Local Renal Aldosterone System and Its Regulation by Salt, Diabetes, and Angiotensin II Type 1 Receptor

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Abstract—CYP11B2 is the enzyme responsible for aldosterone synthesis mainly in the adrenal gland. In this study, we hypothesized that CYP11B2 gene, protein, and aldosterone are produced locally in kidney and regulated by low salt intake, angiotensin II type 1 (AT1) receptor and insulin-deficient diabetes hyperglycemia. We used real-time RT-PCR, immunohistochemistry staining, and microdialysis techniques to monitor changes in renal CYP11B2 mRNA and protein and aldosterone production in normal, adrenalectomized, or streptozotocin-induced insulin-deficient diabetic hyperglycemic rats. In normal kidney, CYP11B2 mRNA and protein were localized mainly in the renal cortex and upregulated by angiotensin II and low salt intake. The angiotensin II effect was reversed by AT1 receptor blocker valsartan. Immunohistochemistry staining demonstrated presence of CYP11B2 in glomeruli. Although aldosterone was absent in plasma of adrenalectomized rats, it was present in renal interstitium and tissue. Diabetes increased renal cortical and total kidney CYP11B2 mRNA and protein. Lowering blood glucose with insulin decreased total renal CYP11B2 mRNA and protein. Despite lack of significant changes in blood glucose, valsartan treatment caused significant reduction in renal CYP11B2 mRNA and protein. In presence of diabetes, there was an increase in CYP11B2 immunostaining in glomeruli and proximal tubules. This expression was abrogated with insulin or valsartan treatment. These results demonstrate the presence of all components of local renal aldosterone system. This system is physiologically active because it is regulated by angiotensin II and low salt intake. In insulin-deficient diabetes hyperglycemia rat model, glucose, insulin, and AT1 receptor modulate CYP11B2 expression in the kidney. (Hypertension. 2005;46:584-590.)

Key Words: aldosterone ■ angiotensin ■ receptors, angiotensin II ■ diabetes mellitus ■ kidney

Aldosterone plays an important role in regulating renal fluid and electrolyte homeostasis and blood pressure regulation.1 Aldosterone secretion is regulated mainly by angiotensin II (Ang II), corticotropin, and potassium.2,3,4 The key enzyme for aldosterone synthesis is CYP11B2, a member of cytochrome P450 family with molecular weight 48.5 kDa. The CYP11B2 gene is ∼45 kb, contains 9 exons and is located on chromosome 8.5

Aldosterone stimulates cellular hypertrophy, matrix formation,6,7 and cell death.8 Although aldosterone can be produced in tissues other than the adrenal gland,9 its production in the kidney has never been reported. The demonstrated benefits of aldosterone receptor blockers in diabetic nephropathy,5,10–12 despite reported normal13–15 or low16–19 levels of plasma aldosterone, suggest that this hormone may be produced locally within the kidney. Thus, we hypothesized that there is a local renal aldosterone system. Identification of different components of an aldosterone system in the kidney would boost this hypothesis. In this study, we confirmed that CYP11B2 gene, protein, and aldosterone production are locally present in kidney and regulated by low salt intake, Ang II type 1 (AT1) receptor, and insulin-deficient diabetes hyperglycemia.

Methods

Animal Preparation

Study protocols were approved by the University of Virginia animal research committee. Sprague-Dawley rats (Harlan Teklad; Madison, Wis) weighing 245 to 255 g were used in this study. Rats were housed in a well-ventilated room (21 ± 1°C; 12-hour light/dark cycle). Animals were weighed at the end of each study week.

Adrenalectomy and Microdialysis Procedures

Bilateral adrenalectomy was performed according to previously published methods.20 A single microdialysis probe was inserted into left renal cortex as described previously.21 Each animal in the control group was instrumented by microdialysis probe but did not have adrenalectomy (sham). After adrenalectomy, rats received dexamethasone (Sigma; 250 mg/kg per day SC). On the fifth day after surgery, renal interstitial fluid (RIF) samples22 were collected from normal (n=6) and adrenalectomized (n=6) rats.

