Evidence for Different Angiotensin II Receptors in Rat Adrenal Glomerulosa and Rabbit Vascular Smooth Muscle Cells

STUDIES WITH COMPETITIVE ANTAGONISTS

By Gordon H. Williams, Lynne M. McDonnell, Marie C. Raux, and Norman K. Hollenberg

ABSTRACT

Angiotensin II (AII) acts as both an endogenous vasoconstrictor agent and a specific trophic hormone that controls aldosterone secretion. The characteristics of vascular and adrenal receptors for AII were examined in vitro by comparing stimulation and blockade induced by two structural analogues of AII. The adrenal response was assessed on the basis of corticosterone and aldosterone production by isolated rat glomerulosa cells; isolated rabbit aortic strips provided an index of the response of vascular smooth muscle. Threshold sensitivity to AII in both tissues was 0.3–1.0 ng/ml with a peak response at about 300 ng/ml. P113 (1-Sar-8-Ala-AII) was a competitive antagonist in both systems; 1–10 ng/ml of P113 produced a significant parallel shift of the AII dose-response curve (P < 0.01). The maximum response was not reduced; increasing doses of AII overcame the blockade, and the responses to other agonists were not influenced. P113 did not stimulate either tissue. P4T8 (4-Phe-8-Tyr-AII) was a partial agonist in the rabbit aorta; 100 ng/ml of P4T8 induced competitive blockade, and contraction followed doses above 1,000 ng/ml. Conversely, P4T8 neither stimulated nor blocked the adrenal receptors, despite very high concentrations. Thus, the results of this study reveal functional differences in the AII receptors in the two systems and provide an approach to designing therapeutic agents that might block only one of its two major biological effects.

KEY WORDS  aldosterone secretion  vascular reactivity  corticosterone
1-Sar-8-Ala-angiotensin II  4-Phe-8-Tyr-angiotensin II  angiotensin analogues

Although angiotensin II (AII) has a variety of pharmacologic effects, the two most physiologically significant are the effect on vascular smooth muscle and the effect on the adrenal glomerulosa cells. Little is known about the comparative characteristics of the AII receptors in these two tissues. Classically, insight into receptors has largely come from the assessment of the response to structural analogues. An especially useful approach has been to examine the effect of competitive inhibitors. For example, epinephrine is the most potent agonist for both alpha- and beta-receptor effects; yet, antagonists that selectively produce alpha- or beta-receptor blockade have helped to dissociate these two types of adrenergic receptors. The development of AII analogues that are competitive inhibitors provides a similar tool for comparing the AII receptors in vascular smooth muscle and glomerulosa cells. Such analogues have already been used to assess the role of AII in the maintenance of blood pressure and to discriminate AII receptors in other tissues (1–4). The present study used this approach to demonstrate that the adrenal and the vascular receptors for AII are functionally different.

Methods

Studies were performed in vitro in the adrenal system (rat glomerulosa cells) and the vascular system (rabbit aortic strips). In each system a dose-response curve for AII was established along with the dose-response relationship for a nonangiotensin agonist—Cortrosyn for the adrenal system and norepinephrine for the vascular system. Two angiotensin analogues were assessed over a wide dose range for their ability to stimulate each system, to block the actions of AII, and to influence the
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responses of each system to the nonangiotensin stimulus.

**PREPARATION OF RAT GLOMERULOSA CELLS**

Normal female rats (200 g) (Charles River Laboratories) were maintained on a normal sodium intake (Purina rat chow). The rats were decapitated, and their adrenal glands were removed and decapsulated as previously described (5). A glomerulosa cell suspension was then prepared according to the techniques of Haning et al. (6). In brief, this technique consisted of incubating the adrenal capsules for 50 minutes at 37°C in a modified Krebs-Ringer’s bicarbonate solution containing 200 mg glucose/100 ml, 4% bovine serum albumin, and 3.7 mEq potassium/liter to which 3.7 mg of crude collagenase and 0.05 mg deoxyribonuclease/ml were added. Following incubation, the cells were mechanically disrupted. Cells from the capsules of eight glands (four rats) were then reincubated in 2 ml of the modified Krebs-Ringer’s solution for 2 hours at 37°C under a 95% O2-5% CO2 atmosphere. Corticosterone and aldosterone output were measured by radioimmunoassay techniques which have been previously described (5). Computations were performed on a General Electric 635 computer. Statistical analysis of the data included a two-way analysis of variance of the means. A t value for the response of each dose level compared with its appropriate control was computed on a log transformation of the data. Homogeneity of variances was assessed by Bartlett’s test (7), and F values were obtained from Dunnett’s tables (8).

