Characterization of a New Selective Antagonist for Angiotensin-(1–7), D-Pro^7-Angiotensin-(1–7)

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Abstract—Angiotensin-(1–7) [Ang-(1–7)] has biological actions that can often be distinguished from those of angiotensin II (Ang II). Recent studies indicate that the effects of Ang-(1–7) are mediated by specific receptor(s). We now report the partial characterization of a new antagonist selective for Ang-(1–7), D-Pro^7-Ang-(1–7). D-Pro^7-Ang-(1–7) (50 pmol) inhibited the hypertensive effect induced by microinjection of Ang-(1–7) [4±1 vs 21±2 mm Hg, 25 pmol Ang-(1–7) alone] into the rostral ventrolateral medulla without changing the effect of Ang II (16±2.5 vs 19±2.5 mm Hg after 25 pmol Ang II alone). At 10^{-7} mol/L concentration, it completely blocked the endothelium-dependent vasorelaxation produced by Ang-(1–7) (10^{-10} to 10^{-6} mol/L) in the mouse aorta. The antidiuresis produced by Ang-(1–7) (40 pmol/100 g body weight) in water-loaded rats was also blocked by its analog [1 μg/100 g body weight; 3.08±0.8 vs 1.27±0.33 mL in Ang-(1–7)–treated rats]. D-Pro^7-Ang-(1–7) at a molar ratio of 40:1 did not change the hypotensive effect of bradykinin. Moreover, D-Pro^7-Ang-(1–7) did not affect the dipsogenic effect produced by intracerebroventricular administration of Ang II (11.4±1.15 vs 8.8±1.2 mL/h after Ang II) and did not show any demonstrable angiotensin-converting enzyme inhibitory activity in assays with the synthetic substrate Hip-His-Leu and rat plasma as a source of enzyme. Autoradiography studies with ^{125}I–Ang-(1–7) in mouse kidney slices showed that D-Pro^7-Ang-(1–7) competed for the binding of Ang-(1–7) to the cortical supramedullary region. In Chinese hamster ovary cells stably transfected with the AT_1 receptor subtype, D-Pro^7-Ang-(1–7) did not compete for the specific binding of ^{125}I–Ang-II in concentrations up to 10^{-6} mol/L. There was also no significant displacement of Ang II binding to angiotensin type 2 receptors in membrane preparations of adrenal medulla. These data indicate that D-Pro^7-Ang-(1–7) is a selective antagonist for Ang-(1–7), which can be useful to clarify the functional role of this heptapeptide. **(*Hypertension*. 2003; 41[part 2]:737-743.)**

Key Words: angiotensin antagonists ■ angiotensin II ■ angiotensin-(1–7) ■ D-Pro^7-Ang-(1–7) ■ diuresis

Increasing evidence indicates that besides angiotensin II (Ang II), other angiotensin peptides, such as angiotensin-(1–7) [Ang-(1–7)], Ang III, and angiotensin-(3–8) (Ang IV), mediate biological actions of the renin-angiotensin system (RAS).[1–9]

Among these putative RAS mediators, Ang-(1–7) is particularly interesting because of its selectivity, probably owing to the absence of the *C*-terminal phenylalanine (Phe).[2,10] The importance of Phe at the *C*-terminal is well illustrated by the lack of an Ang-(1–7) pressor effect after peripheral or intracerebroventricular administration.[11] Ang-(1–7) is a major product derived from Ang I by a route that is independent of the angiotensin-converting enzyme (ACE).[12,13] It can also be formed from Ang II by removal of the *C*-terminal Phe by ACE-2,[12] prolylendopeptidase,[13] and prolylcarboxypeptidase.[14] Ang-(1–7) plasma concentration is usually lower than that of Ang II, ranging in rats from 10 to 40 fmol/mL.[7]

Ang-(1–7) has been shown to produce selective central and peripheral biological effects that can be similar to or often opposite those of Ang II.[2,4–8,15] A role for Ang-(1–7) in the control of hydroelectrolyte balance has been suggested by its vasopressin secretagogue action in vitro,[5] its dense immunostaining in the supraoptic and paraventricular nuclei of the hypothalamus and neurohypophysis,[6] and its potent antidiuretic effect in water-loaded rats.[7] There is also evidence that Ang-(1–7) interacts with kinins and augments bradykinin (BK)-induced vasodilator responses (see Santos et al[2] for a review). In vascular smooth muscle cells, Ang-(1–7) caused an antiproliferative response, inhibiting mitogen-stimulated [^{3}H]thymidine incorporation and reducing serum-stimulated cell growth, counteracting Ang II effects.[8]

Although these observations indicate that Ang-(1–7) is an important mediator of the RAS, a more meaningful evalua-

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tion of its physiological role requires the study of the effects of selective blockade of this angiotensin. We have previously shown that an Ang-(1–7) synthetic analog, D-Ala^7-Ang-(1–7) (A-779), is a potent and selective Ang-(1–7) antagonist.[16] This compound antagonizes several actions of Ang-(1–7), including its antidiuretic effect[7,16] and the changes in mean arterial pressure produced by Ang-(1–7) microinjection into the dorsomedial and ventrolateral medulla.[16] A-779 also produces selective blockade of the Ang-(1–7) stimulatory effect on neuronal activity in the paraventricular nucleus.[17]

With the use of this compound, several findings support the existence of a distinct Ang-(1–7) receptor[2,7,18] and for establishing the biological relevance of endogenous Ang-(1–7).[7,19–22] However, one may argue that some of the effects obtained with A-779 could be due to an agonistic effect or interference with the actions of other peptides, although these are unlikely possibilities.[16] The availability of other Ang-(1–7) antagonists would be an important tool to confirm and extend the evidence obtained with A-779. In this study, we report the partial characterization of a new antagonist selective for Ang-(1–7), D-Pro^7-Ang-(1–7).

