Hypersensitivity of the Adrenal Cortex to Trophic and Secretory Effects of Angiotensin II in Lyon Genetically-Hypertensive Rats

Frédéric Aguilar, Ming Lo, Bruno Claustrat, José Maria Saez, Jean Sassard, and Jacques Yuan Li

Abstract—In Lyon hypertensive (LH) rats, a model of low-renin genetic hypertension, we investigated adrenal sensitivity to angiotensin II in terms of angiotensin II receptor (AT1, and AT2 receptors) regulation, morphological changes, and aldosterone and corticosterone secretion. Twelve-week-old LH rats, compared with normotensive LN and LL rats, were either untreated or treated for 4 weeks with AT1 receptor antagonist irbesartan (50 mg/kg/d), angiotensin-converting enzyme inhibitor perindopril (3 mg/kg/d), or perindopril (3 mg/kg/d) plus angiotensin II infusion (200 ng/kg/min). At 16 weeks, untreated LH rats had high systolic blood pressure (P<0.05), low aldosterone (P<0.05), and increased corticosterone (P<0.05) plasma levels. AT1-receptor binding density in the zona glomerulosa was similar in the three strains. In LH rats, angiotensin II infusion increased the relative adrenal weight from 10.5±0.3 to 16.7±0.7 mg/100g (P<0.05), whereas the change was very modest in normotensive rats. Zona glomerulosa enlarged and plasma aldosterone increased after angiotensin II infusion in the 3 strains, but more markedly in LH versus normotensive rats (2.4- versus 1.3- and 1.6-fold, respectively; 20- versus 10-fold in normotensive rats, P<0.05). Surprisingly, after angiotensin II infusion, despite the absence of angiotensin II receptors in the three strains, the zona fasciculata-reticularis enlarged 1.5-fold and plasma corticosterone increased 1.7-fold only in LH rats (P<0.05), suggesting an indirect control of this compartment by angiotensin II. The hypertrophy and hypersecretory activity of both zona glomerulosa and zona fasciculata-reticularis in LH rats in response to angiotensin II point to the adrenal cortex as a pivotal tissue in the pathophysiology of hypertension in LH rats. (Hypertension. 2004;43:87-93.)

Key Words: rats ■ hypertension, genetic ■ receptors, angiotensin II ■ adrenal gland ■ hypertrophy ■ aldosterone ■ corticosterone

Adult genetically-hypertensive rats of the Lyon strain (LH), contrary to their normotensive (LN) and low blood pressure (LL) controls, exhibit a low plasma and kidney renin content but an efficient blood pressure-lowering response to angiotensin converting enzyme (ACE) inhibition. Renin expression at other locations (eg, the adrenal gland and the brain) was not shown to differ in the Lyon rat strains. The paradox of this low renin form of hypertension could be explained by an increased sensitivity of LH rats to angiotensin II (ANG II) and could implicate an abnormal regulation of the number and/or function of ANG II receptors. ANG II receptors can be divided into at least two subtypes: subtype 1 (AT1R) and subtype 2 (AT2R). In rodents, there are two AT1R subtypes, 1A and 1B, differentially expressed and regulated. ANG II receptors display a discrete localization in various organs, including the cardiovascular system, kidneys, adrenals, pituitary gland, and brain. Most, if not all, known actions of ANG II, such as aldosterone secretion, renal salt reabsorption, vasoconstriction, and cell proliferation, are mediated by AT1R.

The adrenal gland is an important site for the regulation of fluid homeostasis and sympathetic function by ANG II. Stimulation of AT1R contributes to regulate aldosterone formation and release. In the adrenal medulla, although AT1R density is low, they partly control catecholamine release. Like in other tissues, the functions of AT2R are still a matter of controversy. Despite their noticeable density in the zona glomerulosa, AT1R do not participate in aldosterone formation or secretion. The presence of a large number of AT2R in the adrenal medulla has been linked to the control of catecholamine release by only a few authors. No significant amount of ANG II receptors has been reported in the zona fasciculata-reticularis of the rat. However, this compartment of the adrenal gland may contribute to hypertension in rodents and man through an adrenocorticotropic-dependent mechanism.

