Angiotensin 1–7 Is a Negative Modulator of Aldosterone Secretion In Vitro and In Vivo

Gabi Shefer,* Yonit Marcus,* Esther Knoll, Oleg Dolkart, Shulamit Foichtwanger, Nava Nevo, Rona Limor, Naftali Stern

Abstract—Angiotensin (1–7) [Ang 1–7] is a 7 amino acid peptide generated predominantly from Ang II by the action of Ang-converting enzyme 2. We previously showed that Ang 1–7 reduced plasma aldosterone and plasma renin activity in high fructose–fed rats, and that the reduction in circulating aldosterone seemed to accord a parallel reduction in plasma renin activity. Here, we tested the possibility that Ang 1–7 affects aldosterone secretion acting directly in glomerulosa cells. First, as detected by immunofluorescence, the receptor for Ang 1–7, Mas1 is localized predominantly at the rat adrenal subcapsular region. Second, in isolated rat glomerulosa cells incubates, Ang 1–7 attenuated the aldosterone response to Ang II, with the strongest effect seen on Ang II (10−9 M) (control 22±2.5 pg/10⁵ cells; Ang II [10−9 M]+Ang 1–7 [10−6 M] 33±3.6 pg/10⁵ cells; P<0.001) and the largest effect on adrenocorticotropic hormone (10−8 M) (control 30±3.4 pg/10⁵ cells; ACTH [10−8 M] 409±32.5 pg/10⁵ cells; ACTH [10−8 M]+Ang 1–7 [10−6 M] 280±12.5 pg/10⁵ cells; P<0.001). In contrast, Ang 1–7 did not affect the aldosterone response to potassium (K⁺). In rats subjected to a low-salt diet for 7 days, continuous infusion of Ang 1–7 (576 μg/kg per day) resulted in a lesser rise in aldosterone (salt deplete+Ang 1–7, 16.4±4.8 ng/dL) compared with rats receiving vehicle (salt deplete+vehicle, 27.6±5.3 ng/dL; P<0.01) but did not modify plasma renin activity. Taken together, these results indicate that Ang 1–7 can act as a negative modulator of aldosterone secretion in vitro and in vivo.

(Hypertension. 2016;68:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.07088.)

Key Words: aldosterone ■ angiotensins ■ fructose ■ hypertension ■ renin-angiotensin-aldosterone-system

Angiotensin (1–7) [Ang 1–7] is a small peptide, consisting of 7 amino acids, generated predominantly from Ang II by the action of Ang-converting enzyme 2 (ACE2). Originally misconceived as a mere byproduct, evidence accrued during the past 2 decades indicates that Ang 1–7 has many protective effects on the cardiovascular system through its receptor, Mas. Indeed, it has been postulated that some of the effects of ACE inhibitors may be related to the accumulation of Ang 1–7 during ACE inhibition,1–3 owing at least in part, to decreased metabolic clearance of Ang 1–7. Among the multiple and complex reported Ang 1–7 effects, some of the most salient and well-described phenomena ascribed to Ang 1–7 include vasodilation,4 lowering of blood pressure,5 anti-arrhythmic effects6 and inhibition of cardiac fibrosis.7 In addition, Ang 1–7 reportedly opposes many of the adverse cardiovascular effects of Ang II, including hypertension, pregnancy-induced hypertension (preeclampsia), renal disease, heart failure, and cardiac arrhythmia.8–11

In a previous report, we found that Ang 1–7 reduced plasma aldosterone (PA) and plasma renin activity (PRA) in high fructose–fed rats.12 Although in that model the Ang 1–7–induced reduction in circulating aldosterone seemed to accord a parallel reduction in PRA, it remained unclear whether these changes were interdependent or reflected separate effects. Furthermore, in the fructose-fed rat model, we showed that the administration of Ang 1–7 also induced weight loss.12 This was in concordance with a study of Oh et al,11 in which a synthetic Ang 1–7 antagonist prevented weight loss. Because obesity and fat accumulation have been shown to affect the systemic14 and local adipose tissue renin–angiotensin–aldosterone-system15 in a complex manner, it remained unclear whether the inhibitory effects of chronic Ang 1–7 on PA and PRA could reflect, at least in part, indirect effects mediated by fat mass reduction.

In this study, we investigated the effects of Ang 1–7 on aldosterone and renin in vitro and in vivo. The results suggest that Ang 1–7 is a negative modulator of aldosterone secretion acting directly on adrenal glomerulosa cells.

