Aldosterone Receptor Antagonism Normalizes Vascular Function in Liquorice-Induced Hypertension

Thomas Quaschning*, Frank Ruschitzka*, Sidney Shaw, Thomas F. Lüscher

Abstract—The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD2) provides mineralocorticoid receptor specificity for aldosterone by metabolizing glucocorticoids to their receptor-inactive 11-dehydro derivatives. The present study investigated the effects of the aldosterone receptor antagonists spironolactone and eplerenone on endothelial function in liquorice-induced hypertension. Glycyrrhizic acid (GA), a recognized inhibitor of 11β-HSD2, was supplemented to the drinking water (3 g/L) of Wistar-Kyoto rats over a period of 21 days. From days 8 to 21, spironolactone (5.8±0.6 mg · kg⁻¹ · d⁻¹), eplerenone (182±13 mg · kg⁻¹ · d⁻¹), or placebo was added to the chow (n=7 animals per group).

Endothelium-dependent or -independent vascular function was assessed as the relaxation of preconstricted aortic rings to acetylcholine or sodium nitroprusside, respectively. In addition, aortic endothelial nitric oxide synthase (eNOS) protein content, nitrate tissue levels, and endothelin-1 (ET-1) protein levels were determined. GA increased systolic blood pressure from 142±8 to 185±9 mm Hg (P<0.01). In the GA group, endothelium-dependent relaxation was impaired compared with that in controls (73±6% versus 99±5%), whereas endothelium-independent relaxation remained unchanged. In the aortas of 11β-HSD2–deficient rats, eNOS protein content and nitrate tissue levels decreased (1114±128 versus 518±77 µg/g protein, P<0.05). In contrast, aortic ET-1 protein levels were enhanced by GA (308±38 versus 497±47 pg/mg tissue, P<0.05). Both spironolactone and eplerenone normalized blood pressure in animals on GA (142±9 and 143±9 mm Hg, respectively, versus 189±8 mm Hg in the placebo group; P<0.01), restored endothelium-dependent relaxation (96±3% and 97±3%, respectively, P<0.01 versus placebo), blunted the decrease in vascular eNOS protein content and nitrate tissue levels, and normalized vascular ET-1 levels. This is the first study to demonstrate that aldosterone receptor antagonism normalizes blood pressure, prevents upregulation of vascular ET-1, restores NO-mediated endothelial dysfunction, and thus, may advance as a novel and specific therapeutic approach in 11β-HSD2–deficient hypertension. (Hypertension. 2001;37[part 2]:801-805.)

Key Words: 11β-hydroxysteroid dehydrogenase • endothelin-1, endothelium • glycyrrhizic acid • nitric oxide

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) confers mineralocorticoid receptor specificity by metabolizing glucocorticoids to their receptor-inactive 11-dehydro derivatives. Impaired conversion of cortisol to cortisone results in sodium retention and severe hypertension and leads to a complex disease named congenital aldosterone deficiency. Because liquorice-derived glycyrrhizic acid (GA) is a well-known inhibitor of 11β-HSD2, ingestion of GA would result in 11β-HSD2–deficient hypertension. In addition to its role in the kidney, 11β-HSD is present in cardiac fibroblasts, coronary vascular smooth muscle, and endothelial cells, where it is thought to act in a paracrine fashion by modulating local glucocorticoid and mineralocorticoid activity, vascular tone, and endothelial function. Interestingly, reduced urinary excretion of steroid metabolites, suggesting decreased activity of 11β-HSD, occurs in liquorice-induced as well as in untreated essential hypertension.