We hypothesized that, in LH rats, an abnormal expression and/or regulation of ANG II receptor subtypes in the adrenal gland could contribute to enhanced effect of ANG II in this model of low renin hypertension. Therefore, we studied the...
Effects of pharmacological alterations of the renin-angiotensin system on blood pressure, adrenal morphology, ANG II receptors, and steroidogenic response. This was conducted in LH rats compared with their two genetically pure and different normotensive controls, the LN and LL rats.

Materials and Methods

Experimental Protocol

Male Lyon rats bred in the laboratory were housed in controlled conditions (21°C ±1°C; humidity: 60%±10%; lighting 8 to 20 hours) with a standard rat chow containing 0.3% sodium and 0.75% potassium (A03; UAR, Villemonais-sur-Orge, France). Experiments were conducted in accordance with our institutional ethical guidelines.

Twelve-week-old animals were divided into four groups of 10 LH, LN, and LL rats that were untreated or treated for 4 weeks as follows: group C, control untreated animals received buffered water (solution for ibesartan preparation); group I, orally treated with an AT1 antagonist, ibesartan (50 mg/kg/d; Sanofi, Montpellier, France), dissolved in 0.035-mol/L NaH2PO4 and neutralized with 0.035-mol/L NaH2PO4; group P, orally treated with an ACE inhibitor perindopril (3 mg/kg/d; Servier, Neuilly-sur-Seine, France); and group P+ANG II, orally treated with perindopril (3 mg/kg/d) associated with a subcutaneous infusion of ANG II (200 ng/kg/min; Sigma Chemical, St. Louis, Mo) through an osmotic minipump (Alzet 2ML4; ALZA, Palo Alto, Calif) to clamp at a stable level the circulating ANG II. Indirect systolic blood pressure (SBP) was measured weekly by tail-cuff plethysmography (Narco Biosystems, Houston, Tex) on freely moving preheated rats. At 16 weeks of age, animals were euthanized between 10 and 12 AM by decapitation 20 minutes after being tranquilized (diazepam 5 mg/kg; IP). Adrenals were dissected out, weighed, and frozen in liquid nitrogen. Blood collected on heparin at 4°C was centrifuged to obtain plasma.

Steroid Dosages

Plasma aldosterone levels were determined by radioimmunoassay using a commercial kit (Immunotech, Marseille, France). Plasma corticosterone levels were determined by radioimmunoassay.11

Autoradiography of ANG II Receptors

Adrenal 12-μm frozen sections were treated as described.12 Adjacent sections were incubated for 120 minutes with 1 nmol/L of 125I-ANG II (specific activity, 2200 Ci/mmol) alone (total binding) or in the presence of 10-μmol/L ANG II (nonspecific binding). Specific AT1 or AT2R binding was determined in the presence of 0.1-μmol/L ANG II. Indirect systolic blood pressure (SBP) was measured weekly by tail-cuff plethysmography (Narco Biosystems, Houston, Tex) on freely moving preheated rats. At 16 weeks of age, animals were euthanized between 10 and 12 AM by decapitation 20 minutes after being tranquilized (diazepam 5 mg/kg, IP). Adrenals were dissected out, weighed, and frozen in liquid nitrogen. Blood collected on heparin at 4°C was centrifuged to obtain plasma.

Stereological Procedures

Serial adrenal cryosections (12 μm) were fixed, stained with eosin-hematoxylin, observed under light microscope, and photographed with a digital camera. The analysis was made with the NIH image 1.6 software. Every tenth section of each adrenal was measured for area of the medulla. Width of zona glomerulosa and diameter of adrenals were measured on equatorial sections. Volumes of the medulla were calculated from area x distance between sections. Volumes of the entire gland and the zona glomerulosa were calculated according to Nusserdorfer el al.13 Volumes of the zona fasciculata-reticularis were obtained by subtracting the volumes of the zona glomerulosa and of the medulla from the volume of the whole gland.