Methods

General Procedures

Experiments were carried out in male Wistar rats (200 to 280 g) or male mice (BLG-57, 20 to 25 g), bred at the animal facility of the Biological Science Institute (CEBIO, Federal University of Minas Gerais, Minas Gerais, Brazil). Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-D-Pro^7 [D-Pro^7-Ang-(1–7)] was synthesized by a solid-phase method by M.C. Khosla. Ang-(1–7), Ang II, and BK were from Peninsula Laboratories. Bacitracin and 1,10-phenanthroline were from Sigma. For the in vivo experiments, the peptides were dissolved in isotonic sterile saline and for the in vitro experiments, in the assay solution.

In Vivo Experiments

Intravenous Bolus Injections

One day before the experiment the rats were anesthetized with ether, and a polyethylene catheter (PE-10 connected to PE-50) was inserted into the abdominal aorta through the femoral artery for blood pressure measurements. For intravenous injections, a polyethylene cannula was implanted into the femoral vein. The arterial blood pressure was monitored by a solid-state strain-gauge transducer (model TP-200T, Nihon Khoden) while the heart rate was determined with a heart rate counter (model AT-601G, Nihon Khoden), triggered by the arterial pressure wave. All variables were recorded continuously on a direct-writing Nihon Khoden polygraph (model CP-640G) or on a computer through a data acquisition system (CODAS, Dataq Instruments Inc). BK, 1 or 2 μg, was injected alone or combined with 1, 2, or 20 μg D-Pro^7-Ang-(1–7). A minimum interval of 3 minutes was allowed between injections. Each dose of BK or BK associated with D-Pro^7-Ang-(1–7) was injected twice (0.1 mL per injection). The effect obtained with the second injection was used for calculations.

Medullary Microinjection Procedures

The rats were anesthetized with urethane (1.2 g/kg body weight [BW] IP), and a tracheostomy was performed. Catheters were inserted into the femoral artery and vein for blood pressure measurement and drug injection, respectively. Next, the animals were placed in a stereotaxic frame, and the dorsal surface of the medulla oblongata was exposed. The peptides or saline was microinjected (200 nL) into the rostral ventrolateral medulla (RVLM; 2.0 mm anterior, 1.8 mm lateral to the obex) just above the pia mater by using a stainless steel needle (30 gauge), as described elsewhere.[23] The cardiovascular effects produced by (1) Ang-(1–7), 25 pmol (n=6); (2) D-Pro^7-Ang-(1–7), 50 pmol (n=5); (3) Ang-(1–7), 25 pmol combined with D-Pro^7-Ang-(1–7), 50 pmol (n=9); (4) Ang II, 25 pmol (n=4); (5) Ang II, 25 pmol combined with D-Pro^7-Ang-(1–7), 50 pmol (n=6); or (6) saline (0.9% NaCl, 200 nL, n=6) were tested. Each rat received a single microinjection.

Water Diuresis

Water diuresis was induced by water loading (5 mL/100 g BW by gavage) in awake rats. Immediately after water loading, the rats were treated subcutaneously with (1) vehicle (0.9% NaCl, 0.05 mL/100 g BW, n=6); (2) Ang-(1–7), 40 pmol/100 g BW (n=6); (3) D-Pro^7-Ang-(1–7), 1 μg/100 g BW (n=6); or (4) D-Pro^7-Ang-(1–7), 1 μg/100 g BW combined with Ang-(1–7), 40 pmol/100 g BW (n=6). The urinary volume was measured for 60 minutes after water loading. Urine osmolality was measured with a freezing-point Osmometer (Fiske Osmomether, Fiske Associates Inc).

Water Intake

Four days before the experiments, the rats were anesthetized with tribromoethanol (250 mg/kg BW IP) and placed in a stereotactic frame to allow implantation of guide cannulas in the direction of the lateral ventricle. The following coordinates were used: 0.8 mm caudal to the bregma, 1.5 mm lateral to the midline, and 3.6 mm below the skull surface of the bregma. The cannula tip was positioned ≈2 mm above the lateral ventricle. The guide cannula was fixed to the skull with methacrylate and watch screws and then closed with an occluder until the time for the experiments. Intramuscular antibiotic (Pentabiotico, Fontoura-Wyeth, 0.2 mL/rat) was given to assist postoperative recovery. Water intake was measured for a 1-hour period after microinjection of saline (n=5); D-Pro^7-Ang-(1–7), 2 nmol (n=5); Ang II, 25 pmol (n=5); or Ang II combined with D-Pro^7-Ang-(1–7) (n=5) into the lateral ventricle. The total microinjection volume was 2 μL.