Salt Intake and Renal Aldosterone Synthase Expression

The effects of different levels of salt intake on the renal expression of aldosterone synthase were studied by placing animals for 1 week on low-salt (0.05% NaCl), normal-salt (0.5% NaCl), or high-salt (4% NaCl) diet (n=8 for each group).
Effects of Ang II and AT1 Receptor Blockade on Renal Aldosterone Synthase

Three groups of animals were implanted subcutaneously with Alzet Mini-Osmotic Pumps (Model 2001D; Direct Corporation) and filled with 0.9% NaCl (control group; n=8), Ang II (100 ng/kg per minute; n=8), or combined Ang II and valsartan (100 ng/kg per minute Ang II and 10 mg/kg per day valsartan; Novartis Pharmaceuticals; n=8).

Regulation of Renal Aldosterone Synthase Expression in Diabetic Insulin-Deficient Rat Model

Rats were divided randomly into control and diabetic groups (n=88 each group). Diabetes was induced by streptozotocin (STZ; 65 mg/kg IP). Studies were conducted for 8 weeks. Eight diabetic and 8 control animals were euthanized at the end of each week for a total study period of 8 weeks. During the fifth week of the study, 3 diabetic and 3 control groups (n=8 each) were treated with regular insulin (2 to 8 U SC twice daily) to maintain blood glucose range of 60 to 100 mg/dL, valsartan (10 mg/kg per day orally), or 0.9% NaCl (0.5 mL) daily for 1 week.

At the end of each experiment, the kidneys were removed under anesthesia and stored in −80°C for quantitative real-time RT-PCR and immunohistochemistry. In some animals, the adrenal glands were also harvested for immunohistochemistry as positive control for CYP11B2.

Quantitative Real-Time RT-PCR

After kidney removal, renal cortex and medulla were separated under sterile technique. The tissue was weighed promptly and homogenized on ice, and the total renal RNA was extracted using RNeasy kit (Qiagen). The quality of RNA was confirmed by ethidium bromide staining in 2% agarose gel. Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). Gene-specific primers for aldosterone synthase were designed using the Gene Bank. The exon–intron boundaries were determined using the University of California Santa Cruz Genome Bioinformatics site. The corresponding cDNA primers were selected from NM012538 and BC063166, the gene codes for rat CYP11B2 and β-actin sequences. The specificity of the primers was verified by melting curves (iCycler; Bio-Rad) and amplified product size using agarose gel electrophoresis. Quantitative real-time RT-PCR was performed using iCycler (Bio-Rad), and threshold cycle number was determined using iCycler software version 3.0 (Bio-Rad). Reactions were performed in triplicate, and threshold cycle numbers were averaged. Nontemplate control was used as negative control. Samples were calculated with normalization to β-actin or GAPDH. Fold downexpression or upexpression was calculated according to the formula $2^{-\Delta\Delta Ct}$, where $Rt$ is the threshold cycle number for the reference gene observed in the test sample, $Et$ is the threshold cycle number for the experimental gene observed in the test sample, $Rn$ is the threshold cycle number for the reference gene observed in the control sample, and $En$ is the threshold cycle number for the experimental gene observed in the test sample.

Primers

Primers were: (1) aldosterone synthase (CYP11B2): forward sequence: tgagaaccttgtgttccttg; reverse sequence: ggctccaaagctccttgcc; length 126 bp (reference gene NM012538); and (2) β-actin: forward sequence: aagctcatacagactcctctcttg; reverse sequence: accctcatagtgccagctcag; length 115 bp (reference gene BC063166).

