StAR expression and the long-term aldosterone response to high-potassium diet in Wistar-Kyoto and spontaneously hypertensive rats

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Peters B, Teubner P, Clausmeyer S, Puschner T, Maser-Gluth C, Wrede HJ, Kränzlin B, Peters J. StAR expression and the long-term aldosterone response to high-potassium diet in Wistar-Kyoto and spontaneously hypertensive rats. Am J Physiol Endocrinol Metab 292: E16–E23, 2007. First published August 1, 2006; doi:10.1152/ajpendo.00454.2005.—ANG II and potassium are known to increase steroidogenic acute regulatory protein (StAR) levels. However, a corresponding increase in StAR mRNA levels has so far been observed only in response to ANG II. We therefore studied the regulation of adrenal StAR mRNA expression in the context of dietary potassium-stimulated aldosterone production. Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were fed a diet containing either 1 or 4% KCl for 5 days. The high-potassium diet increased StAR mRNA levels within the zona glomerulosa in both strains, as demonstrated by in situ hybridization. However, aldosterone production increased in WKY but not in SHR (WKY: from 22.8 to 4.8 to 137 ± 25 ng/100 ml, P < 0.001, vs. SHR: from 29 ± 3.8 to 51 ± 10.2 ng/100 ml, not significant). This increase was associated with an increase in Cyp11b2 mRNA levels in WKY (3-fold; P < 0.001) but not in SHR. In both strains, the 4% KCl diet was associated with increased plasma renin-independent aldosterone production, as indicated by the marked increase of the aldosterone-to-renin ratios (from 1.4 ± 0.3 to 9 ± 3 in WKY and from 3 ± 1 to 14 ± 5 in SHR; P < 0.002). We conclude that an increase of StAR mRNA levels within the outer cortex is involved in the long-term adrenal response to potassium. This increase alone is not sufficient to increase aldosterone production in the presence of normal Cyp11b2 mRNA levels.

Cyp11b2; zona glomerulosa; steroidogenic acute regulatory protein

ACUTE REGULATION of aldosterone production depends on the rapid translocation of cholesterol to the inner mitochondrial membrane (21). Here, important steroidogenic steps take place, such as the conversion of cholesterol to pregnenolone by Cyp450scc. The translocation of cholesterol is mediated by a factor termed steroidogenic acute regulatory protein (StAR; see Refs. 7 and 32). The role of StAR in the rapid steroidogenic response has been demonstrated in glucocorticoid-producing cells (20). Interestingly, it has been shown that the rapid increase in cholesterol translocation (10- to 100-fold within min) requires newly produced and modified StAR rather than modification of existing StAR protein (4). Because the existence of StAR mRNA is a prerequisite for a rapid increase of de novo synthesis of StAR, regulation of StAR transcript levels may also be involved in the long-term regulation of steroid production.

Steroidogenic cells store minimal amounts of hormone; therefore, hormone levels are regulated primarily at the level of synthesis. Expression of the late-step enzymes of steroidogenesis is zone specific. Cells of the zona glomerulosa express Cyp11b2, producing aldosterone, whereas fasciculata cells express Cyp11b1, producing corticosterone (28). The long-term regulation of aldosterone by potassium or ANG II involves a stimulation of expression of Cyp11b2 (16, 27, 31) and depends on both calcium influx and intact protein synthesis (39, and for a review see Ref. 32). An increase of aldosterone production is further accomplished by an increase in the numbers of aldosterone-producing cells (29). It has been shown that the major regulators of aldosterone production, namely ANG II and potassium, increase StAR protein levels in H295R cells (8). However, in these cells only ANG II, but not potassium, increased StAR mRNA levels as well. This was surprising, since both factors are known to increase intracellular calcium levels, which are thought to mediate the effects on aldosterone production. However, one cannot be sure that a cell line completely represents in vivo physiology. For instance, in NCI-H295R cells, AT1 receptor levels decrease in response to potassium, but, in vivo, AT1 receptor levels increase under potassium load (3). Hence, the response of aldosterone-producing cells to potassium in vivo, in terms of StAR expression, still remains to be investigated.

