of *Mas* abolished the antidiuretic effect of AVE during water loading (0.37±0.10 mL [n=9] versus 0.27±0.03 mL [n=11] in AVE-treated mice). Accordingly, no changes were observed in urine osmolality (684.4±183.2 mOsm/KgH$_2$O versus 696.9±131.7 mOsm/KgH$_2$O in AVE-treated *Mas*-KO mice; Figure 1B).

To exclude the possibility that the antidiuresis produced by AVE was promoted by systemic hemodynamic changes, additional water-load experiments were performed in mice instrumented for blood pressure recording. Only minor, nonsignificant, changes in MAP were observed in vehicle-treated mice (baseline 112±6.1 mm Hg and 116±1.0, 115±4.0, and 114±3.3 mm Hg at 20, 40, and 60 minutes after water load, respectively; $P>0.05$ compared with baseline values; 1-way ANOVA for repeated measurements). A similar observation was made for AVE-treated mice (baseline 109±3.3 mm Hg and 107±3.5, 102±1.5, and 106±2.915 mm Hg at 20, 40, and 60 minutes after water load, respectively; $P>0.05$ compared with baseline values). No differences in heart rate before and after treatment were observed in vehicle-treated mice (baseline 567±50.5 and 629±25.8 bpm at 60 minutes) and AVE-treated mice (baseline 540±59.7 and 618±43.1 bpm at 60 minutes).

Water Diuresis in Swiss Mice
To assure that the antidiuretic effect of AVE was not strain specific, we tested the effects of AVE in another mouse strain. As observed with C57BL/6 mice, administration of

AVE (0.58 nmol/g) in water-loaded Swiss mice also produced a significant decrease of the urinary volume compared with vehicle-treated animals (0.13±0.05 mL [n=16] versus 0.51±0.04 mL [n=40]; $P<0.01$; Figure 1C). In accordance with the results obtained in C57BL/6 mice, the urine osmolality was significantly higher in AVE-treated animals (1382.0±162.9 mOsm/KgH$_2$O [n=8] versus 924.1±104.9 mOsm/Kg H$_2$O in vehicle-treated mice [n=36]; $P<0.05$; Figure 1D). Furthermore, the antidiuretic action of AVE and its effect on urine osmolality were completely abolished by the selective Ang-(1–7) antagonist A-779 (46 pmol/g; 0.58±0.11 mL [n=4] and 852.0±90.19 mOsm/KgH$_2$O [n=4]), whereas A-779 alone did not change the urinary volume and osmolality compared with vehicle-treated animals (0.57±0.13 mL [n=7] and 814.7±184.0 mosm/KgH$_2$O [n=6], respectively).

In Vitro Receptor Autoradiography of ^{125}I-Angiotensin Binding to Mouse Kidney
As summarized in Figure 2A, AVE (10^{-5} mol/L) displaced the binding of ^{125}I-Ang-(1–7) to mouse kidney slices. As demonstrated by the binding in the presence of A-779, AVE produced a displacement of 85% of the specific ^{125}I-Ang-(1–7) binding. In contrast, no displacement of the binding of ^{125}I-Ang II or ^{125}I-Ang IV was observed in the presence of AVE (10^{-5} mol/L; Figure 2B and 2C).