Western Blot Analysis

Kidney tissue (n=8) was homogenized (crude homogenate) and then loaded (50 μg each) and separated on a 10% Criterion Tris-HCl gel (Bio-Rad), followed by blotting of the proteins onto nitrocellulose (povynilnyldene fluoride membrane; Bio-Rad). The blots were blocked with buffer (50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, 2% BSA, and 0.1% Tween 20) for 1 hour at room temperature. Then the blots were incubated with CYP11B2 (Chemicon International, Inc.) and β-actin (Santa Cruz Biotechnology) antibodies for 1 hour at room temperature. The blots were washed 6× with Tris-buffered saline (5 minutes) and then incubated for 1 hour at room temperature with secondary antibody conjugated with horseradish peroxidase (Bio-Rad). The blots were washed 6× with PBS (5 minutes), followed by detection of immunoreactive proteins with enhanced chemiluminescence (Amersham). The bands density was measured by GelDoc Imaging with Quantity One software system (Bio-Rad).

Aldosterone Synthase Immunohistochemistry

Immunohistochemistry was performed according to previously described methods. Brieﬂy, the frozen kidneys and adrenal glands were taken out from −80°C storage and placed in −18°C cryostat chamber for 1 hour. Each tissue sample was mounted with OCT (an embedding medium) on a microtome block with designed cutting orientation. The block was held firmly into cryostat microtome and 2- to 4-μm sections were cut. Sections were then picked up on histological slides and immediately dipped into Strept Tissue Fixative (NE68128; Streck Laboratories) at −4°C for 15 minutes, transferred into PBS buffer at −4°C for 30 minutes, and preabsorbed in 5% goat serum for 20 minutes before the immunostaining process. The immunostaining was performed by incubating with monoclonal CYP11B2 antibody (Chemicon International, Inc.) at −4°C overnight, followed by 1 hour of incubation with secondary antibody conjugated with biotin at room temperature (Sigma). Immunoreactive signal was detected with an avidin-biotin immunoperoxidase reaction (Sigma) and visualized by exposure to diaminobenzidine (Sigma). The nonspecific binding was controlled by using the mouse IgG isotype (Sigma) as a primary antibody for negative comparison. Positive immunostaining was observed under light microscopy.

Aldosterone Assay

Each sample of RIF (50 μL), plasma (50 μL), and homogenized kidney tissue (79 to 94 mg) was extracted by methylene chloride (1:2 volumes). After evaporation of methylene chloride using a vacuum centrifuge, the extract was dissolved into enzyme immunoassay buffer (1:1 volumes) and added to the assay wells (50 μL per well). Each well was added with aldosterone acetylcholinesterase inhibitor tracer (50 μL) and antiserum (50 μL) and incubated at 4°C overnight. Finally, the plate was developed by Ellman’s reagent and read at a wavelength 405 nm. The concentration was calculated according to the assay manufacture standard protocol (Cayman Chemical). The assay has 100% specificity for aldosterone and 0.11% cross-reactivity with corticosterone. The aldosterone assay standard curve detection range is 3.9 to 500 pg/mL with detection limit (80% B/Bo) 5 pg/mL.

Statistical Analysis

Comparisons between normal and diabetes groups were examined by 1-way ANOVA. Comparisons between treatment groups were examined using t test. Data were expressed as mean±SE. Statistical significance was identified at P<0.05.

Results

Renal Aldosterone Synthase mRNA and Protein Expression and Aldosterone Levels in Normal and Adrenalectomized Rats

Aldosterone synthase mRNA was detected in normal rat kidney, mainly in the renal cortex, and its level was ~3-fold higher compared with the renal medulla (Figure 1A). Low salt intake increased total renal aldosterone synthase mRNA level in normal kidney (Figure 1B). In contrast, high salt intake did not influence its expression in the kidney. Ang II increased renal aldosterone synthase mRNA, and this effect was reversed by AT1 receptor blockade with valsartan (Figure 1C). Five days after adrenalectomy, plasma aldosterone levels were undetectable (Figure 2A). However, renal cortical interstitial aldosterone levels were ~75% of the levels ob-
served in normal animals (Figure 2B). Similarly, total renal tissue aldosterone levels were detectable, although much less so than the observed levels in normal animals (Figure 2C).