We recently demonstrated that StAR mRNA is heterogeneously expressed within the rat adrenal gland in vivo (29). StAR mRNA expression is prominent within the zona fasciculata and reticularis. In contrast, basal expression is low within the outer cortex. This area consists functionally of the zona glomerulosa, producing aldosterone, and the undifferentiated cell zone (22). The undifferentiated cell zone represents a steroidogenic inactive pool of cells, which produces neither glucocorticoid nor mineralocorticoid hormones under basal conditions, but which can be recruited to produce steroids (25, 29).

After bilateral nephrectomy in rats, StAR mRNA levels increased in the outer cortex, indicating a role for StAR expression in the long-term regulation of aldosterone production (29). Furthermore, since bilateral nephrectomy is known to increase plasma potassium levels, we wished to address three questions in the present study. First, does potassium regulate StAR expression in the outer adrenal cortex in vivo? Second,
which cell type of the adrenal cortex responds to potassium in terms of StAR expression? Third, is the adrenal response to potassium identical in terms of the regulation of Cyp11b2 and StAR mRNA expression?

To answer these questions, we investigated the effect of a high-potassium diet on the adrenal cortex in two rat strains, namely the Wistar-Kyoto rat (WKY) and the spontaneously hypertensive rat (SHR).

MATERIALS AND METHODS

All animal experiments were conducted in accordance with federal and local laws and institutional regulations. Eight- to 10-week-old age-matched male WKY rats (n = 16) and SHR rats (n = 16) were housed under alternating 12:12-h light-dark cycles at a controlled temperature between 20 and 22°C. Both strains were divided into two groups of eight animals. The rats had free access to tap water and received either a normal diet of standard laboratory chow containing 1% KCl and 0.25% NaCl or a high-potassium diet containing 4% KCl and 0.25% NaCl. After 5 days, animals were anesthetized with ketamine-rompun (75 mg/kg body wt and 6 mg/kg, respectively; Sigma-Aldrich Chemie, Schnelldorf, Germany). Blood samples were taken from the abdominal aorta. One adrenal gland was removed and manually separated into capsular tissue, containing mainly zona glomerulosa and cells of the undifferentiated zone, and decapsular tissue containing zona fasciculata and reticularis according to standard protocols (13). Fixation of the remaining adrenal gland for nonradioactive in situ hybridization was performed by retrograde perfusion from the abdominal aorta with 2% freshly prepared paraformaldehyde in PBS, pH 7.4, for 90 s at a pressure of 800 mosmol/kgH2O for another 3 min at the same pressure level. Adrenal glands were removed, mounted quickly on MATERIALS AND METHODS

MATERIALS AND METHODS

Table 1. Effect of a 5-day high-potassium diet on plasma aldosterone and renin concentrations, serum electrolyte levels, and body weight gain

<table>
<thead>
<tr>
<th></th>
<th>1% KCl</th>
<th>4% KCl</th>
<th>1% KCl</th>
<th>4% KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC, ng ANG·ml⁻¹·h⁻¹</td>
<td>19±4</td>
<td>23±2.7</td>
<td>12±2</td>
<td>6±1§</td>
</tr>
<tr>
<td>K⁺, mmol/l</td>
<td>4.3±0.1</td>
<td>4.9±0.3</td>
<td>4.5±0.2</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>Na⁺, mmol/l</td>
<td>139±2</td>
<td>134±2</td>
<td>136±3</td>
<td>137±2</td>
</tr>
<tr>
<td>Weight gain, %</td>
<td>8±1</td>
<td>5±1</td>
<td>6±2</td>
<td>5±1</td>
</tr>
<tr>
<td>No. of Cyp11b2 mRNA positive cells</td>
<td>120±72</td>
<td>510±156§</td>
<td>112±55</td>
<td>268±105§</td>
</tr>
</tbody>
</table>

WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; PRC, plasma renin concentration. Data represent means ± SE of n = 8 rats in each group. *P < 0.001 between strains on same diet; §P < 0.05 (PRC); P < 0.01 (number of Cyp11b2-positive cells in SHR); P < 0.001 (number of positive cells in WKY) between diets in the same strain.