Statistical Analysis

Results are reported as mean±SEM. Statistical analyses were performed by Student’s t tests, the level of significance being P<0.05. If statistically significant effects were found by the t tests, Bonferroni corrections for multiple comparisons were applied where appropriate. Comparison of indirect SBP with age used two-way analysis of variance with repeated measures over time. P<0.05 was considered as significant.

Results

Systolic Blood Pressure

Control LH rats had a significantly higher SBP than age-matched LN and LL rats (Figure 1). As usual, these two latter did not differ. Ibesartan decreased SBP more markedly in LH than in LN and LL rats. A similar observation was made in perindopril-treated animals with the difference that perindopril lowered more SBP in LN and LL rats than did ibesartan. The infusion of ANG II in perindopril-treated rats induced during the first two weeks a similar increase in SBP in the three strains. During the two last weeks of infusion, SBP continued to increase in LH rats, whereas it remained stable in LN and LL rats.

Trophic Effect of Angiotensin II on the Adrenals

Control LH rats had a significantly (P<0.05) larger body weight (BW) than LN and LL rats (Table 1). Neither ibesartan nor perindopril significantly changed the BW of the three strains of rats. On the contrary, the infusion of ANG II decreased markedly the BW in LH rats (Table 1). The relative adrenal weight (Figure 2) did not differ between control rats. ANG II infusion induced a modest increase in adrenal weight of LL rats whereas, in LH rats, a striking elevation (+60%) was observed. This ANG II-induced increase remained highly significant when absolute instead of relative adrenal weights were considered.

To assess which zones of the adrenal were enlarged during ANG II treatment, we estimated the volumes of the different compartments of the adrenal in perindopril-treated rats which had or not been infused with ANG II (Table 2). Volumes of the entire gland, zona glomerulosa, zona fasciculata-reticularis, and medulla were not significantly different in the three strains treated with perindopril. ANG II infusion led to a much greater increase (2.4-fold) in the zona glomerulosa volume in LH than in normotensive rats (about 1.5-fold in both LN and LL rats). In addition, the volume of the zona fasciculata-reticularis increased only in LH rats. Whatever the strain of rats, the volume of the medulla was unaffected by ANG II infusion.

Plasma Steroids

Basal levels of plasma aldosterone were significantly lower in 16-week-old LH rats (145±13 pg/mL) than in age-matched LN and LL rats (400±58 and 288±53 pg/mL, respectively, Figure 3). Ibesartan or perindopril induced a significant decrease of plasma aldosterone in LN and LL rats, but not in LH rats. ANG II infusion in perindopril-treated rats strongly elevated plasma aldosterone in the three strains, this increase being greater in LH rats (20-fold) than in normotensive rats (about 10-fold for both LN and LL rats). Basal levels of plasma corticosterone were higher in 16-week-old LH than in age-matched LN and LL rats. Plasma corticosterone levels were not altered significantly by AT1 or R blockade with ibesartan in the three strains. Perindopril treatment induced a
modest plasma corticosterone increase in LL rats. ANG II infusion in perindopril-treated rats markedly increased plasma corticosterone in LH rats only.

**AT\textsubscript{1}R and AT\textsubscript{2}R Subtypes**

The affinity of AT\textsubscript{1}R and AT\textsubscript{2}R in adrenal cell membranes from the four experimental groups was comparable in the three strains of rats and unaffected by the various treatments (data not shown). Figure 4 shows the autoradiographic localization of ANG II receptors in adrenal glands of 16-week-old Lyon rats. AT\textsubscript{1}R binding sites were highly expressed in the zona glomerulosa, slightly expressed in the medulla, and undetectable in the zona fasciculata-reticularis. AT\textsubscript{2}R binding sites were mostly present in the adrenal medulla and moderately in the zona glomerulosa. As for AT\textsubscript{1}R, no AT\textsubscript{2}R binding sites were detected in the zona fasciculata-reticularis. In the zona glomerulosa, AT\textsubscript{1}R and AT\textsubscript{2}R-binding site densities were similar in LH, LN, and LL rats, and not altered by irbesartan or perindopril treatments (Table 3). The chronic infusion of ANG II induced an upregulation of AT\textsubscript{1}R and AT\textsubscript{2}R in the zona glomerulosa of comparable intensity in the three strains (Table 3 and Figure 4B). In the adrenal medulla, AT\textsubscript{1}R density did not differ among the groups studied, whereas AT\textsubscript{2}R-binding site density