Materials and Methods

Materials
Ang 1–7 was synthesized by the solid-phase methodology, using a multiplepeptide synthesizer AMS 422 (Abimed Analyzer Technik GmbH).

Received January 7, 2016; first decision January 12, 2016; revision accepted May 3, 2016.
From the Institute of Endocrinology, Metabolism and Hypertension, Tel Aviv-Sourasky Medical Center Medical Center and Sackler Faculty of Medicine, Tel Aviv, Israel (G.S., Y.M., E.K., S.F., R.L., N.N.); Division of Orthopedic Surgery, Shoulder Unit, Tel Aviv Medical Center, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel (O.D.); and Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel (N.N.).
*These authors contributed equally to this work.
Correspondence to Naftali Stern, Institute of Endocrinology, Metabolism and Hypertension, Tel Aviv-Sourasky Medical Center, 6 Weizmann St, Tel Aviv 64239, Israel. E mail naftalis@tlvmc.gov.il
© 2016 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.116.07088
Adrenocorticotropic hormone (ACTH, synacthen 0.25 mg/mL) was purchased from Sigma-tau. The Ang 1–7 receptor (Mas1) antagonist A779 was purchased from Bachem-Israel (product number H-2888-0025). Low-salt rat chow was purchased from Harlan laboratories (Teklad 7034, 0.1% sodium).

Immunofluorescence Studies

The adrenal glands of Wistar rats were taken out under anesthesia and frozen at −80°C in optimal cutting temperature medium (Electron Microscopy Sciences, Hatfield, PA). Cryostat-cut sections (10-μm thick) of the adrenal gland were washed in methanol for 30 s at −20°C and then 3x for 2 minutes in PBS. Sections were blocked in 1% normal horse serum in PBS for 2 hours and subsequently incubated with a polyclonal antigoat Mas1 Ab (1:50; MAS1 Antibody [M-13]; sc-54848) at 40°C overnight. The sections were then washed with PBS and incubated with Cy3-conjugated AffiniPure Donkey antigoat–IgG (1: 100; Jackson ImmunoResearch 705-165-147, West Grove, PA) was performed at 4°C overnight. Afterward, sections were washed 3x (5 minutes each wash) in PBS and mounted on slides with 90% glycerol in 50 mmol/L−1 Tris-HCl, pH 7.4. To validate the staining procedure, adrenal sections were first photographed under a different filter (eq. GFP [green fluorescent protein]) and then incubated with the secondary antibody alone without the primary antibody, after which the inner sections were captured as well to rule out nonspecific staining. Fluorescent images were obtained using Nikon Eclipse 80i using Nikon intensilight C-HGF1 camera.

In Vitro Studies

Adrenal Glomerulosa Cells

Rat adrenal glomerulosa cells were prepared as previously described in detail.16,17 In brief, adrenal glands removed from euthanized rats were trimmed of fat and placed in normal saline. Capsules were separated by gross dissection and incubated with collagenase (3.7 mg/mL) and deoxyribonuclease (0.85 mg/mL) in modified Krebs–Ringer bicarbonate with glucose (KRBG) buffer containing 4.5% bovine serum albumin (Pentex Fraction V, fatty acid free) essential and nonessential amino acids, 1-glutamine and glucose for 50 minutes in a Dubnoff metabolic shaker under 95% O₂ and 5% CO₂ atmosphere with graded concentrations of Ang II, ACTH, or K+ either alone or in the presence of Ang 1–7. Aldosterone, renin activity, and progesterone were measured using commercial assays (ALDO-RIACT; Cisbio Bioassays, GammaCoat Plasma Renin Activity 1125 RIA Kit, DiaSorin, Siemens 06670112 Immulite 2000 Progesterone Kit).

Renal Slices

Rats participating in experiment 1 (see below; Sprague-Dawley rats [Harlan, Rehovot, Israel]), were euthanized and kidneys were removed rapidly, freed of fat, and decapsulated. A Stadie–Riggs microtome (Arthur Thom-as Co, Philadelphia, PA) was used to obtain 2 slices (≈0.5-mm thick), from the superficial cortex of each kidney. The slices (wet weight, 15–30 mg) were placed in 25-mL flasks and washed with KRBG buffer containing 0.01% bovine serum albumin. The washed slices were taken up into fresh KRBG containing (mmol/L) 120 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 KH₂PO₄, 26.8 NaHCO₃, and 10 glucose at pH 7.4. The slices were preincubated for 15 minutes and then incubated in 2 mL of KRBG buffer at 37°C under 95% O₂ and 5% CO₂, in a shaking water bath for 5 consecutive 15-minute intervals. Experiments were designed such that each slice served as its own control: each slice was incubated for two 15-minute baseline periods, after which various agents were added and the response to their own agent was observed for the next 3 15-minute periods. The slices were exposed to agents for only 15 minutes, and the buffer was aspirated and replaced with fresh buffer during each subsequent 15-minute period. Baseline or control renin was derived from the average of 2 consecutive incubates.