Plasma and serum parameters. Aldosterone concentrations (PAC) and renin concentrations (PRC) were measured from EDTA-plasma described previously (30). Serum sodium and potassium were measured by means of flame photometry.

In situ hybridization. Nonradioactive in situ hybridization to detect StAR, Cyp11b2, and Cyp11b1 mRNA was performed as previously described (29). Full-length StAR cDNA was obtained from rat adrenal cDNA. Because the published cDNA sequences of rat StAR differ in their 5’-region (19, 23), we used an adaptor-ligated cDNA, prepared from rat adrenal poly(A)+ RNA using the Marathon cDNA Amplification Kit (Clontech, Heidelberg, Germany) according to the manufacturer’s instructions. First- and second-strand cDNA synthesis was followed by the ligation of an adaptor to both cDNA ends. Amplification of the StAR cDNA was performed using the Expand Long Template PCR system (Roche, Penzberg, Germany), with a specific antisense primer (5’-GTGCAGGTAGATGGTGGGCG-3’), hybridizing immediately downstream of the 3’-end of the StAR coding sequence and a sense primer hybridizing to the adaptor. The resultant fragment was cloned into pBluescript and verified by sequencing. A specific probe for detecting the Cyp11b2 transcript was also generated by PCR from the full-length cDNA, which had been prepared from the rat adrenal cDNA. The PCR used primers 5’-GGGATGTGTCATCCTTGAGTATG-3’ (sense) and 5’-GATTGCTGTCGTGCTGTAAC-3’ (antisense) to yield a fragment of 185 bp, corresponding to nucleotides 779–964 of Cyp11b2 (24). The highest sequence dissimilarity to Cyp11b1 and to Cyp11b3 is found in this region, with a homology of <70%. Amplification was performed with Pwo DNA polymerase (Roche), and the resulting fragment was cloned into pBluescript and sequenced.

DIGI-11-UTP-labeled sense and antisense riboprobes were synthesized from the linearized plasmids using T3 and T7 RNA polymerases, respectively. In situ hybridization was carried out as previously described (29). Cryostat sections (6 μm thick) were transferred onto silane-coated glass slides (Sigma-Aldrich Chemie). Sections were postfixed in 4% paraformaldehyde (in PBS, pH 7.4) for 20 min, rinsed three times in PBS, and washed in diethyl pyrocarbonate-treated bidistilled water for 10 min. A mild deproteinization step was performed by immersing slides in 0.1 M HCl for 10 min, followed by two short rinses (5 min each) in PBS. Slides were then acetylated for digestion.
Table 2. Effect of a 5 day high-potassium diet on the levels of Cyp11b2 and StAR mRNA

<table>
<thead>
<tr>
<th></th>
<th>Capsular Adrenal Tissue</th>
<th>Decapsular Adrenal Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
</tr>
<tr>
<td>Cyp11b2</td>
<td>290.4 ± 106*</td>
<td>88.2 ± 33</td>
</tr>
<tr>
<td>StAR</td>
<td>79 ± 11.7</td>
<td>88.2 ± 24</td>
</tr>
</tbody>
</table>

Values are means ± SE of n = 8 rats in each group. StAR, steroidogenic acute regulatory protein. Data represent transcript levels in the indicated adrenal tissue after a high-potassium diet and are given as %control diet (control diet = 100%). Data were obtained using a light cycler. *P < 0.05 between diets in the same strain; †transcript level below quantification limit.

Effect of diet on body weight, serum potassium, PRC, and PAC. The acceptance of the high- and normal-potassium diets was monitored by weight gain. Weight gain during the 5-day observation period, although slightly lower in rats on the high-potassium diet, was not significantly different between WKY and SHR (Table 1). Serum potassium and sodium levels were not significantly different between WKY and SHR on either normal or high-potassium diets (Table 1).

