Altered regulation of renal nitric oxide, atrial natriuretic peptide and cyclooxygenase systems in aldosterone escape in rats

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ABSTRACT

The present study was aimed to determine whether there is an altered role of local nitric oxide (NO), atrial natriuretic peptide (ANP) and cyclooxygenase (COX) systems in the kidney in association with the aldosterone escape. Male Sprague–Dawley rats were used. Aldosterone (200 μg/day) was infused through entire time course. The control group was kept on a low sodium diet (0.02 mEq/day), and the experimental group was supplied with a higher sodium diet (2.0 mEq/day). Four days after beginning the regimen, the kidneys were taken. The protein expression of NO synthase (NOS) and COX isoforms was determined by semiquantitative immunoblotting. The mRNA expression of components of ANP system was determined by real-time polymerase chain reaction. The activities of soluble and particulate guanylyl cyclases were determined by the amount of cGMP generated in responses to sodium nitroprusside and ANP, respectively. There developed aldosterone escape in the experimental group. Accordingly, the renal content and the urinary excretion of NO increased. The expression of nNOS was increased in the inner medulla. Neither the expression of eNOS nor that of iNOS was changed. The expression and the catalytic activity of soluble guanylyl cyclase remained unaltered. The mRNA expression of ANP was increased. Neither the expression of NPR-A or NPR-C nor the activity of particulate guanylyl cyclase was altered in the papilla. The protein expression of COX-2 was increased in the inner medulla, while that of COX-1 remained unchanged. In conclusion, the upregulation of nNOS, ANP, and COX-2 may be causally related with the aldosterone escape.

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1. Introduction

The regulatory mechanisms which are responsible for overriding the sodium-retaining effects of mineralocorticoids have been termed as “escape” [1,2]. The escape has been attributed to the expansion of the effective vascular volume induced by salt and water retention and the resultant increase in arterial pressure, leading to increased sodium excretion [3]. It has now become clear that there is an activation of various local natriuretic systems including nitric oxides (NO), atrial natriuretic peptides (ANP), and prostaglandins (PG) to facilitate the escape [4–6]. The net effect of NO system in the kidney is to promote natriuresis and diuresis, contributing to the renal adaptation to variations of salt intake [7]. An increased synthesis of NO stimulated by increased renal perfusion pressure has been shown to contribute to the escape from mineralocorticoids [4]. However, there may be a controversy concerning the causative role of NO in aldosterone escape [8]. Moreover, the regulation of different isoforms of NO synthases (NOS) has not been established in aldosterone escape. ANP may influence tubular sodium reabsorption through changes in medullary blood flow and sodium reabsorption [9]. An increase of plasma ANP levels was suggested as a mediator of aldosterone escape [5]. However, the natriuretic response to ANP does not correlate well with its plasma levels after acute intravenous saline infusion [10]. In addition, a substantial increase in circulating ANP for several days by chronic intravenous ANP infusion produces no changes or trivial increases in urinary sodium excretion [11]. These observations suggest a role of local ANP system, rather than circulating ANP, in renal handling of sodium homeostasis.