Mas-Transfected Cell Experiments
In keeping with the receptor autoradiography data and with the functional data in *Mas*-KO mice, AVE competed the specific binding of ^{125}I-Ang-(1–7) to *Mas*-transfected COS cells with an IC$_{50}$ value of 4.75×10^{-8} mol/L (n=3). In addition, the specific binding of rhodamine–labeled Ang-(1–7) to *Mas*-transfected CHO cells was completely abolished in the presence of 10^{-6} mol/L AVE (Figure 3). In accordance with previous observations,[13] no changes were
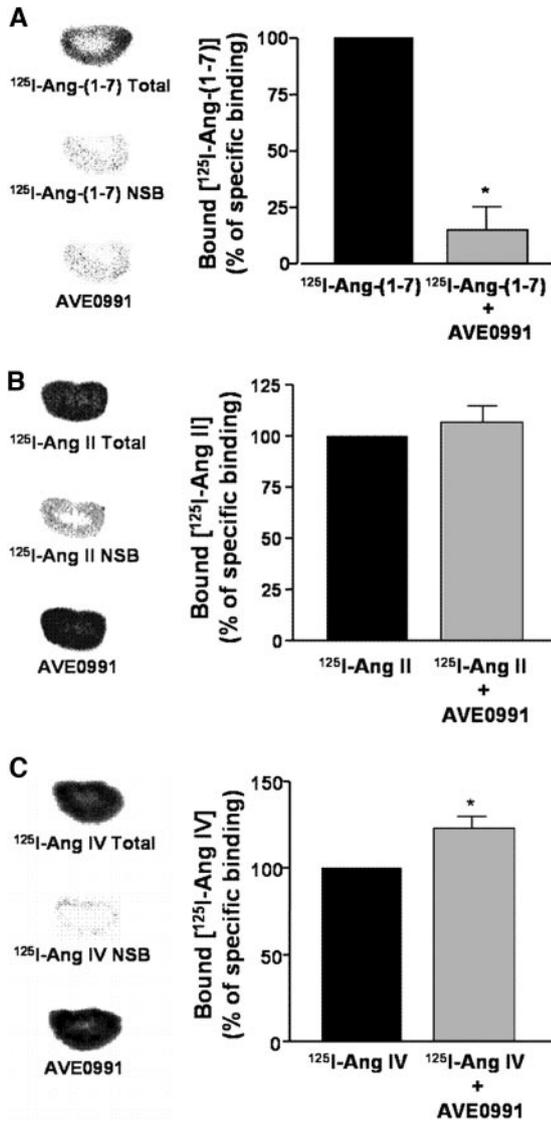
Figure 2. In vitro autoradiographic localization of ^{125}I-Ang-(1–7), ^{125}I-Ang II and ^{125}I-Ang IV binding in C57BL/6 mouse kidney sections. A, ^{125}I-Ang-(1–7) total binding (top), the ^{125}I-Ang-(1–7) NSB in the middle, and the ^{125}I-Ang-(1–7) binding in the presence of AVE 0991 (bottom). The graph shows the effect of AVE on the specific binding of ^{125}I-Ang-(1–7) to mouse kidney sections (n=20 kidney slices from 3 mice from each group). B, ^{125}I-Ang II total binding (top), the ^{125}I-Ang II NSB (middle), and the ^{125}I-Ang II binding in the presence of AVE (bottom). The bars show the effect of AVE on the specific binding of ^{125}I-Ang II to mouse kidney sections (n=8 kidney slices from 2 mice from each group). C, ^{125}I-Ang IV total binding (top), the ^{125}I-Ang IV NSB (middle), and the ^{125}I-Ang IV binding in the presence of AVE (bottom). The graph shows the effect of AVE on the specific binding of ^{125}I-Ang IV to mouse kidney sections (n=12 kidney slices obtained from 3 mice from each group). The concentration of ^{125}I-Ang-(1–7), ^{125}I-Ang II, and ^{125}I-Ang IV was 0.5 nmol/L, whereas A-779 (NSB), Ang II, Ang IV, and AVE 0991 were used at 10^{-5} mol/L. *$P<0.05$ Student t test.

observed in the binding of rhodamine–Ang-(1–7) to *Mas*-transfected CHO cells in the presence of 10^{-6} mol/L CV11974 or 10^{-6} mol/L PD123319. More important, binding of AVE produced a functional response (NO production) in *Mas*-transfected but not in control CHO cells (Figure 4), which was completely blocked by 10^{-6}
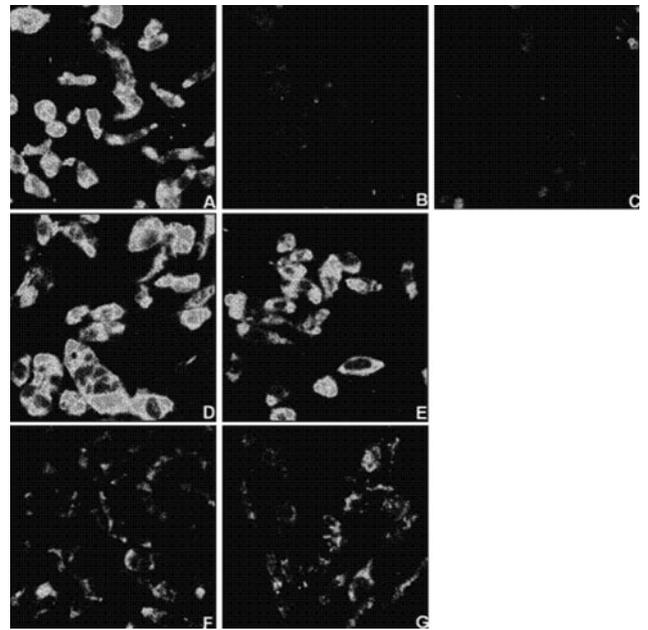