**RABBIT AORTA PREPARATION**

The rabbit aorta preparation has been described in detail (9, 10). In brief, 4 strips cut from each rabbit aorta were mounted with 4 g of tension in muscle chambers with a 10-ml working volume containing a modified Krebs-Ringer’s bicarbonate solution. The solution was maintained at 37 ± 0.5°C and aerated constantly with a gas mixture containing 95% O2-5% CO2. Isotonic contractions were monitored with a force transducer. Cumulative dose-response curves for All or norepinephrine were obtained with an initial dose of 10-11 g/ml and subsequent logarithmic increments until a maximum contraction was achieved; at least 60 minutes were allowed for equilibration prior to administration of the agent. Only a single agonist (angiotensin or norepinephrine) was used on each strip because of the possibility of desensitization. Alternate strips were exposed to antagonists (10-4-10-9 g/ml) for 10 minutes prior to adding the agonist. A contractile response to P113 was never seen. Each combination was studied in at least 12 aortic strips taken from at least four rabbits.

**REAGENTS**

Radioactive Steroids.—1,2H-corticosterone (specific activity approximately 40 c/mmole), and 1,2H-aldosterone-γ-lactone (specific activity approximately 40 c/mmole), and 1,2H-aldosterone (specific activity approximately 50 c/mmole) were obtained from New England Nuclear Corporation. The purity of 1,2H-corticosterone and 1,2H-aldosterone were checked by paper chromatography (benzene-methanol-water solution, 1500:400:400), and the purity of 1,2H-aldosterone-γ-lactone was checked by another system (cyclohexane-benzene-methanol-water solution 500:200:500:100).

Other Reagents.—The following substances were used: Cortrosyn (cosyntropin), All (1-Asp-5-Ile-All, P, T8 (4-Phe-8-Tyr-All derivative), P113 (1-Sar-8-Ala-All derivative), All (Hypertensin, Ciba), norepinephrine bitartrate, bovine serum albumin (fraction V, Pentex Corporation, lot no. 153), dextran 80, activated charcoal, crude collagenase (Worthington Biochemical Corporation, lot CLS 21B), and deoxyribonuclease (Sigma DN-C, lot no. 112C-2720).

**RESULTS**

GLOMERULOSA CELL AND AORTIC STRIP RESPONSE TO ANGIOTENSIN II

Aldosterone production increased significantly (P < 0.01) when 0.25 ng All/ml was added (Fig. 1). The control level (1.53 ± 0.11 ng/rat hour-1) rose to 2.16 ± 0.14 ng/rat hour-1 with 0.25 ng of All and peaked at 3.93 ± 0.27 ng/rat hour-1 with 250 ng All/ml. Control corticosterone production (37 ± 3 ng/rat hour-1), however, did not increase significantly until 2.5 ng All/ml was added. A peak response (72 ± 5 ng/rat hour-1) was again obtained with 250 ng All/ml.

The rabbit aorta showed similar relationships with a threshold response occurring between 0.3 ng/ml and 1.0 ng/ml and a maximal response occurring between 200 ng/ml and 300 ng/ml (Fig. 2).

**EFFECT OF P113 (1-SAR-8-ALA-ANGIOTENSIN II)**

P113 (30 ng/ml) produced a significant (P < 0.01) parallel shift of the All dose-response curve (Fig. 3). However, the maximum aldosterone production induced by All (3.85 ± 0.34 ng/rat hour-1) was not different from that produced in the presence of 30 ng P113/ml (3.98 ± 0.33 ng/rat hour-1). Similarly, maximum corticosterone output with (64 ± 7 ng/rat hour-1) or without (68 ± 6 ng/rat hour-1) P113 was not different. Thus, the blockade was surmountable. In six experiments, the effect of 1 ng P113/ml on the All dose-response curve was assessed. As anticipated, the effect on the aldosterone response was less noticeable, but a significant (P < 0.05) parallel shift of the curve was demonstrated. The effect of P113 on the All dose-response curve in the aortic strip preparation was similar with a significant parallel shift of the curve after the addition of 10 ng P113/ml (Fig. 4). Again, the maximum absolute response induced by All
Figure 1

Aldosterone and corticosterone output from isolated glomerulosa cells in response to increasing doses of angiotensin II. Values are means ± SE of 20 experiments.