In patients with hypertension, endothelial dysfunction precedes the rise in blood pressure and predisposes them to structural vascular changes. The endothelium releases vasoactive mediators such as nitric oxide (NO) and endothelin-1 (ET-1), both of which regulate vascular tone and structure. The aldosterone receptor antagonist spironolactone increases NO bioavailability and vascular relaxation in patients with heart failure. This may contribute to its regulatory effects in the treatment of hypertension, for which aldosterone receptor antagonism is a well-established treatment option. Because spironolactone is beneficial in heart failure, a renaissance for aldosterone receptor antagonism has begun, including the development of new compounds such as eplerenone (Figure 1). Furthermore, there is a good body of evidence for a link between aldosterone and ET, as ET-1 regulates aldosterone secretion from adrenal cells in both healthy individuals and patients with congestive heart failure.
Because alterations in GA-induced hypertension are mineralocorticoid receptor mediated, aldosterone receptor antagonism represents a logical therapeutic approach. Hence, the aim of the present study was to elucidate the impact of the established aldosterone receptor antagonist spironolactone in comparison with the recently developed compound eplerenone on endothelial function in GA-induced hypertension.

Methods

Animals
Male Wistar-Kyoto rats (mean weight 250 g, 13 weeks old) were obtained from RCC, Fueflinsdorf, Switzerland. GA or vehicle was supplemented to the drinking water (3 g/L) over a period of 21 days. From days 8 to 21, the rats were randomly assigned to treatment with either the orally available traditional aldosterone receptor antagonist spironolactone (5.8±0.6 mg · kg⁻¹ · d⁻¹), the novel aldosterone receptor antagonist eplerenone (182±13 mg · kg⁻¹ · d⁻¹), or placebo administered with their chow (n=7 animals per group). Systolic arterial blood pressure and heart rate were measured by the tail-cuff method with a pulse transducer (model LE 5000, Letica). The study design and the experimental protocols were approved by the institutional animal care committee (Komission für Tierversuche des Kantons Zürich, Switzerland) and are in accordance with the American Heart Association guidelines for research animal use.

Tissue Harvesting
Animals were anesthetized with pentobarbital (50 mg/kg IP) after 3 weeks of treatment, and blood samples were collected through puncture of the right vena cava. The thoracic aorta was removed and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution and centrifuged at 5000 rpm at 4°C. The pellet was resuspended in Tris-SDS buffer (Tris-HCl 0.0635 mol/L, pH 6.8, 2% SDS), boiled for 1 minute, and then subjected to 8% SDS–polyacrylamide gel electrophoresis. Equal amounts of protein were used for electrophoresis, and comigrating bands were detected by silver staining. The protein was quantified by reverse-phase high-performance liquid chromatography on an ECE250/4.5 Supersil 100 RP column (Machery & Nagel) with use of a semidyren transfer unit. The membranes were subsequently blocked by using 2% skim milk in phosphate-buffered saline–Tween buffer (0.1% Tween 20, pH 7.5) for 1 hour and incubated with a 1:1000 dilution of rabbit anti–endothelial NO synthase (eNOS) 3 IgG antibody (Santa Cruz Biotechnology Inc).

Using Bio-Rad VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA), the immunoreactive bands were detected by an enhanced chemiluminescence detection system (Amersham). Optical density of eNOS protein bands was detected by NIH imaging software, and optical density in control rats was regarded as 100%.

Calculations and Statistical Analysis
Relaxations to agonists in isolated arteries are given as percent preconstriction in rings that were precontracted with NE to ~70% of the contraction induced by KCl (100 mmol/L). Contractions are expressed as percentages of 100 mmol/L KCl–induced contractions, which were obtained at the beginning of each experiment. Results are presented as mean±SEM. In all experiments, n equals the number of rats per experiment. For statistical analysis, the sensitivity of the vessels to the drugs was expressed as the negative logarithm of the concentration that caused half-maximal relaxation or contraction (pD₂). Maximal relaxation (expressed as a percentage of preconstriction) or contraction was determined for each concentration–response curve by nonlinear regression analysis with the use of MatLab software. For comparison between 2 values, the unpaired Student’s t test or the nonparametric Mann-Whitney test was used when appropriate. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni’s correction. Pearson’s correlation coefficients were calculated by linear regression. A value of P<0.05 was considered significant.

Results

Body Weight, Systolic Blood Pressure, and Heart Rate
Body, kidney, liver, and heart weights did not differ between groups either before or after GA treatment nor during treatment with the aldosterone receptor antagonists. Systolic blood pressure was increased after GA treatment and returned to baseline on administration of either aldosterone receptor antagonist, whereas heart rate was unaltered (Figure 2).