The urinary PG excretion is also increased along with the mineralocorticoid escape, and a transient sodium retention may be induced by indomethacin [12]. The synthesis of PG is catalyzed by cyclooxygenases (COX), two different isoforms which have been characterized [13]. Although COX-1 and COX-2 share similar enzymatic properties, they differ markedly with respect to cellular expression pattern and regulation [14,15]. They may be differentially regulated in aldosterone escape.
Table 1
Renal functional data (Protocol 1).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Escape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>258 ± 7</td>
<td>264 ± 21</td>
</tr>
<tr>
<td>UO (mL/d)</td>
<td>11.9 ± 1.3</td>
<td>11.6 ± 2.1</td>
</tr>
<tr>
<td>Pcr (mg/dL)</td>
<td>0.20 ± 0.05</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>Ccr (mg/min)</td>
<td>2.3 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Pnu (mEq/L)</td>
<td>142.6 ± 0.9</td>
<td>144.7 ± 0.8*</td>
</tr>
<tr>
<td>UNa × UO (mEq/d)</td>
<td>0.22 ± 0.02</td>
<td>1.82 ± 0.47</td>
</tr>
<tr>
<td>FENa (%)</td>
<td>0.05 ± 0.01</td>
<td>0.35 ± 0.11*</td>
</tr>
<tr>
<td>Plasma aldosterone (ng/dL)</td>
<td>878 ± 209</td>
<td>912 ± 153</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. These values are measured at the last day of experiments (on day 4). UO, urine output; Pcr, plasma creatinine; Ccr, creatinine clearance; Pnu, plasma sodium; UNa × UO, daily urinary sodium excretion; FENa, fractional excretion of sodium. *p<0.05 compared with control.

The present study was aimed to determine whether there is an altered regulation of local NO, ANP and COX systems in the kidney in association with the aldosterone escape.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 180–200 g were used. Rats were kept in a temperature-controlled room regulated on a 12:12-h light–dark cycle. The study was approved by the Ethics Committee of Chonnam National University Medical School.

In Protocol 1, the escape was induced as described by previous investigators [16]. The rats were anesthetized with ketamine (90 mg/kg)/xylazine (10 mg/kg) and subcutaneously implanted with osmotic minipumps (Alzet, Model 2ML2; Palo Alto, CA, USA) delivering 200 μg xylazine (10 mg/kg) per day of aldosterone (Sigma; St. Louis, MO, USA). The minipump was sustained throughout the experiment. The intake of sodium was initially maintained at a very low level (0.02 mEq/d) by ration feeding of low-Na food (Ziegler Brothers; Gardner, PA, USA) and water. After 3 days, they were then divided into 2 groups: the one was switched to a higher Na intake (2.0 mEq/d, NaCl-replete group). The kidneys were taken and processed for immunoblotting. The left kidney was removed and assayed for mRNA expression by real-time polymerase chain (PCR) reaction.

In Protocol 2, the experiment was made to investigate the effects of different levels of sodium intake, with no aldosterone infused. The intake of sodium was initially maintained at a very low level (0.02 mEq/d) for 4 days. Thereafter, they were divided into 2 groups: the one was kept on low sodium intake (0.02 mEq/d, NaCl-restricted group), and the other was switched to a higher sodium intake (2.0 mEq/d, NaCl-replete group). The kidneys were taken and processed for immunoblotting and real-time PCR.

2.2. Semiquantitative immunoblotting

The dissected cortex/OSOM, ISOM and inner medulla were homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM ethylenediaminetetraacetic acid (EDTA), 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), with pH 7.2. The homogenates were centrifuged at 10000 g for 15 min at 4 °C to remove whole cells, nuclei and mitochondria. The total protein concentration was measured by BCA assay kit (Pierce; Rockford, IL, USA). All samples were adjusted with isolation solution to reach the same final protein concentrations. They were then dissolved at 65 °C for 15 min in SDS-containing sample buffer and stored at −20 °C. To confirm equal loading of protein, an initial gel was stained with Coomassie blue. SDS-PAGE was performed on 9 or 12% polyacrylamide gels.

The proteins were transferred by gel electrophoresis (Bio-Rad, Mini Protein II; Hercules, CA, USA) onto nitrocellulose membranes (Amersham Pharmacia Biotech, Hybond ECL RPN3032D; Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBS with tween 20 (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4 °C with primary antibodies, followed by incubation with secondary anti-rabbit (Dako; Glostrup, Denmark) or anti-mouse (Dako) horseradish peroxidase-conjugated antibodies. The labeling was visualized by an enhanced chemiluminescence system. The monoclonal antibodies to eNOS, nNOS, and iNOS (Transduction Laboratories; Lexington, KY, USA), soluble guanylyl cyclase (sGC; Santa Cruz Biotechnology; Santa Cruz, CA, USA), and COX-1, and -2 (Cayman Chemical; Ann Arbor, MI, USA) were commercially obtained.