Basal PAC were not different between WKY and SHR (Fig. 1). However, the increase of PAC in animals on the high-potassium diet significantly differed between the strains (P < 0.004). PAC was fivefold elevated in WKY, but not in SHR, on the high-potassium diet when compared with the normal-potassium diet (Fig. 1). PRC was not significantly different between WKY and SHR on the normal diet, although the mean PRC value was lower in SHR (Table 1). The high-potassium diet selectively decreased PRC in SHR (P < 0.03) but not in WKY. The PRC was about fourfold lower in SHR than in WKY on the high-potassium diet (Table 1). Although the high-potassium diet did not elevate PAC in SHR, a prominent increase in the PAC-to-PRC ratio was observed with the high-potassium diet in both strains with no differences in potassium responses between the strains (Fig. 1).

Expression of Cyp11b2 and StAR mRNA in adrenal capsule as measured by real-time RT-PCR. There was no difference in basal levels of Cyp11b2 mRNA between WKY and SHR in
adrenal capsular tissue, which represents the outer cortex. RT-PCR revealed a different response to potassium between the strains \((P < 0.002)\): Cyp11b2 mRNA was increased by the high-potassium diet in WKY but not in SHR (Table 2). In contrast, according to the RT-PCR, the high-potassium diet apparently had no effect on StAR mRNA expression in the capsular tissue of both strains (Table 2). However, the high-potassium diet decreased the number of fasciculata cells in the capsular tissue, as indicated by the decrease in Cyp11b1 mRNA levels in the capsules (Fig. 2). Although StAR mRNA levels in the capsule did not change with high-potassium intake, there were fewer fasciculata cells (as indicated by the decrease in Cyp11b1 mRNA levels) and more glomerulosa cells (as indicated by the increase in Cyp11b2 mRNA levels) in the capsule in response to potassium. We previously demonstrated that the fasciculata cells contain much higher levels of StAR mRNA than the glomerulosa and cells of the undifferentiated zone (29). Because the high-potassium diet had no effect on StAR expression in the decapsular tissue (Table 2), the different proportion of fasciculata cells in the capsular tissue may thus have led to a severalfold overestimation of glomerulosa StAR mRNA expression in rats on control diets, masking an increase of glomerulosa StAR mRNA.

**Expression of Cyp11b2 and StAR mRNA in the adrenal cortex analyzed by means of nonradioactive in situ hybridization.** Because it was not possible to quantify the expression levels of Cyp11b2 and StAR correctly by means of real-time RT-PCR of manually separated homogenized tissue, we next investigated the expression levels by means of nonradioactive in situ hybridization.

Distribution patterns of positive signals were specific for each antisense probe used (Fig. 3), as described previously (29). Sense probes did not produce any detectable signal (Fig. 3, E, G, and H). No difference in the basal expression of Cyp11b2 in WKY and SHR was observed. The location and the number of cells expressing aldosterone synthase was similar (Table 1).
The high-potassium diet increased the number of aldosterone-producing cells in both strains, as indicated by the expression of Cyp11b2. The increase was two times higher in WKY than in SHR (Table 1 and Fig. 4), indicating less growth of the zona glomerulosa in SHR. In rats on the high-potassium diet, still some cells of the zona glomerulosa and the cell layers of the undifferentiated zone did not express Cyp11b2 (Fig. 5). This was true for the outer cortex from both strains.

StAR expression was detected mainly in the zona fasciculata and reticularis of rats on the control diets, as expected from previous results (Fig. 5, A and D). The high-potassium diet increased the expression of StAR prominently in the zona glomerulosa and in the undifferentiated cell zone (Fig. 5, B and E). There was no difference in the expression pattern between WKY and SHR in basal or potassium-induced expression of StAR.