<table>
<thead>
<tr>
<th>Strain</th>
<th>C</th>
<th>I</th>
<th>P</th>
<th>P+ANG II</th>
</tr>
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<tbody>
<tr>
<td>LH</td>
<td>430±4*†</td>
<td>415±6*†</td>
<td>417±5*†</td>
<td>368±9*§</td>
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<tr>
<td>LN</td>
<td>318±4</td>
<td>314±3</td>
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<tr>
<td>LL</td>
<td>388±4*</td>
<td>379±6*</td>
<td>382±4*</td>
<td>372±5*‡</td>
</tr>
</tbody>
</table>

Values are means±SEM, n=9–10 rats per group. LH indicates Lyon genetically hypertensive rats; LN, Lyon normotensive rats; and LL, Lyon low blood pressure rats.

*P<0.05 vs LN rats; †P<0.05 vs LL rats; ‡P<0.05 vs C; and §P<0.05 P+ANG II vs P.
TABLE 2. Volumes (mm$^3$) of the Entire Adrenal Gland and the Zona Glomerulosa, Fasciculata-Reticularis, and Medulla in 16-Week-Old LH, LN, and LL Rats Treated With Perindopril Alone (P) or Associated With Angiotensin II (P+ANG II)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Strain</th>
<th>P</th>
<th>P+ANG II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire adrenal gland</td>
<td>LH</td>
<td>15.7±1.2</td>
<td>24.6±1.6†‡</td>
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<tr>
<td></td>
<td>LN</td>
<td>13.7±0.7</td>
<td>14.4±1.0</td>
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<tr>
<td></td>
<td>LL</td>
<td>14.4±1.5</td>
<td>17.7±1.8</td>
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<tr>
<td>Glomerulosa</td>
<td>LH</td>
<td>2.32±0.20</td>
<td>5.58±0.20†‡</td>
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<tr>
<td></td>
<td>LN</td>
<td>2.21±0.10</td>
<td>2.79±0.14‡</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>2.09±0.17</td>
<td>3.28±0.28‡</td>
</tr>
<tr>
<td>Fasciculata-reticularis</td>
<td>LH</td>
<td>12.3±1.0</td>
<td>17.8±1.3†‡</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>10.3±0.7</td>
<td>10.3±1.0</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>11.2±1.2</td>
<td>13.4±1.5</td>
</tr>
<tr>
<td>Medulla</td>
<td>LH</td>
<td>1.12±0.04</td>
<td>1.24±0.03</td>
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<tr>
<td></td>
<td>LN</td>
<td>1.15±0.04</td>
<td>1.23±0.04</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>1.08±0.03</td>
<td>1.05±0.08</td>
</tr>
</tbody>
</table>

Values are means±SEM. n=4 rats per group. LH indicates Lyon genetically hypertensive rats; LN, Lyon normotensive rats; and LL, Lyon low blood pressure rats.

*P<0.05 vs LN rats; †P<0.05 vs LL rats; ‡P<0.05 P+ANG II vs P.

was higher in LN than in LH and LL rats in the control groups (Table 3 and Figure 4A). Suppression of AT$_1$R and AT$_2$R stimulation with perindopril did not modify the density of AT$_2$R binding sites. On the contrary, AT$_2$R overstimulation either as a result of irbesartan treatment or ANG II infusion significantly decreased their density in the three strains of rats (Table 3 and Figure 4B).

Discussion

The major finding of this study was that the high and sustained rise in blood pressure induced in LH rats by ANG II infusion was associated with an impressive enlargement of the adrenals and a marked increase of both plasma aldosterone and corticosterone levels.