In Vitro Experiments

Mouse Aortic Ring Preparation and Mounting

Rings (2 to 3 mm) from the descending thoracic aorta, free of fat and connective tissue, were set up in gassed (95% O_2 and 5% CO_2) Krebs-Henseleit solution (mmol/L): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, NaH_2PO_4 2.33, and glucose 11.51, at 37°C under a tension of 0.5 g for a 1-hour equilibration period. During this period, the incubation medium was changed every 15 minutes. After the equilibration period, 2 contractile responses were evoked by submaximal concentrations of phenylephrine (0.3 μmol/L) to elicit reproducible responses. The effect of D-Pro^7-Ang-(1–7) (0.1 μmol/L) on the vasorelaxant activity of Ang-(1–7) was measured in aortic rings containing a functional endothelium precontracted with 0.1 μmol/L phenylephrine. Ang-(1–7) (0.0001 to 0.3 μmol/L) was added in increasing cumulative concentrations once the response to phenylephrine had stabilized. The effect of D-Pro^7-Ang-(1–7) was determined by adding the analog to the bath 1 minute before starting the Ang-(1–7) dose-response curve. The presence of a functional endothelium was assessed by the ability of acetylcholine (10 μmol/L) to induce >70% relaxation of vessels precontracted with phenylephrine (0.3 μmol/L). Mechanical activity, recorded isometrically by a force transducer (World Precision Instruments Inc) was fed to an amplifier-recorder (model TMB-4, World Precision Instruments Inc) and a personal computer equipped with an analog-to-digital converter board (AD16JR, World Precision Instruments Inc) equipped with CVMS data acquisition/recording software (World Precision Instruments Inc).

ACE Assay

Plasma ACE activity was measured by a fluorometric method with Hip-His-Leu as the substrate, as previously described (Santos et al[24]). Duplicate aliquots of a pool of rat plasma (10 μL) were incubated with 490 μL of 1.25 mmol/L Hip-His-Leu in 0.4 mol/L sodium borate buffer, pH 8.3, containing 0.9 mol/L NaCl for 15 minutes at 37°C. The reaction was stopped by the addition of 1.2 mL of 0.34 mol/L NaOH; 100 μL orthophthaldehyde (20 mg/mL in methanol) was added, and after 10 minutes at room temperature, 200

μL of 3N HCl was added. After centrifugation at 800*g* for 5 minutes, fluorescence of the supernatant solution (365-nm excitation and 495-nm emission) was measured against water. Blanks were prepared by inverting the order of addition of rat plasma and NaOH. To test the ACE inhibitory activity of D-Pro^7-Ang-(1–7), the peptide at final concentrations ranging from 10^{-8} to 10^{-5} mol/L was added to the assay solution before addition of the rat plasma.

Binding of ^{125}I-Angiotensins in Whole Cells

Binding of ^{125}I–Ang II was performed by incubating angiotensin type 1 (AT_1) receptor–stably transfected Chinese hamster ovary cells (a gift from Dr José E. Krieger, INCOR-USP, São Paulo, Brazil) at 25°C with 100 pmol/L ^{125}I–Ang II (2200 Ci/mmol) for 1 hour in HEPES-buffered RPMI containing 0.1% bovine serum albumin, 1 mmol/L 1,10-phenanthrolene, and 1 mmol/L bacitracin. Nonspecific binding and displacement were determined in parallel culture dishes by using the same concentration of ^{125}I–Ang II in the presence of a 1000-fold excess of nonradioactive Ang II. The ability of D-Pro^7-Ang-(1–7) to displace ^{125}I–Ang II binding was tested by preincubating (10 minutes) the peptide with D-Pro^7- Ang-(1–7). The incubation period was terminated by 3 1-mL washes with ice-cold buffer and lysis of the cells with 3% (wt/vol) Triton X-100 in 10 mmol/L HEPES and 10 mmol/L EGTA (pH 7.4). The amount of bound ^{125}I–Ang II was determined after 1 hour of incubation by counting the γ-radiation in a Packard Cobra II gamma counter.

Binding of ^{125}I-Angiotensins in Membrane Preparation

Crude membrane fractions of rat adrenal glands were prepared as follows. Adrenal medulla tissue was homogenized in 40 volumes of ice-cold, hypotonic Tris-HCl buffer (50 mmol/L, pH 7.4, containing 5 mmol/L Na_2EDTA). The homogenates were centrifuged at 1000*g* for 10 minutes at 4°C. The supernatant was then centrifuged at 40 000*g* for 30 minutes. The pellet was resuspended in 40 volumes of hypotonic buffer and recentrifuged. The pellet was then resuspended in 40 volumes of assay buffer (50 mmol/L Tris-HCl buffer, pH 7.4, containing 150 mmol/L NaCl, 5 mmol/L Na_2EDTA, 10 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL bacitracin, and 0.1% bovine serum albumin) and recentrifuged at 40 000*g* for 30 minutes. The pellet was resuspended in 8 volumes of assay buffer with a final protein concentration of 0.4 mg/mL. Protein concentration was estimated by the method of Bradford.[25] Binding assays were performed by incubating aliquots of freshly prepared membrane fractions with 100 pmol/L ^{125}I–Ang II and 1 μmol/L angiotensin antagonists in a final volume of 0.25 mL assay buffer. After incubation for 60 minutes at 25°C, the bound and free radioactivity was rapidly separated by filtration through glass fiber filters (GF/B, Whatman). The trapped radioactivity was determined with a gamma counter.