**DISCUSSION**

The mineralocorticoid aldosterone promotes hypertension, stroke, cardiac fibrosis, ventricular hypertrophy, and myocardial necrosis (for review, see Ref. 35). The characterization of the regulation of aldosterone production is therefore of crucial importance for the understanding of the pathophysiology of cardiovascular diseases. Steroidogenic cells store minimal amounts of hormone; therefore, regulation is primarily at the level of hormone synthesis (32). More recently, it became evident that the availability of substrate also plays an important role, at least as shown for progesterone and glucocorticoid synthesis. The StAR protein facilitates the transport of cholesterol to the inner mitochondrial membrane, where it is converted to pregnenolone (33). StAR protein expression has been shown to increase in primary cells of the outer adrenal cortex in vitro by the same factors that also regulate aldosterone production, such as potassium and ANG II (8). However, only ANG II, but not potassium, increased StAR mRNA levels in NCI-H295R cells in vitro. In the present study, we asked whether or not this also holds true in vivo and, furthermore, whether or not expression of Cyp11b2 and StAR was zone specifically and differentially regulated in vivo.

A note of caution is required regarding the method of choice to answer these questions. In this respect, one aim of this paper is methodological: the values obtained using real-time RT-PCR to assay homogenized tissue can be misleading if the relative contribution of different cell types within a given tissue changes between experimental samples. Cells expressing the genes of interest will then be diluted or enriched in the homogenate. The decapsulation of the adrenal gland for preparation of glomerulosa cells (13) is still a widely used method, even with preceding growth stimulatory interventions. Our study demonstrates that, after growth of the zona glomerulosa, the capsular portion of the adrenal contains fewer fasciculata cells than in control animals. Such an effect made it impossible...
to quantify StAR mRNA by RT-PCR in aldosterone-producing cells of capsular tissue cDNA. The effect can be explained by the prominent growth response of the outer adrenal cortex together with the fact that StAR is expressed to a higher degree in the outer fasciculata than the zona glomerulosa in the nonstimulated context. In this instance, the method of nonradioactive in situ hybridization is superior to the PCR analysis.

Based on the results obtained with in situ hybridization, this study shows that a potassium-rich diet, resulting in only transitory elevations of serum potassium levels, increases StAR mRNA prominently in the outer cortex, i.e., the zona glomerulosa and the undifferentiated cell zone in both rat strains. In contrast, after the potassium-rich diet, Cyp11b2 mRNA positive cells increased in the zona glomerulosa, but Cyp11b2 was still not expressed in the undifferentiated cell zone. This shows that there is differential regulation of the expression of StAR and Cyp11b2 in the outer cortex.

At present, we do not know whether the potassium-induced increase of StAR expression is a direct effect of extracellular potassium or a secondary event. Zona glomerulosa size and aldosterone production remain affected by a high-potassium diet even when plasma potassium levels are not affected. This holds true also for StAR expression in our study. Secondary events may involve, for example, activation of an intra-adrenal renin-angiotensin system. This idea is supported by evidence that adrenal renin expression increases in response to small and transient increases of serum potassium levels; this increase has been shown to contribute to the rise in aldosterone production (10, 26). Furthermore, also in human NCI-H295 cells, a local renin-angiotensin system is involved in potassium-induced aldosterone secretion (14).

Whereas in the zona glomerulosa of WKY the high-potassium diet increased StAR expression and Cyp11b2 and aldosterone production, in SHR the high-potassium diet increased StAR expression but had no considerable effect on aldosterone production, despite the fact that Cyp11b2 was expressed at similar or even slightly higher levels than on the control diet. The SHR is a model of genetic hypertension known to exhibit deranged aldosterone regulation in response to sodium (5). Reports about basal aldosterone levels in SHR are controversial. Some groups report that aldosterone levels are in the normal range (17), and others observed elevated levels exclusively in young SHR (7–11 wk old; see Ref. 12) or in old SHR (18–25 wk old) or decreased levels at all ages tested (18). Age, stress, strain differences, and sodium balance may explain the differences in results. In the present study, basal aldosterone