Figure 1. Structural formula of eplerenone.

which were purchased from Novabiochem/Calbiochem AG. After the experiments, vessel rings were blotted dry and weighed.

Tissue ET-1 Levels
Aortic tissue and samples of renal medulla and cortex were snap-frozen in LN₂ and kept at −80°C until assayed. ET-1 was extracted as previously described.

Nitrite and Nitrate Tissue Levels
Homogenized aortic tissue was diluted 1:4 in sterile, distilled water and deproteinized (Millipore 10 ultrafiltration membranes). Nitrites and nitrates, the stable end products of NO oxidation, were quantified by reverse-phase high-performance liquid chromatography on an ECE250/4.5 Supersil 100 RP column (Machery & Nagel) by using ion-pairing chromatography with photodiode-array detection at 210, 215, and 220 nm as described before.

Aortic Endothelial NO Synthase Protein Content
After incubation with collagenase for 15 minutes at 37°C, the aortic endothelium was scraped off with a surgical blade. Cells were suspended in Krebs-Ringer bicarbonate solution and centrifuged at 5000 rpm at 4°C. The pellet was resuspended in Tris-SDS buffer (Tris-HCl 0.0635 mol/L, pH 6.8, 2% SDS), boiled for 1 minute, and then subjected to 8% SDS–polyacrylamide gel electrophoresis. Equal amounts of protein were used for electrophoresis, and comigrating bands were confirmed by silver staining. The protein was then transferred onto ImmobilonTM-P filter papers (Millipore AG) with use of a semidyren transfer unit. The membranes were subsequently blocked by using 2% skim milk in phosphate-buffered saline–Tween buffer (0.1% Tween 20, pH 7.5) for 1 hour and incubated with a 1:1000 dilution of rabbit anti–endothelial NO synthase (eNOS) 3 IgG antibody (Santa Cruz Biotechnology Inc). Immunoreactive bands were detected by an enhanced chemiluminescence system (Amersham). Optical density of eNOS protein bands was detected by NIH imaging software, and optical density in control rats was regarded as 100%.
Alterations in the ET System

**ET-1 Tissue Concentrations**
Aortic tissue levels of ET-1 were elevated (Figure 3; \( P < 0.05 \) vs controls) and were normalized by either aldosterone receptor antagonist (Figure 3; \( P < 0.05 \) versus GA).

**Vascular Reactivity to ET-1**
ET-1–induced concentration-dependent contractions were enhanced after chronic GA feeding and were ameliorated by both aldosterone receptor antagonists (Figure 4; \( P < 0.05 \) versus GA-fed rats).

**Contractions to NE and KCl**
Concentration-dependent contractions to NE were unaffected by feeding with GA and by treatment with aldosterone receptor antagonists (the Table). Contractile responses to 100 mmol/L KCl did not differ between the groups (data not shown).

Alterations of the NO System

**eNOS Protein Levels**
Western blot analysis revealed a decrease in aortic eNOS protein levels in 11\( \beta \)-HSD–deficient rats. Aortic eNOS protein levels were normalized in a similar fashion by both aldosterone receptor antagonists (Figure 5).

**Nitrate Tissue Concentrations**
Aortic tissue nitrate concentrations, the stable end product of NO metabolism, were reduced by 50% by GA feeding and were normalized by both aldosterone receptor antagonists (Figure 6; \( P < 0.05 \)).

**NO-Mediated Endothelial Function**
Endothelium-dependent relaxations of aortic rings to ACh were blunted after GA treatment and were normalized by both aldosterone receptor antagonists (Figure 7). Relaxations to ACh were blocked by \( \text{N}\text{G}-\text{nitro-L-arginine methyl ester} \) and unaffected by superoxide dismutase or indomethacin (data not shown). Endothelium-independent relaxations to SNP were comparable in all groups (the Table).