Binding of ^{125}I-Angiotensins in Kidney Slices

Mouse kidneys (n=3) were sectioned in a cryostat at a temperature of −22°C. Serial slices starting from the central area of the kidney with a thickness of 16 μm were prepared, mounted on gelatinized glass microscope slides, and dried at 4°C before the binding assay. Experiments were performed in duplicate or quadruplicate. All slices were preincubated with 400 μL of 10 mmol/L sodium phosphate buffer, pH 7.4, containing 120 mmol/L NaCl, 5 mmol/L $MgCl_2$, 0.2% bovine serum albumin, and 0.005% bacitracin for 10 minutes. Thereafter, the slices were immersed in the same buffer containing 10^{-7} mol/L phenylmethylsulfonyl fluoride and 5×10^{-7} mol/L orthophenanthrolene. For slices (n=20) used to determine nonspecific binding, 10^{-5} mol/L A-779 or Ang-(1–7) was added to the preincubation and incubation buffers. D-Pro^7-Ang-(1–7) (10^{-5} mol/L) was added to the preincubation and incubation buffers to determine the displacement of ^{125}I–Ang-(1–7) (n=20 slices). Total binding was determined by incubation of the slices (n=20) in 400 μL of 10 mmol/L sodium phosphate buffer, pH 7.4, complemented with the same enzyme inhibitors as above and 0.5 nmol/L ^{125}I–Ang-(1–7) for 60 minutes. Peptides were labeled with ^{125}I by the chloramine T method and purified by high-performance liquid chromatography, as described by Neves et al.[26] The binding reaction was stopped by washing the slices 3 times (30 seconds each) in 250 mL of ice-cold Tris-HCl, 50 mmol/L, pH 7.4, and finally for 30 seconds in ice-cold deionized water. After being dried at room temperature, the slices were exposed to autoradiographic film (Fuji Image Plate) for 1 to 2 days at room temperature. After exposure, the films were scanned (Bio-Imaging Analyzer, Fujifilm), and the optical density was quantified (Fujifilm BAS 1800 II). The illustrations were made with Osiris Medical Imaging software (version 3.12, University Hospital of Geneva, Switzerland).

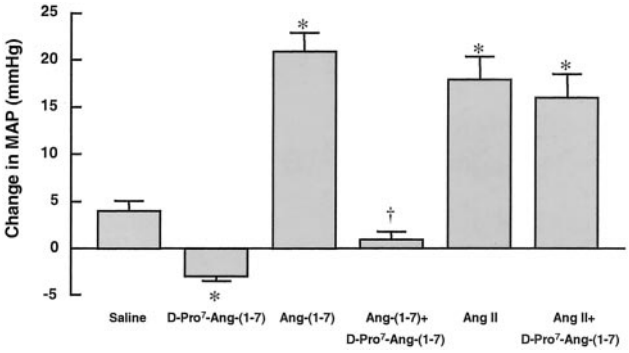

Figure 1. Average changes in mean arterial pressure (MAP, mm Hg) produced by microinjection of angiotensin peptides into the rostral ventrolateral medulla of urethane-anesthetized rats. This figure shows the effect of microinjection of saline (n=6), of 25 pmol Ang-(1–7) (n=6) alone or associated with 50 pmol D-Pro^7-Ang-(1–7) (n=9), and of 25 pmol Ang II (n=4) alone or associated with 50 pmol D-Pro^7-Ang-(1–7) (n=6). *$P<0.05$ compared with microinjection of saline. †$P<0.05$ compared with microinjection of peptide alone.

Statistical Analysis

Results are presented as mean±SEM. Data were analyzed by 1-way ANOVA or Student *t* test. The level of significance was set at $P<0.05$.

Results

Blockade of Physiological Effects of Ang-(1–7) by D-Pro^7-Ang-(1–7)

In the first set of experiments, we evaluated the effect of D-Pro^7-Ang-(1–7) as an Ang-(1–7) antagonist at the RVLM. As expected, 25 pmol Ang-(1–7) microinjected into the RVLM produced a significant increase in MAP compared with vehicle (21±2.2 vs 4±1.0 mm Hg, $P<0.05$; Figure 1). Microinjection of 50 pmol D-Pro^7-Ang-(1–7) into the RVLM produced a small but significant decrease in baseline MAP (−3±0.37 compared with 4±1.0 mm Hg after vehicle microinjection, $P<0.05$). D-Pro^7-Ang-(1–7) completely blocked the pressor effect produced by microinjection of Ang-(1–7) [1±0.85 vs 21±2.2 mm Hg after Ang-(1–7) alone, $P<0.05$]. In contrast, the pressor effect produced by Ang II microinjection into the RVLM was also unaffected by D-Pro^7-Ang-(1–7) (16±2.5 vs 19±2.5 mm Hg after Ang II alone).