Fig. 5. Effect of a high-potassium diet on StAR expression in the adrenal gland. Nonradioactive in situ hybridization with a StAR-specific riboprobe on representative sections of adrenal glands (n = 8/group). A–C: WKY; D–F: SHR. StAR expression after normal potassium diet (A and D) compared with high-potassium diet for five days (B and E). C and F: cohybridization with Cyp11b1 and Cyp11b2 antisense probes counterstained with Meyers Hemalaun indicate cell layers without expression of these enzymes after high-potassium diet. Bar = 50 μm.
levels were not significantly different between SHR and WKY. Nevertheless, we demonstrate here that the adrenal response to potassium is markedly impaired in SHR. The most likely explanation for the blunted aldosterone response in SHR is the decrease of the circulating renin-angiotensin system. It has been reported that levels of active renin in plasma decrease with a high-potassium diet (10, 26). In our study, this response was stronger in SHR compared with WKY. There are two possible explanations for this: first, renal regulation of renin secretion in response to elevation of serum potassium levels is more sensitive in SHR; second, in SHR the aldosterone action on its targets (e.g., the sodium-potassium ATPase or the epithelial sodium channel ENaC) is more effective, leading to a higher renal sodium reabsorption, a condition known to inhibit renin release. It has just recently been shown that the expression of the ENaC is increased in SHR (17) so that less aldosterone is needed to control electrolyte levels.

Several studies in human and animal models of hypertension, including studies in SHR, have found a protective effect of a high-potassium diet on survival, stroke, kidney disease, and vascular oxidative stress (15, 37, 40). Although our short-term study was not designed to investigate the protective role of potassium on blood pressure or end organ damage, it may be noteworthy that in our study in SHR the high-potassium diet was accompanied by reduced circulating renin levels and a reduced increase of aldosterone concentrations. Both renin and aldosterone increase blood pressure and are known to induce, or at least aggravate, end organ damage (35). In this respect, the blunted aldosterone response to potassium in SHR may be regarded as protective. Thus, although we did not measure blood pressure in the present study, the decreased renin levels and diminished response of aldosterone levels to potassium in SHR may contribute to a drop in blood pressure observed with a high-potassium diet (36).

By including SHR in this study, we had no intention to describe yet another pathological response in a model of hypertension. Instead, comparison of the WKY and SHR strains gave us the opportunity to observe that an increase in StAR expression in the zona glomerulosa, despite unchanged or even slightly elevated levels of Cyp11b2 mRNA, will result in increased aldosterone production in vivo only in cooperation with other stimulators such as the circulating renin-angiotensin system. From these results, we conclude that an increase of StAR expression renders the cells of the glomerulosa more sensitive to rapid stimulation but appears not to be a factor for direct long-term regulation of aldosterone production. The aldosterone response to ANG II depends on the sodium balance (38). ANG II increases aldosterone release within minutes from adrenal cells in vitro. Under sodium depletion, the same amount of ANG II elicits a greater increase of aldosterone production than under sodium load (6). The reasons for this long-term modulation of adrenal sensitivity to rapid stimulation are complex. The activity of an intra-adrenal renin-angiotensin system (10, 26) and the regulation of angiotensin receptors (11) contribute to the adrenal sensitivity. After a high-potassium diet, cells obtained from adrenal capsules contain more aldosterone-producing glomerulosa and fewer fasciculata cells so that a greater proportion of the cells in the primary cultures respond to ANG II. Additionally, the increase of StAR expression renders the cells more capable of responding rapidly, with increased de novo synthesis of StAR protein and subsequent transport of cholesterol to the mitochondria.

In glucocorticoid-producing cells, StAR has been shown to mediate a rapid steroidogenic response. Furthermore, increased de novo synthesis of StAR, rather than preformed and stored StAR, was required for the effect (1, 4). Consequently, the increased StAR mRNA levels observed in our experiment are an important prerequisite for the cells of the outer cortex to be more responsive to stimulation.

In summary, in this study, we demonstrated that high potassium intake increases StAR mRNA levels in the outer adrenal cortex in vivo. The increase in StAR mRNA alone is not sufficient to increase aldosterone production. Other factors, such as the activity of the circulating or local renin-angiotensin systems or an increased expression of Cyp11b2, are needed as well. Compared with WKY, SHR respond equally well to potassium in terms of regulating capsular StAR expression, but the aldosterone response in SHR is markedly blunted and their plasma renin response is more sensitive.

ACKNOWLEDGMENTS

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