Relaxin ameliorates salt-sensitive hypertension and renal fibrosis

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Abstract

**Background.** Although relaxin (RLX) has potent vasodilatory and anti-fibrotic properties, there is no information on its effects on salt-sensitive hypertension.

**Methods.** We investigated the effects of short-term treatment with RLX on blood pressure (BP) and nitric oxide synthase (NOS) protein in the kidneys of male Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) rats after 1 week consumption of an 8% NaCl diet. We also evaluated the inhibitory effects of each specific NOS inhibitor on BP during 1-week RLX treatment under high-salt diet. Next, we examined the long-term effects of RLX treatment for 6 weeks on renal histology and transforming growth factor-beta1 (TGF-\(\beta\)) expression in male DS and DR rats placed on the 8-week high-salt diet.

**Results.** The short-term RLX treatment significantly attenuated the high-salt diet-induced rise in BP in DS rats with increasing neuronal NOS and endothelial NOS protein in kidneys. Selective inhibition of each of the three NOS isoforms significantly blocked the anti-hypertensive effects of RLX in DS rats after 1-week high-salt diet. The long-term treatment of DS rats with RLX for 6 weeks significantly reduced systolic BP, lessened glomerular and tubulo-interstitial changes and reduced TGF-\(\beta\) signaling compared to saline-treated controls.

**Conclusions.** The results suggested that RLX converted salt sensitivity to salt resistance, at least in part, by up-regulating NOS. RLX is a potentially useful therapeutic agent for salt-sensitive hypertension.

Keywords: NOS; TGF-\(\beta\); SMAD

Introduction

Hypertension, which is highly sensitive to changes in sodium chloride intake is classified as salt-sensitive hypertension [1, 2]. Although the exact cause of salt sensitivity is unknown, the prevalence of hypertension increases after menopause and the loss of ovarian hormones increases the occurrence of salt sensitivity among healthy premenopausal women [3, 4]. Salt-sensitive hypertension is generally associated with increased cardiac output and peripheral vascular resistance [5]. However, normal pregnant women usually experience a modest fall in blood pressure (BP), while circulating blood volume, cardiac output, renal plasma flow (RPF) and glomerular filtration rate (GFR) all increase by 30–70% [6]. Nitric oxide (NO) is implicated in vasodilatation and hypotension during pregnancy [7]. It is reported that relaxin (RLX) essentially mediates the increased vasodilation and NO synthesis during pregnancy [7]. Since both NO synthesis and renal neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) expression levels increase during pregnancy [8], it is plausible that RLX increases NO synthesis and NOS expression. On the other hand, impaired NO activity has been implicated as a possible etiology in salt-sensitive hypertension [10–12]. Both the salt-sensitive
patients and Dahl salt-sensitive (DS) rats show reduced NO production [11–13]. Moreover, inhibition of NO activity induces the development of salt sensitivity in Dahl salt-resistant (DR) rats [11, 14–16].

Renal fibrosis is characteristic of salt-sensitive hypertension and DS rats show vascular sclerosis, glomerulosclerosis and tubulointerstitial fibrosis [17, 18]. Transforming growth factor-β (TGF-β) is the most important fibrosis-promoting cytokine and its signaling is mainly regulated via the Smad proteins [19]. Phosphorylation of Smad2 and nuclear translocation of phosphorylated Smad2 are critical steps in the signaling pathway [19]. The expression of TGF-β is increased in the DS rat kidney [20]. RLX has potent anti-fibrotic properties by down-regulating TGF-β and Smad signaling, as demonstrated in both in vitro and in vivo studies [21–23].

In this study, we hypothesized that RLX is therapeutically useful for salt-sensitive hypertension based on its vasodilatory and anti-fibrotic properties. To test the hypothesis, we determined the short-term effects of RLX treatment on BP, three NOS isoforms and the inhibitory effects of each specific NOS isoform inhibitor in experimental salt-sensitive hypertension. We also examined the long-term effects of RLX on renal histology and TGF-β and Smad signaling in kidneys.

Materials and methods

The experimental protocol was approved by the Ethics Committee for Animal Experimentation, School of Food and Nutritional Sciences, University of Shizuoka.