We also examined the effect of D-Pro^7-Ang-(1–7) in water-loaded awake rats. Confirming previous observations,[7] Ang-(1–7) produced a significant decrease in water diuresis compared with vehicle-treated rats (1.27±0.33 vs 3.58±0.43 mL, $P<0.05$; Figure 2). The antidiuretic effect of Ang-(1–7) was associated with an increase in urine osmolality (287±23 compared with 170±16 mOsm/kg in saline-treated rats, $P<0.05$). In contrast, treatment of water-loaded animals with D-Pro^7-Ang-(1–7) (1 μg/100 g BW) did not change water

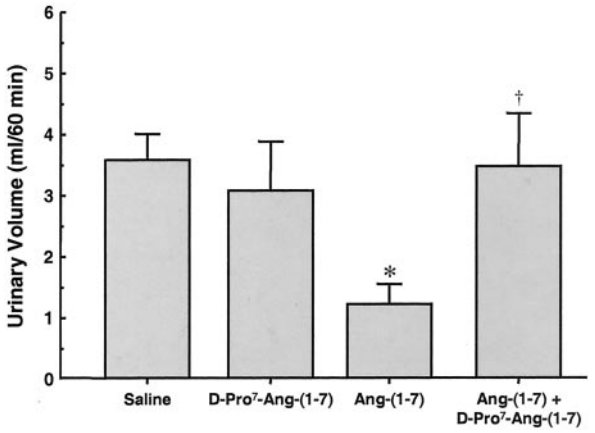

Figure 2. Effect of D-Pro^7-Ang-(1–7) on antidiuresis induced by Ang-(1–7) in water-loaded rats. D-Pro^7-Ang-(1–7) (1 μg/100 g BW, SC, n=6) was administered immediately before Ang-(1–7) (40 pmol/100 g BW, SC, n=6) or saline (NaCl 0.9%, 0.1 mL/100 g BW, SC, n=6). Control animals were injected with saline immediately before administration of D-Pro^7-Ang-(1–7). *$P<0.05$ compared with the group treated with saline. †$P<0.05$ compared with the group treated with Ang-(1–7) alone.

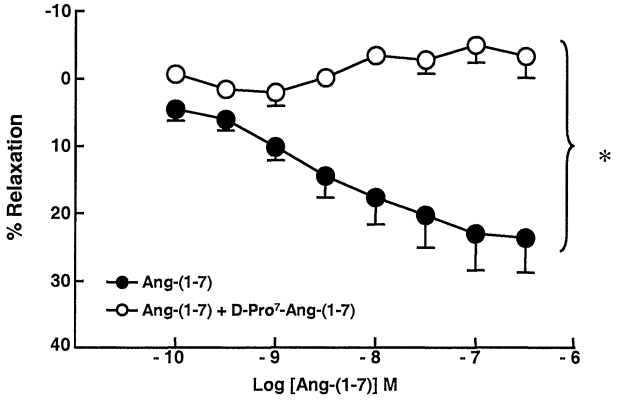

Figure 3. Effect of D-Pro^7-Ang-(1–7) on percent relaxation produced by increasing concentrations of Ang-(1–7) in mice aortic rings (n=4) containing functional endothelium precontracted with 0.1 μmol/L phenylephrine. The effect of D-Pro^7-Ang-(1–7) (10^{-7} mol/L) was determined by adding this analog to the bath 1 minute before starting the Ang-(1–7) dose-response curve. *$P<0.001$

diuresis (3.08±0.80 vs 3.58±0.43 mL in saline-treated rats, $P>0.05$) and urine osmolality (174±20 vs 170±16 mOsm/kg in saline-treated rats, $P>0.05$). However, D-Pro^7-Ang-(1–7) (1 μg/100 g BW) essentially abolished the antidiuretic effect of Ang-(1–7) (3.47±0.87 vs 1.27±0.33 mL, $P<0.05$), producing a significant reduction in urine osmolality compared with Ang-(1–7) alone (168±22 vs 287±23 mOsm/kg, $P<0.05$).

We next tested the effect of D-Pro^7-Ang-(1–7) on the endothelium-dependent relaxation produced by Ang-(1–7) in mouse aortic rings. As shown in Figure 3, the relaxation produced by Ang-(1–7) in the mouse aorta was completely blocked in the presence of 0.1 μmol/L D-Pro^7-Ang-(1–7). The antagonist by itself was without effect in this preparation.

Effect of D-Pro^7-Ang-(1–7) on ^{125}I–Ang-(1–7) and ^{125}I–Ang II Binding

Binding assay experiments substantiated the evidence that D-Pro^7-Ang-(1–7) is a specific Ang-(1–7) antagonist. As shown in Figure 4, D-Pro^7-Ang-(1–7) (10^{-5} mol/L) displaced the binding of ^{125}I–Ang-(1–7) to mice kidney slices. As demonstrated by the slightly negative specific binding in the presence of the antagonist, the displacement produced by D-Pro^7-Ang-(1–7) was even higher than that obtained with an equimolar concentration of D-Ala^7-Ang-(1–7).