ANG II Receptor Regulation in Control and Treated Animals

Binding studies on adrenal sections shown in the present study indicated that the general pattern of expression of the ANG II receptors in the adrenal gland was similar in the three strains of the Lyon rats and reflected the classical profile reported in the rodent adrenal gland.

The design of the pharmacological protocol was aimed at creating in each group a clear-cut status of the renin-angiotensin system: in group C, ANG II receptors were under the control of endogenous ANG II; in group I, AT$_1$R were blocked by irbesartan whereas AT$_2$R were likely to be over-stimulated by the increase of endogenous circulating ANG II resulting from the lack of AT$_1$R-mediated negative feed-back on renin release; in group P, ACE inhibition abolished the stimulation of both ANG II receptor subtypes; and in group P+ANG II, both ANG II receptor subtypes were over-stimulated at a constant level by an infusion of exogenous ANG II in animals deprived of endogenous and thus variable formation of ANG II via perindopril treatment.

These various pharmacological treatments induced subtle differences in the levels of expression of the ANG II receptors in the three strains of Lyon rats.

In the zona glomerulosa, over-stimulation of both AT$_1$R and AT$_2$R by ANG II infusion induced an upregulation of AT$_1$R and AT$_2$R in the three strains of Lyon rats. In rodents, upregulation of AT$_2$R in the zona glomerulosa by ANG II is well documented, and that of AT$_1$R has been reported in a model in which ANG II was increased by low sodium diet.

At 16 weeks of age, the medullas of control LN rats displayed a higher AT$_2$R density than those of the LH and LL rats. High density of AT$_2$R binding in the LN rats seems to be a peculiar feature of this strain. No significant differences of AT$_2$R-binding site densities were reported in spontaneously hypertensive rats (SHR) versus Wistar Kyoto (WK) rats, AT$_2$R binding sites in Lyon rats were down-regulated by an increased production of ANG II resulting either from AT$_1$R blockade or ANG II infusion. This is at variance with the regulation of AT$_1$R, reported above, in the zona glomerulosa by the same treatments. These differences illustrate in vivo the complexity of the regulation of AT$_2$R expression by ANG II which resorts to specific mechanisms with regard to the cell type as reported in several in vitro models. Interestingly, biochemical evidence suggests that AT$_1$R and AT$_2$R make oligomers, which alter cellular responses to ANG II. The differences of expression of the two receptor subtypes we

![PLASMA ALDOSTERONE](http://hyper.ahajournals.org/)

![PLASMA CORTICOSTERONE](http://hyper.ahajournals.org/)

Figure 3. Plasma steroids levels in 16-week-old LH, LN and LL rats control (C), treated with irbesartan (I), perindopril alone (P), or perindopril associated with angiotensin II (P+ANG II). Values are means±SEM, n=4 to 6 rats per group. †P<0.05 versus LN rats. §P<0.05 versus C.
Adrenal Gland Induced by the Treatments

Morphological and Functional Alterations of the Adrenal Cortex Proliferated in Response to ANG II

The volume of the zona glomerulosa or the absolute number of AT1R, the amount of plasma aldosterone per volume unit of zona glomerulosa or per AT1R is lower in the LH than in the normotensive rats. Although the balance between the rates of aldosterone synthesis and excretion were not determined, such a calculation suggests a low sensitivity of the zona glomerulosa in the LH rats to the secretory effects of ANG II. In comparison to the normotensive controls, the LH rats display an imbalance between the trophic and the secretory effects of ANG II with a shift in favor of the growth-promoting effect of the AT1R.