Renal Aldosterone Synthase Expression in Normal and Diabetic Rats

Animals treated with STZ had a significant increase in blood glucose and failed to gain weight (Figure 3A and 3B). In diabetic animals, there was significant and progressive increase in renal aldosterone synthase mRNA (Figure 4A) compared with normal animals throughout the study. By the end of 1 week and 8 weeks after development of diabetes, there were 155% and 12-fold increase in total renal aldosterone synthase expression, respectively, compared with normal animals (Figure 4A). In diabetic animals, the increase in total renal aldosterone synthase mRNA was mainly attributable to its upregulation in the renal cortex (Figure 4B). After 6 weeks of diabetes development, there was $\approx700\%$ increase in renal cortical aldosterone synthase compared with nondiabetic animals (Figure 4B). Diabetes did not cause significant changes in this enzyme expression in renal medulla, and its levels were similar to those of nondiabetic animals (Figure 4B).

Regulation of Renal Aldosterone Synthase by Insulin and AT$_1$ Receptor

After 4 weeks of diabetes development, insulin treatment for 1 week caused significant reduction in blood glucose from $319\pm112$ mg/dL to $69\pm25$ mg/dL and decreased renal aldosterone synthase mRNA (Figure 5A) and protein (Figure 5B) by $\approx50\%$ and $40\%$, respectively. During valsartan treatment (week 5 of diabetes), there were no significant changes in blood glucose levels ($312\pm102$ mg/dL in diabetes group versus $289\pm95$ mg/dL in diabetes valsartan group). However, valsartan treatment caused an $\approx75\%$ and $70\%$ decrease in aldosterone synthase mRNA (Figure 5A) and protein (Figure 5B), respectively.

Localization of Aldosterone Synthase Protein in Normal and Diabetic Kidneys

Figure 6 shows representation of immunohistochemistry staining for aldosterone synthase in the adrenal gland in normal and diabetic rat kidneys at the end of 5 weeks of diabetes. Using adrenal glands as positive control, CYP11B2 immunostaining was present in the zona glomerulosa cells
(Figure 6B), although negative in the same anatomic region using mouse IgG (Figure 6A). Similarly, immunostaining for CYP11B2 with mouse IgG was negative in renal cortex (Figure 6C) and medulla (Figure 6D). In normal kidney, immunostaining for aldosterone synthase protein was detected mainly in glomeruli (Figure 6E and 6F). This staining was more visible in the nucleoli of renal glomeruli. Diabetes caused significant increase in this protein expression in renal cortex in glomeruli and proximal tubules (Figure 6G), but no increase was observed in renal medulla (Figure 6H). Treatment with insulin (Figure 6I) or valsartan (Figure 6K) reduced the immunohistochemistry staining of the aldosterone synthase in glomeruli and proximal tubules. Insulin (Figure 6J) or valsartan (Figure 6L) did not cause significant changes in aldosterone synthase protein in renal medulla.

**Discussion**

In this study, we report for the first time the local expression of the aldosterone synthase CYP11B2 and aldosterone production in the kidneys of normal rats. The presence of aldosterone in the kidney in adrenalectomized animals confirms the hypothesis that aldosterone is produced locally in the kidney. Previous studies suggested the presence of aldosterone synthase in the blood vessels, heart, and brain. Extra adrenal regulation of this enzyme is largely unknown. In our study, majority of aldosterone synthase expression is present in the renal cortex, mainly in the glomeruli. Although CYP11B2 is known to be a mitochondrial enzyme, in this study, we observed most of this enzyme immunostaining to be mainly localized in the nucleoli of renal glomeruli. We confirmed the specificity of the CYP11B2 antibody by...
Western blot analysis. The significance of the nuclear staining for CYP11B2 is not clear at this time.