Figure 3. Confocal microphotographs of the rhodamine–Ang-(1–7) [Rho-Ang-(1–7)] binding on *Mas*-transfected CHO cells or nontransfected CHO cells. A, Rho-Ang-(1–7) total binding (2×10^{-9} mol/L) on *Mas*-transfected CHO cells. B, Rho-Ang-(1–7) binding in the presence of 10^{-6} mol/L AVE on *Mas*-transfected CHO cells. C, Rho-Ang-(1–7) NSB [10^{-6} mol/L Ang-(1–7)] on *Mas*-transfected CHO cells. D and E, Rho-Ang-(1–7) binding on *Mas*-transfected CHO cells in the presence of AT$_2$ antagonist PD123319 (10^{-6} mol/L) or AT$_1$ antagonist losartan (10^{-6} mol/L), respectively. F and G, Rho-Ang-(1–7) total and NSB on nontransfected CHO cells, respectively. For color figure, see data supplement, available online at http://www.hypertensionaha.org.

mol/L A-779 but not by AT$_1$ (CV11974) or AT$_2$ (PD123319) antagonists (Figure 4).

Effect of AT$_1$ and AT$_2$ Antagonists on Water Diuresis

To further assess the specificity of the AVE action, we tested the effects of Ang II antagonists on the antidiuresis produced by AVE in water-loaded mice. Water diuresis experiments were performed in Swiss mice tested with AVE (0.58 nmol/g), the AT$_1$ antagonists losartan or valsartan, or the AT$_2$ antagonists PD123319 or PD123177. In contrast with the CHO cell data described above, the antidiuretic action of AVE was completely blocked by concomitant administration of AT$_2$ antagonists (PD123319 0.61 ± 0.28 mL versus 0.59 ± 0.26 in PD123319+AVE; PD123177 0.33 ± 0.19 mL versus 0.48 ± 0.29 mL in PD123177 plus AVE; supplemental Table I, available online at http://www.hypertensionaha.org), whereas losartan or valsartan produced only a partial inhibition (losartan 0.85 ± 0.15 mL versus 0.43 ± 0.11 mL in losartan+AVE versus 0.13 ± 0.05 mL in AVE-treated mice, $P<0.05$; valsartan 0.97 ± 0.26 mL versus 0.50 ± 0.07 in valsartan plus AVE; supplemental Table).

Discussion

After the description of AVE[15] as a nonpeptide agonist of Ang-(1–7) in the bovine endothelium,[16] this compound has become of great potential for studies related to Ang-(1–7) actions. Among the advantages to use AVE instead of
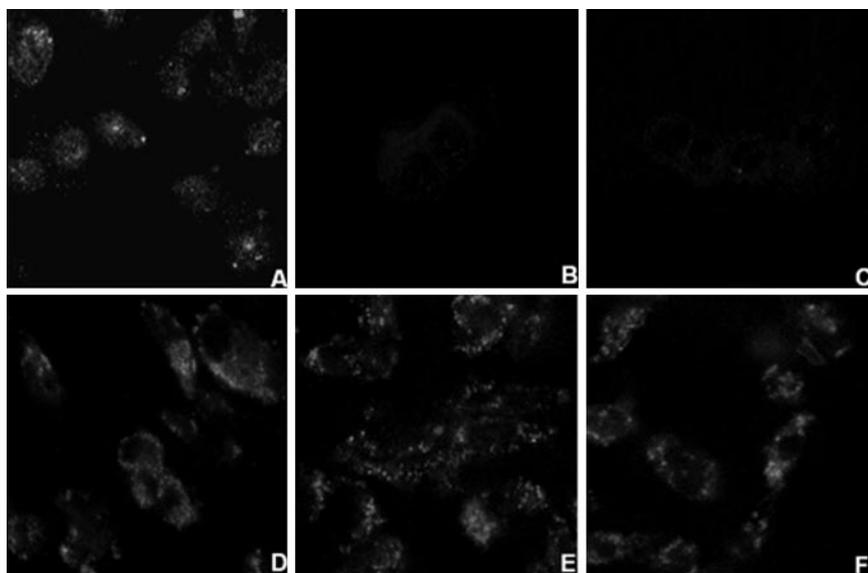
Figure 4. Confocal microphotographs of *Mas*-transfected CHO cells treated with AVE (10^{-8} mol/L; A and D), with AVE (10^{-8} mol/L) in presence of Ang-(1–7) antagonist A-779 (10^{-6} mol/L; B), or non-transfected CHO cells treated with AVE 0991 (10^{-8} mol/L; C). E and F, Microphotographs of *Mas*-transfected CHO cells treated with AVE (10^{-8} mol/L) in presence of AT_2 antagonist PD123319 (10^{-6} mol/L) or AT_1 antagonist CV11974 (10^{-6} mol/L), respectively. NO was visualized using the DAF-2 DA probe. For color figure, see online data supplement.