In Chinese hamster ovary cells stably transfected with AT_1 receptors, D-Pro^7-Ang-(1–7) did not compete with the specific binding of ^{125}I–Ang II, even at concentrations up to 10^{-6} mol/L (Figure 5A). The IC_{50} of D-Pro^7-Ang-(1–7) was higher than 10^{-5}mol/L. In the same experiments, nonradioactive Ang II competed with ^{125}I–Ang II binding with an IC_{50} of 2.39 nmol/L. In the experiments performed to determine the competition for angiotensin type 2 (AT_2) binding sites of Ang II in adrenal gland membranes (Figure 5B), we observed that in the presence of losartan, an AT_1 receptor antagonist, ≈30% of Ang II binding was inhibited. The remaining Ang II binding was completely abolished when 10^{-7}mol/L CGP42112A, an AT_2 ligand, was added together with losartan. No further inhibition was seen when 10^{-6}mol/L D-Pro^7-Ang-(1–7) was added to samples containing losartan.

Specificity of D-Pro^7-Ang-(1–7)

The evidence for D-Pro^7-Ang-(1–7) specificity was further evaluated by in vivo and in vitro experiments. The effect of D-Pro^7-Ang-(1–7) on the hypotensive response induced by BK is shown in Figure 6A. D-Pro^7-Ang-(1–7) at 1, 2, or 20 μg did not change the BK hypotensive response, contrasting with the well-established BK-potentiating effect of Ang-(1–7).[15] As observed for the BK hypotensive effect, D-Pro^7-Ang-(1–7) did not change rat plasma ACE activity (basal values were 93±8 nmol His-Leu · min^{-1} · mL^{-1}) at concentrations up to 10^{-5} mol/L. The estimated IC_{50} was ≥100 μmol/L.

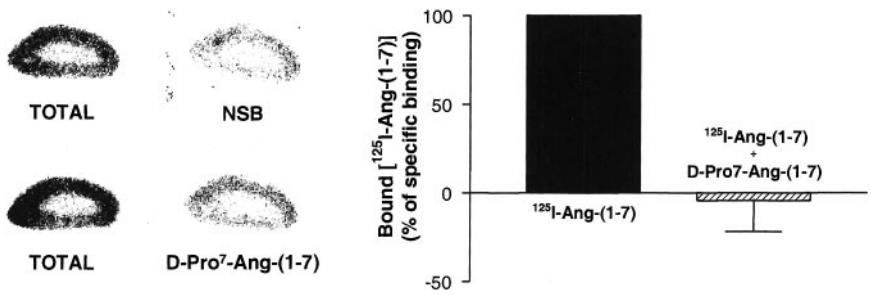

Figure 4. In vitro autoradiographic localization of ^{125}I-Ang-(1–7) binding in mice kidney slices. Figure shows total binding on the left slice (total) and nonspecific binding (NSB) on the top right slice. On the lower right slice, ^{125}I–Ang-(1–7) binding in the presence of D-Pro^7-Ang-(1–7) is shown. Concentration of ^{125}I–Ang-(1–7) was 0.5 nmol/L, concentration of A-779 was 10 μmol/L (NSB), and concentration of D-Pro^7-Ang-(1–7) was 10 μmol/L. The bar graph at right shows the quantitative data of the effect of D-Pro^7-Ang-(1–7) on the specific binding of Ang-(1–7) to mouse kidney slices.

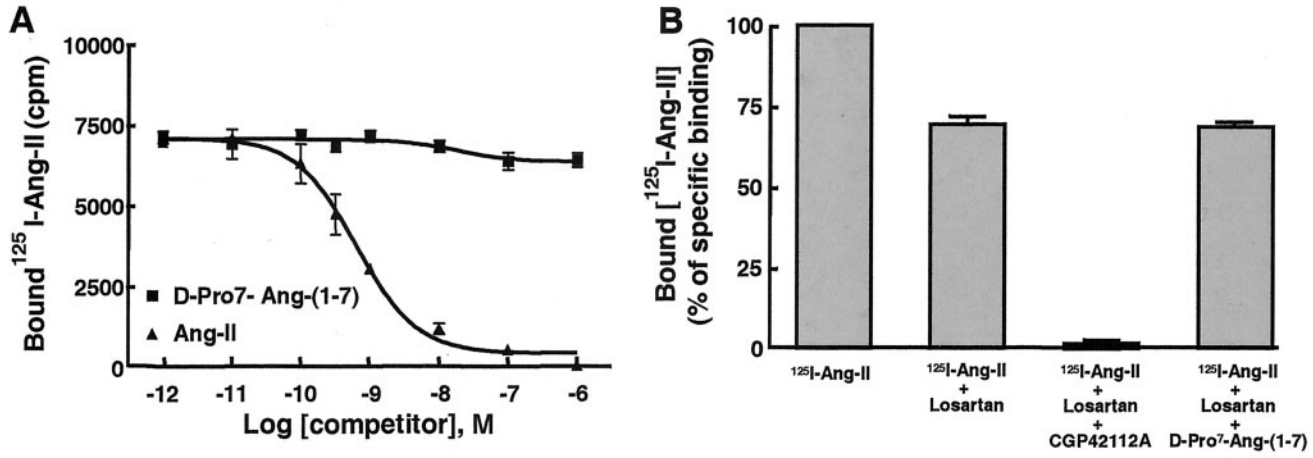

Figure 5. A, ^{125}I–Ang II binding in Chinese hamster ovary AT_1-transfected cells. Chinese hamster ovary cells stably transfected with AT_1 receptor were incubated with 100 pmol/L ^{125}I–Ang-II plus the indicated concentrations of unlabeled Ang-II (▲) or D-Pro^7-Ang-(1–7) (■). Amount of bound ligand was determined after 1 hour of incubation. Data represent mean±SE of individual determinations from 3 separated experiments. B, Competition for AT_2 binding of Ang-II to adrenal gland membrane crude preparations. Membrane proteins (50 μg) of adrenal glands were incubated with 100 pmol/L ^{125}I–Ang-II in the absence or presence of 1 μmol/L of the indicated antagonists for 1 hour at room temperature. These data show mean±SEM of individual determinations from 3 separate experiments.