2.3. Colorimetric assay of nitrite/nitrate

As an index of synthesis of NO, its stable metabolites (nitrite/nitrate, NOx) were measured by a colorimetric NO assay kit (Oxford Biochemical; Oxford, MI, USA). Microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrate with griess reagent, 80 µl MOPS (50 mM)/EDTA (1 mM) buffer and

Fig. 1. Semiquantitative immunoblotting of nNOS, eNOS and iNOS in the inner medulla. *p<0.05 compared with control.
5 µl samples were added to the wells. Nitrate reductase (0.01 U) and 10 µl NADH (2 mM) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were added, and absorbance values were read at 540 nm in a microtitre plate reader (Bio-Rad, Model 3550).

2.4. Expression of natriuretic peptide system

The mRNA expression of ANP, natriuretic peptide receptor (NPR)-A, and NPR-C was determined by real-time PCR. cDNA was made by reverse transcribing 5 µg of total RNA using oligo (dT) priming and supercript reverse transcriptase II (Invitrogen; Carlsbad, CA, USA). cDNA was quantified using Smart Cycler II System (Cepheid; Sunnyvale, CA, USA) and SYBR Green was used for detection. PCR was done using Rotor-Gene™ 3000 Detector System (Corbette Research; Mortlake, New South Wales, Australia). Primers were prepared as described previously [17].

2.5. Guanylyl cyclase activity

The biological effect of NO is mediated by activation of soluble guanylyl cyclase (sGC) and subsequent generation of cGMP, whereas most of the biological effects of ANP are mediated by secondary formation of cGMP catalyzed by particulate guanylyl cyclase (pGC) coupled with NPR-A [18]. Therefore, the tissue was fractionated into cytosolic and membrane portions.

The papilla was homogenized in an ice-cold homogenization buffer (50 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM PMSF, and 250 mM sucrose). The homogenate was centrifuged at 1000 g for 15 min at 4 °C and the supernatant was recentrifuged at 100,000 g for 60 min at 4 °C. The supernatant was used as soluble fraction for the measurement of sGC activity, and the resulting pellet as membrane preparation for the measurement of pGC activity. Protein concentrations were measured by BCA assay kit (Pierce).

For the measurement of sGC activity, the protein samples were incubated for 15 min at 37 °C in 50 mM Tris–HCl (pH 7.6), containing 1 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 1 mM ATP, and 15 mM MgCl₂, in the presence of sodium nitroprusside (SNP, 10⁻⁷ to 10⁻³ M). Incubation was stopped by adding ice-cold 50 mM sodium acetate (pH 5.0) and boiling for 5 min. Samples were then cooled on ice. The reaction was terminated by addition of 100 µl of 0.25 M trichloroacetic acid. The samples were centrifuged at 10,000 g for 15 min at 4 °C, and 1 ml of supernatant was mixed with 2 ml of concentrated HCl.

Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control (n=5)</th>
<th>Escape (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex/OSOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>1.00±0.02</td>
<td>1.12±0.10</td>
</tr>
<tr>
<td>nNOS</td>
<td>1.00±0.11</td>
<td>0.70±0.06</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.00±0.11</td>
<td>1.15±0.05</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>1.00±0.06</td>
<td>1.40±0.22</td>
</tr>
<tr>
<td>nNOS</td>
<td>1.00±0.11</td>
<td>1.11±0.06</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.00±0.08</td>
<td>1.09±0.18</td>
</tr>
<tr>
<td>Inner medulla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>1.00±0.03</td>
<td>1.10±0.05</td>
</tr>
<tr>
<td>nNOS</td>
<td>1.00±0.30</td>
<td>1.06±0.08</td>
</tr>
</tbody>
</table>

Values are mean±SEM. OSOM, outer stripe of outer medulla; ISOM, inner stripe of outer medulla. *p<0.05 compared with control.