Ang-(1–7) are the facts that it is orally active and it would be resistant to proteolytic enzymes, circumventing an important problem associated with the use of peptides. In this study, we further documented the Ang-(1–7)–like activity of AVE, using in vitro and in vivo preparations. More important, we have obtained clear evidence that AVE is an Ang-(1–7) receptor Mas agonist.

The ^{125}I-Ang-(1–7) binding to kidney slices of C57BL/6 mice was essentially abolished in the presence of AVE. In contrast, the ^{125}I-Ang II and ^{125}I-Ang IV binding were not altered. These results are in keeping with a previous report[16] showing that AVE was void of significant inhibition of the specific Ang II binding to AT_1 or AT_2 receptors (only $\approx 12\%$ inhibition at 10^{-5} mol/L).

AVE efficiently displaced the binding of rhodamine-labeled or ^{125}I-labeled Ang-(1–7) to *Mas*-transfected cells. These observations reinforce the binding data obtained in kidney slices and are in agreement with our recent description that Ang-(1–7) is an endogenous ligand for the G protein–coupled receptor Mas.[13]

The binding of AVE to the Mas receptor is capable to elicit functional responses as illustrated by our observation that AVE induced NO release in *Mas*-transfected cells. This effect was completely blocked by the Ang-(1–7) antagonist A-779 but not by AT_1 (CV11974) or AT_2 (PD123319) antagonists. Together, these observations clearly indicate that AVE is an Ang-(1–7) receptor agonist essentially devoid of binding to AT_1 or AT_2 receptors.

AVE produces a decrease in urinary volume in control mice associated with a rise in urine osmolality, indicating that the antidiuretic effect of AVE, as that observed with Ang-(1–7),[13] also involves an increase in water reabsorption. Similar data were obtained in rats (M. Bleich, unpublished data, 2002). In accordance with our previous data,[13] the antidiuretic effect of AVE was blunted in *Mas*-deficient animals. Furthermore, the effects of this nonpeptide compound on the urinary volume and urinary osmolality of Swiss mice were completely blocked by A-779. These findings add further support to the evidence that AVE mimics Ang-(1–7)

renal actions by acting on the Ang-(1–7) receptor Mas. Although we cannot exclude at this stage the possibility that other renal or cardiovascular actions of Ang-(1–7) could also be mediated by other receptor(s) or that other peptides could bind to Mas, our present findings give further support to the concept that Mas is an Ang-(1–7) receptor. Of note is the fact that using a bioinformatic approach, Lio and Vannucci[19] classified Mas and the apelin receptor in the angiotensin receptor family.[19]

It has been shown that Ang-(1–7) increases osmotic water permeability in isolated toad skin,[20] a tissue with functional properties similar to those of the distal mammalian nephron.[21] This finding is in keeping with the antidiuretic action of Ang-(1–7) in rats[9,10,18] and in mice.[13] Moreover, it was demonstrated recently that Ang-(1–7) but not Ang IV produces a significant dose-dependent increase in water permeability in the IMCDs accompanied by an increase in cAMP levels.[12] A-779 completely blocked these effects, suggesting a specific receptor-mediated action.[12] Interestingly, pretreatment with an AVP V_2-receptor antagonist inhibited the Ang-(1–7) effect on water permeability. Similarly, A-779 inhibited the effect of AVP on water transport.[12] Surprisingly, both antagonists significantly diminished the increase in water reabsorption induced by forskolin, an adenylate cyclase stimulator, or its effect on cAMP production in IMCD cells. These data suggest that Ang-(1–7) interacts via its receptor with the AVP V_2 system through a mechanism involving activation of adenylate cyclase. In addition, these findings together with our observation that the antidiuretic effect of Ang-(1–7) and its agonist AVE are blunted in *Mas*-deficient animals suggest that Mas is the receptor involved in adenylate cyclase–dependent IMCD actions of Ang-(1–7). Further studies are obviously needed to confirm this possibility.