Figure 6B shows the effect of D-Pro^7-Ang-(1–7) on the water intake induced by Ang II. The dipsogenic response produced by 25 pmol Ang II microinjection into the lateral ventricle (8.8±1.2 mL/h) was not changed by 2 nmol D-Pro^7-Ang-(1–7) (11.4±1.15 mL/h). Microinjection of D-Pro^7-Ang-(1–7) alone produced no significant effects on water intake compared with the saline microinjection group (0.3±0.05 vs 0.7±0.17 mL/h).

Discussion

After the original observation that Ang-(1–7) is a major Ang I metabolite produced by a pathway independent of ACE,[27] we and others have shown that this angiotensin exerts an important subset of angiotensinergic activities, particularly influencing the cardiovascular system[2,11,15,18,23,28,29] and fluid homeostasis.[2,7] In this study, we have found that the heptapeptide Asp-Arg-Val-Tyr-Ile-His-D-Pro, D-Pro^7-Ang-(1–7), is a potent Ang-(1–7) antagonist, without demonstrable intrinsic agonistic activity in the several biological preparations examined. More important, our data provide strong evidence for the existence of a specific binding site for Ang-(1–7).

Antagonists are very useful tools for identification of receptors and for elucidating the physiopathological relevance of endogenous substances. For example, the development of peptide and nonpeptide Ang II antagonists, such as CGP 42112A, losartan (DUP 753), PD 123177, and related analogs, provided convincing evidence for the existence of at least 2 subtypes of angiotensin receptors.[30] The AT_1 receptor

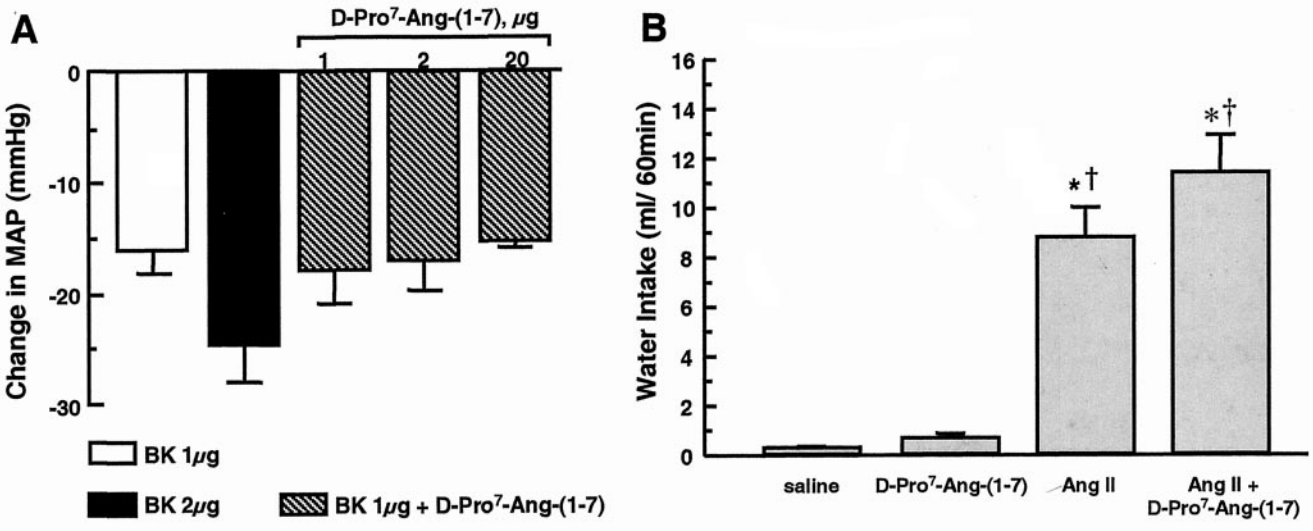

Figure 6. A, Change in mean arterial pressure (MAP, mm Hg) in response to intravenous bolus injection of Bradykinin alone (BK, 1 and 2 μg, n=8) or BK (1 μg) combined with D-Pro^7-Ang-(1–7) (1, 2, or 20 μg, n=8). B, Water intake (mL) during 60 minutes after microinjection into lateral ventricle with saline (n=5), Ang II (25 pmol, n=5), D-Pro^7-Ang-(1–7) (2 nmol, n=5), or Ang II plus D-Pro^7-Ang-(1–7) (n=5). *P<0.05 compared with the group treated with saline. †P<0.05 compared with the group treated with D-Pro^7-Ang-(1–7) alone.