In the zona fasciculata-reticularis, a highly unexpected piece of information was found. In LH rats only, the zona fasciculata-reticularis was prominently enlarged after ANG II infusion. This 1.5-fold increase in volume accounted for 63% of the hypertrophy of the whole gland. Available data do not favor the hypothesis of a growth-promoting effect of ANG II on the zona reticularis. Clues that the zona fasciculata was enlarged in LH rats in group P+ANG II were drawn from the measurements of plasma corticosterone concentrations in Lyon rats. Corticosterone is the main steroid secreted by the zona fasciculata. ANG II infusion induced a 1.7-fold increase in plasma corticosterone in LH rats compared with a limited 1.2-fold increase in the two normotensive strains. This is quite surprising because this compartment does not display detectable levels of either AT1R or AT2R under none of the pharmacological treatments. One cannot exclude that, according to the migration theory, cells in the outer regions of the adrenal cortex proliferate in response to ANG II, then migrate centripetally and acquire the differentiated functions of zona fasciculata cells. A more likely alternative explanation relies on the ability of ANG II to stimulate ACTH release by acting on central ANG II receptors, probably in the anterior pituitary, as shown by several studies. ACTH would bind to ACTH receptors and induce fasciculata cell growth and corticosterone secretion. Our results suggest that LH rats could be hypersensitive to ANG II-induced ACTH secretion. Because ACTH receptors are expressed in both the zona fasciculata and the zona glomerulosa, increased levels of plasma ACTH could also take part in the stimulation of
zona glomerulosa hypertrophy and aldosterone secretion in the LH strain. This possible link between ANG II and ACTH secretion is in line with recent findings showing that, in SHR but not in their normotensive controls, a peculiar pattern of expression of \( \text{AT}_{1} \alpha \text{R} \) and \( \text{AT}_{1} \beta \text{R} \) in the anterior pituitary could be responsible for increased plasma levels of ACTH and corticosterone in response to ANG II. Another specific alteration of the hypothalamo-pituitary-adrenal axis in LH control rats was the higher levels of plasma corticosterone they displayed at 16 weeks of age in comparison with age-matched normotensive rats. The elevated values of plasma corticosterone in control animals above 100 ng/mL are probably consecutive to IP injection of diazepam. The stress associated with the injection is certainly involved. A direct or indirect effect of diazepam on steroidogenesis through its receptors in the adrenal cortex or the anterior pituitary gland is unlikely because the dose we used was reported to be ineffective. However, our finding of higher corticosterone plasma levels in control LH rats is in keeping with the elevated urinary corticosterone levels found in 20-week-old LH rats maintained under unstressed physiological conditions. High plasma corticosterone levels in LH rats raise the question of the impact of glucocorticoids in the physiopathology of hypertension in this strain. It is established that mineralocorticoid receptors have the same affinity for both aldosterone and corticosterone. However, LH rats have been shown to exhibit an impaired 11\( \beta \)-hydroxysteroid dehydrogenase type I (11\( \beta \)-HSD1) activity, a situation that should theoretically decrease the availability of corticosterone to the mineralocorticoid receptors. The hypothesis of glucocorticoid-dependent hypertension has also been raised in SHR. Such convergent results in two independent models of genetically-hypertensive rats are an incentive to explore further the relevance of glucocorticoids in the physiopathology of hypertension in LH rats.

**Perspectives**

The massive adrenal enlargement in response to long-term ANG II infusion in LH rats is one of the hallmarks of the sensitivity of this genetically hypertensive rat strain to the effects of ANG II. This hypertrophic response does not depend on an alteration of the affinity nor an abnormal pattern of the regulation of the ANG II receptor subtypes in the adrenal gland. However, functional hypertrophy of both zona glomerulosa and zona fasciculata-reticularis is underlain by ANG II-dependent mechanisms, specific to or exacerbated in the LH rats. In the zona glomerulosa of LH rats, compared with normotensive controls, the proliferative effect of ANG II seems to prevail over its role on aldosterone production. In the zona fasciculata-reticularis, ANG II-dependent functional hypertrophy appears to be the result of an indirect mechanism that could involve the central control by ANG II of the hypothalamo-pituitary-adrenal axis. Our future direction will allow us to analyze more closely the possible anomalies of ANG II signaling or control in the zona glomerulosa and zona fasciculo-reticularis in the LH rats. Evidence for relevant alterations in this animal model would help deciphering forms of low-renin essential hypertension or ACTH-dependent hypertension in human beings.

**References**

15. Aguilar et al Adrenal Hypertrophy in Lyon Hypertensive Rats
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