In Vivo Experiments

All experimental protocols were approved by the animal research committee of the Tel Aviv-Sourasky Tel Aviv Medical Center. We used 12-week-old male Sprague–Dawley rats (200–225 g) purchased from Harlan (Harlan, Rehovot, Israel).

Experiment 1

Animals were housed at the Tel Aviv-Sourasky Tel Aviv Medical Center animal house under controlled temperature (26°C), humidity (45%–50%), and lighting (14:10 cycle) for a 10-day habituation period before the initiation of the experiment, after which they were randomly divided into 2 groups, one receiving regular rat chow (n=6) and the other low-salt chow (n=7; Teklad Rat Diet 7034 Harlan, Israel; low-sodium water, Neviot) for a 1-week period. An Alzet osmotic minipump (model number 2ML4) was delivered continuously at a constant rate for 1 week. This dose was chosen based on previous studies showing that Ang 1–7 administered chronically, via a minipump such as the one used by us, is clearly bioactive in the cardiovascular system16 and can lower PRA and PA.15

Experiment 2

Eighteen to 21-month-old female Sprague–Dawley rats, fed on regular rat chow diet (1DIETUS no. 2018C by Harlan) throughout their life and during the infusion experiment, were divided into 2 groups, one receiving Ang 1–7 (576 mg/kg rat per day in PBS; n=7) and the other (n=7) receiving the vehicle (PBS) through Alzet osmotic minipumps (model number 2ML4) implanted at the interscapular area under the skin. Insulin was carried out for 2 months, before and after which blood samples were collected for PRA and PA.

Statistical Analysis

Data were compared by means of analysis of variance, with or without repeated measures (the latter was performed when several measures were done on the same specimen). These tests were followed by an Unequal N HSD post hoc test. Significance was determined at P<0.05. All statistical analysis was performed using STATISTICA version 6.0 (StatSoft, Tulsa, OK). Data are presented as means±SEM.

Results

In Vitro Studies

Figure 1 depicts a typical series of images visualizing the distribution of Mas1 receptors, detected by immunofluorescence microscopy. Whereas the adrenal medulla seems entirely devoid Mas, Mas-positive immunofluorescence is apparent at the adrenal cortex with the denser staining seen at the subcapsular region, that is, the zona glomerulosa.

As shown in Figure 2A, Ang 1–7 (10⁻² M) had no effect on basal aldosterone production in glomerulosa cells. However, the aldosterone response curve to Ang II is strongly shifted to the right in the presence of Ang 1–7: Ang 1–7 entirely blocked the aldosterone response to Ang II (10⁻⁸ M) and markedly attenuated the response to a 10-fold higher concentration (10⁻⁷ M) of Ang II. When the cells were exposed to a high concentration of Ang II (10⁻⁷ M), Ang 1–7 was unable to affect the aldosterone response. Ang 1–7 also decreased the progesterone response to Ang II (Figure 2B), but this effect had a somewhat different profile: the increase in response to
Ang II was shifted downwards, except for the highest Ang II concentration (10^{-7} M), under which progesterone production was not modified by Ang 1–7.

In response to ACTH, there was a marked and dose-related increase in aldosterone production (Figure 3A), which was attenuated in the presence of Ang 1–7, such that measured levels were lower at each of the ACTH concentrations which increased aldosterone production (10^{-10} to 10^{-8} M), an effect which reached statistical significance with ACTH at 10^{-9} to 10^{-8} M. Unlike the effect of Ang 1–7 on Ang II–induced stimulation, which was most prominent with lower Ang II concentrations, ~40% inhibition was still seen with the highest ACTH concentration used. In fact, the degree of Ang 1–7–related inhibition seemed to be rising with increasing concentrations of ACTH. In parallel, Ang 1–7 also induced an attenuation of the progesterone response to ACTH, which was evident at ACTH concentrations of 10^{-9} to 10^{-8} M, but not with 10^{-10} M (Figure 3B).