Fig. 2. Nitric oxide metabolites (nitrite/nitrate, NOx) in the inner medulla and urine. *p<0.05 compared with control.

Fig. 3. A) Semiquantitative immunoblotting of soluble guanylnyl cyclase (sGC) in the kidney. B) cGMP production in response to sodium nitroprusside (SNP) in the papilla. Each point represents mean±SEM of experimental rats.
centrifuged (10,000 g, 4 °C, 10 min), of which supernatant was used to measure cGMP by equilibrated radioimmunoassay.

pGc activities were measured by the method of Winquist et al. [19], with a slight modification. The aliquots were incubated for 15 min at 37 °C in 50 mM Tris–HCl (pH 7.6), containing 1 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 1 mM ATP, and 15 mM MgCl2, in the presence of ANP (10^{-10} to 10^{-6} M). The reaction was stopped by adding ice-cold 50 mM sodium acetate (pH 5.0) and boiling for 5 min. Samples were then centrifuged (10,000g, 4 °C, 5 min). cGMP was measured in the supernatant by equilibrated radioimmunoassay.

Standards and samples were introduced in a final volume of 100 µl of 50 mM sodium acetate buffer (pH 4.8) with 100 µl of dilute cGMP antiserum (Calbiochem-Novabiochem; San Diego, CA, USA) and iodinated cGMP (10,000 cpm/100 µl). Results are expressed as pmol cGMP/mg protein per min.

2.6. Statistical analyses

Results are expressed as mean ± SEM. The statistical significance of differences between the groups was determined using an unpaired t-test. Differences with values of \( p < 0.05 \) were considered significant.

3. Results

3.1. Aldosterone escape model

Table 1 shows the body weight, creatinine clearance, and urinary excretion of sodium. The escape was noted in the experimental group, in which the sodium excretion rate rose virtually to the level of the intake. There were no changes in creatinine clearance, indicating a decreased tubular reabsorption.

Semiquantitative immunoblotting revealed increased abundance of nNOS proteins in the inner medulla in aldosterone escape group (Fig. 1, Table 2), with no changes in the expression of eNOS and iNOS (Fig. 1, Table 2). NOx contents in the inner medulla and the amount of urinary NOx excretion were increased (Fig. 2). Neither the expression of sGC nor the SNP-stimulated generation of cGMP changed (Fig. 3).

Fig. 4A shows the expression of ANP, NPR-A and NPR-C mRNA. The expression of ANP was increased in the aldosterone escape group. Neither the expression of NPR-A nor that of NPR-C was changed significantly. Fig. 4B shows cGMP generation in response to graded doses of ANP in the papilla, which did not differ between the groups.

Fig. 5 shows the protein expression of COX-1 and COX-2. In the aldosterone escape group, the expression of COX-2 was increased in the inner medulla, but not in the cortex/OSOM. The expression of COX-1 was not changed either in the cortex/OSOM or in the inner medulla.

3.2. Effects of different levels of sodium intake

Table 3 shows the body weight and urinary parameters in rats without aldosterone infusion. In NaCl-replete group, the urinary sodium excretion increased, while the plasma aldosterone levels were markedly decreased compared with those in NaCl-restricted rats (Table 3). The protein expression of NOS and COX isoforms in the inner medulla did not differ between the groups. Neither the expression of ANP nor that of NPR-A differed between the groups, while that of NPR-C decreased in the NaCl-replete group (Fig. 6).

4. Discussion

In the present study, there occurred aldosterone escape with no changes of glomerular filtration rate, indicating a decreased tubular reabsorption. Accordingly, the expression of nNOS was increased in the inner medulla, while that of eNOS or iNOS remained unaltered.