In sharp contrast to the binding data in the kidney and the binding and functional data in *Mas*-transfected cells, the antidiuretic effect of AVE was completely blocked by the AT_2 receptor antagonists PD123319 and PD123177 and partially blocked by the AT_1 antagonists losartan and valsartan. Strikingly, our in vivo results with AT_1 and AT_2 blockers

are very similar to those of Wiemer et al in bovine aortic endothelial cells.[16] However, in our condition, A-779 completely blocked the AVE effect, whereas only a 50% inhibition was observed for the NO release induced by this compound in bovine aortic endothelial cells.[16] The preincubation time of 20 minutes used in the cell culture may explain this difference. In addition, the fact that bovine angiotensins presented a valine at the fifth position instead of isoleucine (rat, human) provides a structural clue to explain these discrepancies. Actually, the biological effects of Val⁵-Ang II and Ile⁵-Ang II are not always the same.[22]

Although the exact mechanisms linking AT_1, AT_2, and Mas receptors in water diuresis remains to be elucidated, several hypotheses could be considered. In the case of AT_1 antagonists, the effect of AVE on water transport could be partially attenuated by the increase in the urinary flow induced by AT_1 blockade (supplemental Table). A similar effect of AT_1 blockade was observed previously for Ang-(1–7) in rats.[18] An interference of AT_1/AT_2 blockade with the water reabsorption in the peritoneum is unlikely, considering that they did not decrease water diuresis when given alone. Among other possibilities to explain the interference of AT_1 and AT_2 antagonists with the AVE 0991 effect are the existence of oligomers,[23,24] including Mas–Mas sensitive to AT_1 or AT_2 antagonists or AT_1-Mas, AT_2-Mas, or AT_1-AT_2-Mas oligomers or cross-talk mechanisms,[25] as described for the AVP V_2 receptor. Indeed, recent observations favor the oligomer interpretation[26–28] (W. Sampaio and R.A.S. Santos, unpublished data, 2004).

It should be pointed out that ¹²⁵I-Ang-(1–7) binds to kidney slices of AT_2-KO and AT_1-KO mice,[13] indicating that the presence of these receptors is not necessary for the binding of Ang-(1–7) to the Mas receptor. However, they appear to be important for functional renal responses evoked by Ang-(1–7) through Mas in the kidney. Our previous observations showing blockade of the antidiuretic effect of Ang-(1–7) in water-loaded rats, with losartan indicated that these interactions are not restricted to mice.[18] The related observations showing blockade of some Ang-(1–7) effects by AT_1 or AT_2 antagonists[16,29] are also in keeping with the oligomerization or cross-talk mechanism hypothesis. On the other hand, in other conditions/tissues, Mas-mediated Ang-(1–7) effects are independent of a functional interaction with AT_1 or AT_2 mechanisms, taking into account that they are blocked by A-779 but not AT_1 or AT_2 antagonists (eg, blood vessels, rat ventrolateral medulla).[2]

In conclusion, our findings indicate that AVE is an Ang-(1–7) receptor Mas agonist. This novel nonpeptide compound could be useful for studies in vitro and in vivo aimed to elucidate the physiopathological role of Ang-(1–7).

Perspectives

The recent report that Ang-(1–7) is an endogenous ligand for the G protein–coupled receptor Mas[13] opened new possibilities for clarifying the biological significance of this heptapeptide. The nonpeptide AVE was described recently as an Ang-(1–7) mimic in bovine endothelial cells.[16] In the present study, we presented clear evidence that this compound is an Ang-(1–7) receptor Mas agonist. Evidence was also obtained

that the antidiuretic effect of AVE is dependent on an interaction of Mas-mediated with AT_2 and, at lesser extent, with AT_1-mediated mechanisms. Considering our findings and the fact that some actions of Ang-(1–7) are also blocked by AT_1 or AT_2 antagonists,[29–31] an indirect interaction of Ang-(1–7) or AVE 0991 with AT_1 and AT_2 receptors via Mas should be explored in future studies. However, we cannot exclude the possibility that in some circumstances, Ang-(1–7) can interact directly with AT_1 or AT_2 receptors as it does with angiotensin-converting enzyme.[32,33] The availability of a nonpeptide Ang-(1–7) receptor agonist opens many exciting possibilities that may lead to the development of new therapeutic strategies for cardiovascular and renal diseases by interfering with the Ang-(1–7)–Mas axis.

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