was defined by its sensitivity to DUP 753 (losartan) or its active metabolite EXP-3174, whereas AT_2 receptors were initially characterized by their sensitivity to CGP 42112A, PD 123177, PD 123319, or related analogs.[30] There is increasing evidence for the existence of other subtypes of angiotensin receptor.[2,8,9,11,15–18,31] In the present study, we have observed that D-Pro^7-Ang-(1–7) blocked several effects of Ang-(1–7): the pressor effect at the RVLM, the antidiuretic effect in water-loaded rats, and the endothelium-dependent relaxation in aortic rings of mice, without interference with Ang II or BK effects, indicating the existence of a different angiotensin receptor. Ongoing experiments are showing that D-Pro^7-Ang-(1–7) inhibits the BK-potentiating effect of Ang-(1–7) in awake rats (data not shown).

We[18,23,29] and others[28] have shown that Ang-(1–7) microinjection into the RVLM induces a significant increase in arterial pressure in anesthetized[23,28] and conscious[18] animals. This pressor effect is not affected by microinjection of losartan, an AT_1 receptor antagonist, or PD 123319/CGP 42112A, AT_2 receptor antagonists.[2,18] As observed previously with A-779,[16] D-Pro^7-Ang-(1–7) completely blocked the pressor effect of Ang-(1–7) in the RVLM. Furthermore, D-Pro^7-Ang-(1–7) did not interfere with the RVLM effect of Ang II. These data further support the existence of a selective receptor, different from classic AT_1/AT_2 receptor subtypes, at least for the effect of Ang-(1–7) at the RVLM.[2] In addition, D-Pro^7-Ang-(1–7) produced a significant hypotensive effect at the RVLM. This effect was similar to that observed for A-779 in a previous study[16] and is probably related to the antagonism of endogenous Ang-(1–7) in this area.

In this study, we have confirmed our previous observation that Ang-(1–7) possesses a potent antidiuretic effect in water-loaded rats.[7] Furthermore, we have shown that this effect can be completely blocked by the heptapeptide D-Pro^7-Ang-(1–7). The fact that the Ang-(1–7) antagonists, A-779 and D-Pro^7-Ang-(1–7), exert similar effects in water-loaded rats reinforces the existence of a kidney Ang-(1–7) receptor.[2,7,16]

We have recently obtained evidence that the G protein–coupled receptor Mas is necessary for the actions of Ang-(1–7) in the mouse aorta and kidney,[32,33] suggesting that it is a functional Ang-(1–7) receptor. The vasorelaxation produced by Ang-(1–7) in aortic rings[2,31,34] was abolished in Mas-deficient mice.[33] Similarly, the antidiuretic effect of Ang-(1–7) administration after water loading is absent in Mas-knockout mice.[32] The fact that D-Pro^7-Ang-(1–7) blocked the vasorelaxation and the antidiuresis produced by Ang-(1–7) strongly suggests that this Ang-(1–7) analog is a Mas receptor antagonist or that it interferes with the Mas–Ang-(1–7) interaction. The same appears to be true for A-779. The specific ^{125}I–Ang-(1–7) binding to mouse kidney slices was completely displaced by D-Pro^7-Ang-(1–7). In contrast, this analog did not compete for the binding of ^{125}I–Ang II to AT_1-transfected Chinese hamster ovary cells or to adrenal medulla, rich in the AT_2 receptor subtype. These findings further substantiate the specificity of D-Pro^7-Ang-(1–7) for the Ang-(1–7) binding sites. Whether the central effects of Ang-(1–7) are mediated through interaction with Mas or another receptor remains to be elucidated.

Functional experiments also aimed to evaluate the specificity of D-Pro^7-Ang-(1–7) showed that this compound did not change the BK hypotensive effect, plasma ACE activity, the pressor effect of Ang II at the RVLM, or the central dipsogenic action of Ang II in rats. These observations combined with data obtained in binding experiments clearly indicate that D-Pro^7-Ang-(1–7) is a selective Ang-(1–7) antagonist. The lack of well-known actions of Ang-(1–7), ACE inhibitory activity[35] and BK-potentiating activity,[15] of D-Pro^7-Ang-(1–7) illustrates the key role of the *C*-terminal L-proline in the Ang-(1–7) effects.

Perspectives

In the present study, we described the Ang-(1–7) analog, D-Pro^7-Ang-(1–7), as a new Ang-(1–7) antagonist. This peptide blocked several actions of Ang-(1–7) without changing Ang II or BK effects. Most of our current knowledge about the functional relevance of Ang-(1–7) is derived from experiments with the Ang-(1–7) antagonist A-779, and, to a lesser extent, with an Ang-(1–7) antibody. The availability of a new antagonist will be an important tool to confirm and extend the findings obtained with A-779. The recent description of AVE 0991,[36] a nonpeptide mimic of the effects of Ang-(1–7), and the evidence that at least some Ang-(1–7) actions are dependent on interaction with the G protein–coupled receptor Mas give further support for the evidence that most if not all of the actions of Ang-(1–7) are receptor mediated. The use of D-Pro^7-Ang-(1–7) will be important to clarify this issue.

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Characterization of a New Selective Antagonist for Angiotensin-(1–7), d-Pro^7-Angiotensin-(1–7)

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