**Discussion**
These data demonstrate for the first time that in 11\( \beta \)-HSD hypertension, chronic aldosterone receptor blockade by either spironolactone or the novel compound eplerenone not only normalizes blood pressure but also prevents upregulation of vascular ET-1 and endothelial dysfunction, as reflected by reduced eNOS expression, reduced NO production, and impaired endothelium-dependent relaxations. The activity of 11\( \beta \)-HSD, which regulates corticosterone (cortisol in humans) access to its receptors and prevents inappropriate

**Maximal Response and Sensitivity (pD\( \text{2} \)) of Concentration-Dependent Contractions to NE and Relaxations to SNP in Aortic Rings of GA-Fed Rats**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Control</th>
<th>GA + Spironolactone</th>
<th>GA + Eplerenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max, %</td>
<td>121±9</td>
<td>122±11</td>
<td>125±7</td>
</tr>
<tr>
<td>pD( \text{2} )</td>
<td>8.1±0.2</td>
<td>8.2±0.2</td>
<td>8.2±0.1</td>
</tr>
<tr>
<td>SNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max, %</td>
<td>108±5</td>
<td>110±6</td>
<td>106±4</td>
</tr>
<tr>
<td>pD( \text{2} )</td>
<td>9.2±0.2</td>
<td>9.1±0.1</td>
<td>9.4±0.2</td>
</tr>
</tbody>
</table>

Max (%) indicates maximal response; pD\( \text{2} \), \(-\log\) concentration in (mol/L) of substance causing half-maximal response. Data are given as mean±SEM (n=7 rats in each group).

No differences between groups were statistically significant (ANOVA and Bonferroni's correction).

Figure 2. Systolic blood pressure on treatment with different regimens. Blood pressure increased after 1 week of GA feeding compared with placebo and returned to baseline after treatment with the aldosterone receptor antagonists spironolactone and eplerenone. Heart rate remained unchanged. *\( P < 0.05 \) vs control animals. Results are given as mean±SEM (n=7 animals per group).

Figure 3. Vascular ET-1 content. Aortic tissue concentrations of ET-1 were augmented after chronic feeding of GA and were normalized by aldosterone receptor antagonism, *\( P < 0.05 \) vs controls. Results are given as mean±SEM (n=6 animals per group). Spiro indicates spironolactone; EP, eplerenone.

Figure 4. Vascular reactivity to ET-1. In the aortas of GA-fed rats, ET-1–induced contractions were enhanced by feeding of GA and were normalized after aldosterone antagonist treatment with either spironolactone or eplerenone. *\( P < 0.05 \) vs controls. Results are given as mean±SEM (n=7 animals per group).

Figure 5. Protein levels were normalized in a similar fashion by both aldosterone receptor antagonists.

Figure 6. Nitrate tissue concentrations were reduced by 50% by GA feeding and were normalized by both aldosterone receptor antagonists (Figure 6; \( P < 0.05 \)).

Figure 7. NO-mediated endothelial function. Endothelium-dependent relaxations of aortic rings to ACh were blunted after GA treatment and were normalized by both aldosterone receptor antagonists (Figure 7). Relaxations to ACh were blocked by \( \text{N}\text{G}-\text{nitro-L-arginine methyl ester} \) and unaffected by superoxide dismutase or indomethacin (data not shown). Endothelium-independent relaxations to SNP were comparable in all groups (the Table).
occupancy of type I mineralocorticoid receptors by glucocorticoids, is decreased in patients with liquorice-induced as well as salt-sensitive hypertension. In dexamethasone hypertension, the glucocorticoid-induced increase in vascular reactivity precedes the rise in blood pressure, independent of renal mineralocorticoid receptor activation, suggesting that 11β-HSD, which is expressed in vascular smooth muscle and endothelial cells, may play a role in mediating changes in vascular function. Indeed, we provide here the first evidence that impaired NO formation as well as activation of the vascular ET-1 system contributes to the development of hypertension induced by administration of the 11β-HSD inhibitor GA.