(1.99 ± 0.33 g) was not reduced when P113 was present (1.91 ± 0.34 g). This blockade was further accentuated with doses of 30 ng/ml, but there was no consistent effect with 1 ng/ml.

There was no evidence of intrinsic activity, i.e., no increase in aldosterone and corticosterone production occurred, when glomerulosa cells were incubated with increasing concentrations of P113 (Fig. 5). In fact, a dose of 25 ng P113/ml significantly decreased aldosterone production to 71 ± 6% of control. The corticosterone response mimicked that of aldosterone, but to a lesser degree. Similarly, P113 at doses as high as 3 μg P113/ml did not induce contraction of the aortic strip.

The specificity of the P113 blockade was assessed by its effect on Cortrosyn-stimulated aldosterone and corticosterone production and on norepinephrine-stimulated smooth muscle contraction. P113 (30 ng/ml) had no effect on the Cortrosyn-stimulated outputs of aldosterone and corticosterone at doses of 0.16 munits/ml and 2.56 munits/ml. These doses increased aldosterone output two- and fivefold, respectively. Similarly, 10 ng P113/ml did not alter the response of the aorta to norepinephrine over a dose range of 1 ng/ml to 300 ng/ml.
P$_4$T$_8$ in doses as high as 2.5 µg/ml did not alter the AII-stimulated aldosterone output from glomerulosa cells (Table 1). In contrast, P$_4$T$_8$(100 ng/ml) blocked AII-mediated contraction of the aorta (Fig. 6). Furthermore, the blockade was competitive, since both lines on the Lineweaver-Burke plot intercept the ordinate at the same point, and the maximum response induced by AII with P$_4$T$_8$ (2.04 ± 0.43 g) was the same as the response induced without it (1.99 ± 0.33 g).

In three experiments, neither 25 ng/ml or 2,500 ng/ml of P$_4$T$_8$ consistently changed aldosterone or corticosterone production from the control levels. However, in the smooth muscle preparation, doses of 1-30 ng/ml, i.e., levels 10- to 300-fold greater than that producing blockade, produced a consistent dose-related response (Fig. 7).

Discussion

Ultimately, the characterization of receptors will demand their isolation and the definition of their structure. However, this approach may not be possible if the receptor is an integral part of a membrane, since destruction of the membrane may alter the structure of the receptor. An alternative, albeit indirect, approach is to assess the relative biologic effect (intrinsic activity) of a parent compound and a variety of structural analogues. Thus,
the structural determinants of an agent's interaction with its receptor can be determined through these structure-activity relationships. This approach has been used by several investigators (11-16) to define the structural aspects of AII that are essential for activity.

A second way to assess a receptor's characteristics is to determine the effect of various structural analogues that may or may not possess intrinsic activity but are competitive inhibitors. Both types of assessments assume a complementary relationship between the parent compound, its analogues, and the receptor. Recent studies suggest that such a relationship does exist for AII. In a preliminary report (17), both AII and its analogue P113 displaced radioactive angiotensin from adrenal binding sites, but only AII increased adenylyl cyclase activity, implying an interaction with a common receptor but the production of different biochemical events. Since Khairallah et al. (12) demonstrated that the 5-Ile-8-Ala-analogue of AII blocked the action of AII on strips of guinea pig il- eum, various analogues that are competitive inhibitors of the smooth muscle effect of AII have been synthesized (1-4, 16, 18-21). However, in only a few instances has the effect of these analogues on the adrenal cortex been assessed (2,17, 22, 23), and only rarely has the effect of analogues on different angiotensin receptors been specifically compared (1, 22). Peach et al. (1) and Khairallah (24), who used various structural analogues, have suggested that angiotensin receptors on sympathetic nerve endings are structurally different from those on heart muscle.

A similar approach in the present study demonstrated similarities and differences in the effect of AII and two of its analogues on the aorta and isolated glomerulosa cells. The angiotensin sensitivity of the two systems was similar (0.25-1.0 ng/ml for threshold and about 300 ng/ml for maximal responses).