The renal content and urinary excretion of NO metabolites increased. The expression of ANP and that of COX-2 was also increased.

Previous studies indicated that the medulla is the important site of action of NO in the kidney. Selective stimulation or inhibition of medullary NO synthesis led to increases or decreases in medullary blood flow, with parallel changes of sodium and water excretion [20]. Western blot analysis revealed the highest level of nNOS expressed in the medulla, especially in the inner medulla [21,22]. Furthermore, nNOS is mainly expressed in the principal cells, which are responsible for sodium, potassium and water transport, with a minimal expression in the acid-secreting intercalated cells in the collecting duct [23]. Therefore, our findings are in line with those in a previous study, in which a high salt diet for 3 days increased nNOS expression...
in the inner medullary collecting duct cells in the absence of changes in iNOS expression [21]. A selective activation of medullary nNOS and resultant increase of NO synthesis may be involved in the aldosterone escape. Nevertheless, there may be a controversy concerning a causative role of NO in aldosterone escape. It was shown that NG-nitro-L-arginine methyl ester (L-NAME) had no effect on the escape, despite an upregulation of eNOS [8]. In this study, the downregulation of thiazide-sensitive Na–Cl cotransporters of the distal tubule, which is supposed to be related to aldosterone escape, was not further affected by L-NAME. In addition, there were no changes in the expression of sGC or SNP-stimulated generation of cGMP in the present study. The complex molecular events involved in the regulation of NO system associated with aldosterone escape remain to be further elucidated.

An increased plasma ANP level has been suggested to mediate the aldosterone escape [5]. However, the natriuretic response to ANP does not necessarily correlate with its plasma levels [10], but the
natriuretic activity may also be affected by local synthesis of ANP. The present study showed that the expression of ANP mRNA was increased in the kidney in aldosterone escape. Neither the expression of NPR-A or NPR-C nor the catalytic activity of pGC changed significantly. While most of the biological effects of ANP are mediated by activation of NPR-A [24], NPR-C has been considered as a clearance receptor [25]. However, a recent study also suggested a role of NPR-C, in which ANP increases NOS activity via activation of NPR-C in the medulla [26]. In this context, it is plausible that the increased synthesis of ANP may also activate NOS in aldosterone escape. The increased synthesis of ANP may allow a prolonged natriuresis, leading to aldosterone escape.

The expression of COX-2 was increased in the inner medulla in the present study. It has been known that a high salt diet may increase renal medullary COX-2 expression to play a role in maintaining sodium homeostasis [27]. The increases in medullary COX-2 expression with resultant increase of PGE2 contribute to the promotion of sodium excretion and maintenance of normal blood pressure in the setting of high salt intake [28]. Our finding is consistent with an important role of medullary COX-2 to promote natriuresis in aldosterone escape.

Our second set of experiment was done to examine the effect of different levels of salt intake per se, with no aldosterone infused. The NaCl-replete group showed an increased excretion of sodium, along with a decrease of plasma aldosterone levels. The expression of either NOS, COX or ANP in the kidney was not altered, while NPR-C expression was decreased. Therefore, the natriuresis may be attributed to decreased aldosterone effects and an enhanced natriuretic activity via decreased NPR-C expression in this experimental setup, being different from that in aldosterone escape.

In conclusion, the upregulation of nNOS, ANP and COX-2 in the kidney may represent an adaptive mechanism that induces natriuresis in aldosterone escape.

Acknowledgments

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References

[17] Bae EH, Kim IJ, Park JW, Ma SK, Choi KC, Lee JU, Kim SW. Altered regulation of COX-2 expression in renal medulla [26]. In this context, it is plausible that the increased synthesis of ANP may allow a prolonged natriuresis, leading to aldosterone escape.

In conclusion, the upregulation of nNOS, ANP and COX-2 in the kidney may represent an adaptive mechanism that induces natriuresis in aldosterone escape.

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