Activation of the ET system has previously been shown in human hypertension, and indirect evidence suggests that increased vascular ET-1 content may be related to hypertensive end-organ damage and remodeling. In the present study, aortic protein levels of mature ET-1 were enhanced after GA treatment. Although we previously showed simultaneous activation of the vascular ET system in the aorta and mesenteric resistance arteries in another model of hypertension, the glucocorticoid-induced increase in vascular reactivity precedes the rise in blood pressure, independent of renal mineralocorticoid receptor activation, suggesting that 11β-HSD, which is expressed in vascular smooth muscle and endothelial cells, may play a role in mediating changes in vascular function. Indeed, we provide here the first evidence that impaired NO formation as well as activation of the vascular ET-1 system contributes to the development of hypertension induced by administration of the 11β-HSD inhibitor GA.

Figure 5. eNOS protein levels. Western blot analysis revealed that aortic eNOS protein levels decreased in rats on GA feeding. Most interestingly, eNOS protein levels were normalized after aldosterone receptor antagonist treatment. *P<0.05 vs controls. Results are given as mean±SEM (n=6 animals per group). Spiro indicates spironolactone; EP, eplerenone.

Figure 6. Nitrite and nitrate tissue concentrations. After 11β-HSD inhibition with GA, aortic tissue nitrate content was reduced by and normalized after aldosterone receptor blockade. *P<0.05 vs controls; †P<0.05 vs GA feeding. Results are given as mean±SEM (n=6 animals per group). Spiro indicates spironolactone; EP, eplerenone.

down products of NO, and endothelium-dependent relaxation to ACh were markedly reduced. In contrast to a general reduction in NO bioavailability, nitrate levels in the renal medulla remained stable, which may be ascribed to local regulatory processes.

Because relaxations to ACh were completely blocked by N-nitro-L-arginine methyl ester and unaffected by superoxide dismutase, these data are compatible with impairment of the endothelial L-arginine/NO pathway in GA-induced hypertension. Therefore, hypertension induced by 11β-HSD inhibition may involve not only glucocorticoid and mineralocorticoid receptor- mediated modulation of renal function but also marked alterations of the cardiovascular ET-1 and NO systems.

To further elucidate the impact of aldosterone receptor antagonism on GA-induced alterations, the animals were treated with either the established aldosterone receptor antagonist spironolactone or the recently developed aldosterone receptor antagonist eplerenone. Both compounds were equally effective in the treatment of 11β-HSD-deficient hypertension: they normalized blood pressure and reversed activation of the vascular ET-1 system as well as NO bioavailability. The impact of aldosterone receptor antagonism in the treatment of heart failure and hypertension, in particular in states of sodium retention, has been described. In this study, we provide the first evidence of full reversibility of the vascular changes that are induced by the 11β-HSD inhibitor GA by chronic treatment with either spironolactone or eplerenone.

Recent evidence indicates that elevated aldosterone levels play an important role in the development and progression of myocardial fibrosis and hypertrophy in congestive heart failure, and that sodium retention is not the primary mechanism of cortisol-induced hypertension. These findings may be particularly relevant to the present study, because the current data suggest that reduced activity of 11β-HSD, due to the generation of endogenous inhibitors or gene defects, could represent an important additional aldosterone-independent mechanism through which inappropriate access of glucocorticoids to vascular receptors may influence vascular tone. The fact that aldosterone receptor antagonism has recently been proved to decrease mortality in severe heart
failure\(^1\) emphasizes the importance of this therapeutic principle and may further contribute to attempts in evaluation of its underlying mechanisms.

In conclusion, this study demonstrates that the 11β-HSD inhibitor GA mediates the development of hypertension via decreased bioavailability of NO and activation of the vascular ET-1 system. Because both aldosterone receptor antagonists, spironolactone and eplerenone, normalize blood pressure, prevent upregulation of vascular ET-1, and restore NO-mediated endothelial dysfunction, aldosterone receptor antagonism may provide a novel approach in the treatment of cardiovascular disease associated with reduced activity of 11β-HSD.

Acknowledgments
The study was supported by Swiss National Science Foundation grants 32-49648.96 (to S.S.), 32-57365.99 (to S.S. and F.R.); and Deutscher Akademischer Austauschdienst (to T.Q.).

References
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Hypertension. 2001;37:801-805
doi: 10.1161/01.HYP.37.2.801

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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