In this study, 5 days after adrenalectomy and complete absence of aldosterone in the plasma, we were able to detect aldosterone in the kidney. Considering the short half-life of aldosterone in the circulation (<30 minutes), it is highly unlikely that renal aldosterone is picked up from the circulation. Low-sodium diet and Ang II administration increased renal aldosterone synthase expression. AT1 receptor blockade reversed the Ang II upregulation of aldosterone synthase in normal kidneys. These results confirm that AT1 receptor regulates renal production of this enzyme. The influence of low salt and Ang II on renal aldosterone synthase mimics their effects on this enzyme in the adrenal gland.

We also demonstrated the upregulation of renal aldosterone synthase in a model of insulin-deficient diabetes hyperglycemia. This increase in aldosterone synthase was partially reversed by the correction of elevated blood glucose levels by insulin, suggesting that type I diabetes hyperglycemia is a stimulator of this enzyme in the kidney. However, these animals may have other intermediate mechanisms related to hyperglycemia, such as osmotic diuresis, and changes in sodium balance that could modulate the expression of this enzyme.

In diabetes, plasma aldosterone levels have been reported to be low. Suppressed plasma aldosterone levels in diabetes in presence of increased aldosterone synthase expression in the kidney strongly suggest a paracrine role for aldosterone in the kidney. In this study, we report an early increase in renal aldosterone synthase gene expression in diabetes.

We have demonstrated that stimulated renal aldosterone synthase expression of insulin-deficient diabetic hyperglycemic rats can be suppressed significantly by the AT1 receptor blockade in the absence of effects on blood glucose. These results suggest that AT1 receptor is a positive regulator of
aldosterone synthase in normal and diabetic kidneys. This finding adds another function for the local renal renin angiotensin system and expands on our recent findings of increased renal production of Ang II in diabetes. The influence of Ang II on renal aldosterone synthase is confirmed by reduction of this enzyme expression during AT1 receptor blockade, despite continued elevation of blood glucose. Normalization of blood glucose with insulin treatment reduced aldosterone synthase expression. This effect could be attributable to reduction in renal Ang II production secondary to normalization of blood glucose, but we cannot rule out direct insulin effects on aldosterone synthase expression.

The expression of CYP11B2 mRNA and protein and the presence of aldosterone in normal and diabetic kidney provide evidence for the presence of a local renal autocrine or paracrine aldosterone system in which aldosterone production and its receptors exist in close proximity to influence renal structure and function. In addition, we proved that this system is functional because it is regulated by sodium, AT1 receptor, hyperglycemia, and insulin. At the present time, the role of renally produced aldosterone in normal kidney is not clear or whether the observed increase in renal aldosterone synthase initiates or contributes to the progression of diabetes complications in the kidney. Future studies are needed to evaluate the role of this enzyme in health and disease.

Perspectives

By demonstrating the presence of aldosterone synthase gene, protein, and their regulation, and the presence of aldosterone in the kidney of adrenalectomized animals, we firmly established the presence of local renal aldosterone system. Understanding the role of renally produced aldosterone may help elucidate some of the pathophysiological mechanisms involved in development of kidney diseases and could lead to development of new treatment strategies.

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References


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Figure 6. Representative immunohistochemical staining for aldosterone synthase of rat adrenal glands and kidneys. A and B represent adrenal gland; C, E, G, I, and K represent renal cortex; whereas D, F, H, J, and L represent renal medulla. Adrenal cortex (B) was positively immunostained with CYP11B2 monoclonal antibody but negatively with mouse IgG (negative control) in the sections of adrenal gland (A), renal cortex (C), and medulla (D). In renal cortex, CYP11B2 immunostaining was seen in normal rat (E), while significantly increased in diabetes (G). Insulin (I) or valsartan (K) decreased the CYP11B2 immunostaining in the diabetic animals. In renal medulla, there was only faint staining in the sections, but there were no significant differences between the normal (F) and diabetic animals (H). Insulin (J) or valsartan (L) did not affect the enzyme expression in this region.


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