The excellent steroid response probably resulted from the preparation method; the cells had been under the influence of adrenocorticotropin in vivo but they had been incubated without it in vitro. Likewise, the responses of these two tissues of P113 were essentially similar. In both tissues, P113 antagonized the action of AII at doses of 1-10 ng/ml; the blockade was competitive and specific, and intrinsic activity was not demonstrated in either tissue, even at doses as high as 2.5-3.0 μg/ml. The response of the aortic strip to angiotensin was blocked, although the concentration required was considerably higher than that required for P113 (100 ng/ml). In addition, at higher concentrations, P4T8 possessed intrinsic activity and induced aortic contraction. Conversely, P4T4 neither stimulated nor blocked the glomerulosa cells, suggesting that the AII receptors are different in the two tissues. It is possible that higher doses of P113 might have stimulated smooth muscle or that higher doses of P4T8 might have increased adrenal steroid output; however, even if these possibilities are true the difference in the concentrations required would still suggest a major difference in the two receptors. This proposal is also supported by a recent preliminary report (22) in which a different analogue (l-Sar-8-Ile-AII) was used. Because changes in the 8 position significantly altered the interaction of the analogues with these two receptors, the critical difference between the vascular and the adrenal receptors probably is their relationship with the carboxy end of the molecule. The differences in the receptors may also account for

### Table 1

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Aldosterone output (ng/rat hour⁻¹)</th>
<th>From control</th>
<th>From pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4 ± 0.2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AII (0.25 ng/ml)</td>
<td>2.0 ± 0.3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AII + P₄T₈</td>
<td>1.9 ± 0.3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AII (25 ng/ml)</td>
<td>3.2 ± 0.6</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>AII + P₄T₈</td>
<td>2.6 ± 0.4</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>AII (25 ng/ml)</td>
<td>3.7 ± 0.5</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>AII + P₄T₈</td>
<td>4.0 ± 0.6</td>
<td>0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

All values are means ± SE. NS = not significant. P₄T₈ was given in a dose of 2.5 μg/ml.
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Double reciprocal (Lineweaver-Burke) plot of the response of the aorta to angiotensin II with and without $P_4T_8$ (100 ng/ml). The lines meet at ordinate, which is characteristic of competitive kinetics. Each point represents the mean of eight determinations.

![Graph showing double reciprocal plot]

The opposite effects that salt intake has on the responses of the two systems to Angiotensin II; restriction of sodium intake blunts vascular responses to angiotensin, but it potentiates the responses of the adrenal glands (25).

The difference in the response of the two systems to $P_4T_8$, documented in this paper possibly reflects a species difference. The tissues were selected because it is important to work in vitro; the rat provides an excellent in vitro adrenal system, but isolated vascular tissue from the rat develops angiotensin tachyphylaxis too rapidly (9) for studies such as those performed in this investigation. However, in the rat, $P_4T_8$ acts as a partial agonist both in nonvascular isolated smooth muscle (26) and on pressor responses to Angiotensin II (18) used as an index of the integrated vascular response. Moreover, $P_113$ shows remarkably similar effects in both systems, free of any apparent species influence in rat, rabbit, dog, and man (2, 3, 22). It seems unlikely, therefore, that the differences reported in this paper can be attributed to species. Further studies are necessary to delineate the characteristics of adrenal and vascular Angiotensin II receptors in various species.

The most extensive studies with $P_4T_8$ (18, 26, 27) have demonstrated specific inhibition of Angiotensin II-induced responses in isolated uterine muscle and in the blood pressure of the intact rat. There have been no previous reports on the effect of $P_4T_8$ on glomerulosa cells. $P_{113}$ was first studied by Pals et al. (3) who demonstrated specific competitive inhibition of Angiotensin II responses of rabbit aortic strips; these findings are reproduced in the present study. Brunner et al. (4), Johnson and Davis (2), and Mimran et al. (28) have also shown that $P_{113}$ is a potent antagonist of the pressor effect of Angiotensin II in vivo in the rat, the dog, and the rabbit. Furthermore, Johnson and Davis (2) have demonstrated that $P_{113}$ decreases aldosterone secretion in the intact dog and Peach and Chin (23), using isolated glomerulosa cells, have reported that $P_{113}$ is a competitive inhibitor of Angiotensin II. Their demonstration of different structure-activity relationships of adrenal stimulation among several angiotensin analogues in the rabbit adrenal glands further supports the concept of functionally different receptors and makes species differences less likely in this study.

Thus, the present study confirms some previous observations and demonstrates a previously unrecognized phenomenon: a striking difference in the action of $P_4T_8$ in the two systems studied. This difference in the responses of adrenal glands and vascular smooth muscle suggests that there are functional differences in their Angiotensin II receptors; this...
characteristic may be useful in designing therapeutic agents to block only one of the two major physiological effects of All. Moreover, the difference in the receptors may account for the difference in their responses to restriction of sodium intake (25).

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References


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