In parallel to the absence of any effect of Ang 1–7 on basal aldosterone synthesis, the Mas1 receptor antagonist A779 per se had no effect on aldosterone production (Figure 4). However, A779 effectively antagonized the inhibitory effect of Ang 1–7 on both Ang II– and ACTH-stimulated aldosterone secretion in glomerulosa cells. These results support the notion that Ang 1–7 inhibits Ang II–mediated as well as ACTH

Figure 1. Histological sections of a typical series of images visualizing the distribution of Mas1 receptors in the adrenal cortex (A, B, D, and E) and adrenal medulla (C) tissues from rats (n=6). A, x10 magnification of a representative adrenal cortex tissue that was reacted with DAPI (4′,6-diamidino-2-phenylindole) to show nuclei. C, D, and E, x10, x20, and x40 magnification of the same adrenal cortex tissue that was also reacted with an antibody specific to the angiotensin 1–7 receptor, Mas1. All magnifications show clear presence of Mas. C, A x10 magnification of the adrenal medulla reacted with the Mas1 antibody. No expression of Mas1 was detected in slices of adrenal medulla.

Figure 2. Stimulation of zona glomerulosa cells with increasing levels of angiotensin II (Ang II, n=6 rats). Levels of aldosterone (A) and progesterone (B) produced in zona glomerulosa cells. Data are presented as the mean value±SEM.
receptor–mediated induction of aldosterone synthesis via the Mas1 receptor.

As shown in Figure 5A, in contrast to the blunted aldosterone response to Ang II and ACTH in the presence of Ang 1–7, this peptide had no effect on the rise in aldosterone elicited by increasing concentrations of extracellular K⁺, from 3.7 to 10.7 mmol/L. These K⁺ concentrations lead to a rise in aldosterone from ≈12.5 to ≈175 to 187.5 pg/10⁵ cells (Figure 5A). Similarly, Ang 1–7 had no significant effect on the levels of progesterone in the cells challenged with increasing levels of K⁺, as shown in Figure 5B.

In Vivo Experiment 1: Ang 1–7 on PRA and PA During a Low-Salt Diet

At the onset, before the exposure to diets which differed in their salt content, PRA and PA were similar in the rats, which were then divided into a group consuming regular chow and a group placed, after the baseline measurement, on a low-salt diet. After 1 week of dietary intervention, PRA was unchanged in rats on normal salt intake, regardless of whether they received vehicle or Ang 1–7. In contrast, in the group of rats fed with a low-salt diet, (Figure 6), PRA rose within 1 week both in rats receiving a vehicle solution and in the rats receiving a constant infusion of Ang 1–7. The outcome was rather different as regarded PA (Figure 6): expectedly PA rose on low salt (1 week), but not on normal chow. However, in the low-salt–fed rats that were Ang 1–7–infused PA was ≈35% lower than in low-salt rats receiving vehicle (Figure 6).

We also collected blood samples from these rats to analyze serum electrolytes. The average level of Na (in mmol/L) was 139.67 in the normal chow+saline group and 140 in the other 3 groups (normal chow+Ang 1–7, low salt+saline, and low salt+Ang 1–7); average K level (in mmol/L) was 4.8 in the low salt+saline group and 4.9 in all the other 3 groups (normal chow+saline, normal chow+Ang 1–7, and low salt+Ang 1–7).

To better elucidate the role of endogenous renin in the attenuated aldosterone response to salt-restriction during Ang 1–7 infusion, renin secretion from kidneys removed from rats participating in experiment 1 were also studied ex vivo. In this setting, incubates of renal slices from salt-restricted mice...
Shefer et al  Angiotensin 1–7 and Aldosterone

showed ≈2-fold more renin activity than slices from rats consuming regular diet (Figure 7). Notably, Ang 1–7 treatment administered for 1 week did not modify ex vivo renin activity measured in renal slices prepared from rats on normal salt diet, but clearly enhanced renin activity of renal tissue harvested from rats, which were salt restricted.

In Vivo Experiment 2: Ang 1–7 on PRA and PA in Old Obese Rats

Old (average age, 19 months) rats raised in captivity (animal house) receiving regular rat chow, normally gain considerable fat mass. Their mean weight before 2 months of vehicle (saline)/Ang 1–7 infusion was 680±44 and 677±38 g, respectively (P=NS), which increased after 2 months to 839±47 and 807±52 g, respectively (±SEM; P=NS). Ang 1–7 infusion had little effect on PRA (Figure 8A) but resulted in significantly lower PA (Figure 8B) by the end of the infusion period compared with the vehicle-infused animals (P=0.017; Figure 8).

Discussion

The key finding in this report is that Ang 1–7 can act as a negative modulator of aldosterone secretion in vitro and in vivo and can attenuate the aldosterone response to Ang II and ACTH, 2 of its classical secretagogues, acting directly on rat zona glomerulosa cells. Of note is also the observation that under our experimental conditions, Ang 1–7 did not affect potassium-induced aldosterone secretion. We cannot presently offer an explanation for the dichotomous inhibitory effect of Ang 1–7.
receptor-controlled steroidogenesis in glomerulosa cells, rather than specific inhibition of the final pathway of aldosterone synthesis only. The ability to inhibit only Ang II– and ACTH-mediated but not potassium-stimulated aldosterone synthesis is notable because this selective interference with receptor-operated aldosterone secretion has not been previously shown with established circulating inhibitors of aldosterone secretion such as atrial natriuretic peptide or dopamine, which can act directly on glomerulosa cells: both have been shown to nonselectively inhibit all 3 major stimulators of aldosterone secretion, Ang II, K+; and ACTH.19,20 Because we provide evidence that the inhibitory effect of Ang 1–7 on receptor-mediated aldosterone synthesis is exerted through the Mas1 receptor, as it can be effectively blocked by the Mas1 receptor antagonist A779, it seems that activation of this receptor cannot affect potassium-dependent aldosterone synthesis. Although Ang II– and ACTH-dependent steroidalogenesis in the zona glomerulosa use distinct signaling cascades, it is noteworthy that signaling through the AT1R, which mediates Ang II’s action in the glomerulosa as well as ACTH-induced steroidalogenesis were previously shown to operate via transactivation of the epidermal growth factor receptor,21,22 which is apparently expressed in adrenal glomerulosa cells.23 Furthermore, recent evidence suggests that signaling through Mas1 can inhibit AT1R-dependent transactivation of epidermal growth factor receptor.24 However, whether inhibition of this pathway plays a role in Ang 1–7–dependent inhibition of receptor activated aldosterone secretion remains to be directly studied.

Our study further suggests that the inhibitory effect of Ang 1–7 on aldosterone synthesis shown in vitro can be also exerted in vivo. As expected, short-term (1 week) salt restriction elicited a rise in both PRA and PA. Under concomitant continuous infusion of Ang 1–7, however, this response was modified such that the rise in PRA was fully preserved, whereas the increase in aldosterone was markedly attenuated. This supports the notion that under salt restriction, the observed lowering of PA is not secondary to suppression of circulating renin. More likely, it represents a direct inhibitory effect such as observed in the preceding in vitro experiments. However, whether endogenously produced Ang 1–7, reaching the adrenal via classical systemic/endocrine routes or operating locally in a paracrine manner, can mimic the effects shown in the present report with exogenously administered Ang 1–7 cannot be established based on our experiments.

**Perspectives**

The present report broadens the list of endogenous circulating hormones that directly affect aldosterone secretion. Because aldosterone excess per-se has been linked to multiple unfavorable sequelae, such as the evolution of hypertension,25 cardiac and vascular fibrosis,26,27 insulin resistance,28 and worse prognosis after myocardial infarction,29 the present report should instigate further studies of whether/how this previously unrecognized inhibitor of aldosterone secretion can be used under conditions in which high circulating aldosterone is a likely contributor to disease.

**Sources of Funding**

This work was supported by The Sagol Foundations for the Metabolic Syndrome Research Center.
Disclosures

None.

References


In salt-restricted rats, Ang 1–7 attenuates the aldosterone response to salt depletion independent of plasma renin activity.

What Is Relevant?

Because aldosterone excess apparently plays a role not only in the pathogenesis of hypertension but also in target organ damage, this new insight can be now explored as a tool to attenuated aldosterone-related tissue injury.

Summary

Ang 1–7, a formerly unrecognized inhibitor of Ang II–stimulated aldosterone secretion in vitro and in vivo, acts directly at the glomerulosa cell level.
Angiotensin 1−7 Is a Negative Modulator of Aldosterone Secretion In Vitro and In Vivo
Gabi Shefer, Yonit Marcus, Esther Knoll, Oleg Dolkart, Shulamit Foichtwanger, Nava Nevo, Rona Limor and Naftali Stern

Hypertension, published online May 31, 2016;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2016/05/31/HYPERTENSIONAHA.116.07088

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/