Experimental animals and protocols

Effects of short-term RLX treatment on NOS expression in the kidney of DS and DR rats. Male DS (n = 14) and DR (n = 8) rats (SLC, Shizuoka, Japan) were fed a 0.3% NaCl (low salt) diet until the age of 6 weeks and then switched to 8% NaCl (high salt) diet for 1 week. The rats were paired with the same amount of dietary salt intake. Tap water was provided ad libitum throughout the experiment. Each rat group was randomly divided into two groups: the RLX treatment group (DS: n = 7, DR: n = 4) and the untreated group (DS: n = 7, DR: n = 4). Porcine RLX was prepared by the method of Sherwood and O’Byrne [24]. All rats were implanted with an osmotic minipump (model 1007D; Alzet, Cupertino, CA) subcutaneously, which was filled with RLX dissolved in 0.9% saline in the RLX treatment group (500 ng/h/rat) and with 0.9% saline alone in the untreated group. The selected dose of RLX was equivalent to that determined in a previous study that induced the production of serum RLX levels comparable to that observed during pregnancy [25, 26]. BP was measured by the tail-cuff method (BP-98; Softron, Tokyo, Japan) at baseline and after 6 days. At the end of 1-week observation, all rats were anesthetized with pentobarbital sodium, and both kidneys were perfused from the abdominal aorta with ice-cold phosphate-buffered saline (PBS), removed and sectioned longitudinally in half. One-half of the right kidney was snap frozen in liquid nitrogen and stored at −80°C for immunoblotting and the other half was fixed with 4% paraformaldehyde or methyl Carnoy’s solution. Other parts of the kidney tissues were prepared for histological examination by fixation with 4% paraformaldehyde or methyl Carnoy’s solution. Other parts of the kidney were snap frozen and stored at −80°C for immunoblotting and renin content assay.

The rats were randomly divided into five groups: RLX treatment control group (n = 7), RLX and nNOS inhibitor treatment group (n = 5), RLX and iNOS inhibitor treatment group (n = 5), RLX and eNOS inhibitor treatment group (n = 5) and RLX-untreated control group (n = 7). RLX treatment was applied as described above. After 5 days, BP was measured again and then each NOS inhibitor was injected subcutaneously. RLX-untreated and RLX-treated control groups were injected with saline instead of NOS inhibitors. The next day, rats were injected again with each NOS inhibitor or saline before BP measurement.

Effects of long-term RLX treatment on salt-sensitive hypertension. DS (n = 12) and DR rats (n = 3) were housed as described above. All rats were fed high-salt diet from 6 weeks of age for 8 weeks. After 2 weeks of the high-salt diet, DS rats were randomly divided into two groups: the RLX treatment group (n = 6) and the untreated group (n = 6). All rats were subsequently implanted with an osmotic minipump (model 2ML2; Alzet). The minipump was filled with RLX dissolved in 0.9% saline in the RLX treatment group (500 ng/h/rat) and with 0.9% saline alone in the untreated group. The minipump was replaced every 2 weeks. BP was measured again by the tail-cuff method. After 8 weeks observation, plasma and 24-h urine samples were collected for measurement of creatinine clearance. Each rat was anesthetized, and blood was sampled from the abdominal aorta. Kidneys were perfused with ice-cold PBS and harvested. Parts of the kidney tissues were prepared for histological examination by fixation with 4% paraformaldehyde or methyl Carnoy’s solution. Other parts of the kidney were snap frozen and stored at −80°C for immunoblotting and renin content assay.

Fig. 1. (A) In the short-term experiment, serial changes in SBP in the four rat groups. After feeding high-salt diet for 1 week, SBP increased significantly in DS rats compared to DR rats and RLX-treated DR and DS rats. Data are mean ± SEM of 4–7 rats in each group. *P < 0.01, DS versus other groups. (B) Serial changes in SBP in five DS rat groups. High-salt intake for 5 days increased SBP in DS rats and treatment with RLX abrogated such increase. Each of the selective NOS inhibitors blocked the anti-hypertensive effects of RLX. Data are mean ± SEM of 5–7 rats in each group. †DS versus other groups, P < 0.05. **DS + RLX versus other groups, P < 0.01.
Analytical procedures

Plasma and urine creatinine levels were measured by standard laboratory techniques, and urine protein was determined by Micro TP test (Wako Pure Chemical, Osaka, Japan). Renal renin content was measured using Renin-Riabead (Dinabot Japan, Tokyo), as described previously [28].

Histology and immunohistochemistry

In the short-term experiment, immunohistochemistry was performed on paraffin-embedded 3-μm tissue sections. The tissue sections were deparaffinized and pretreated by steaming in 10 mM citrate buffer (pH 6.0) at 120°C. Endogenous peroxidase activity was blocked with 3% H2O2 solution in methanol. After blocking with 10% normal goat serum, the slides were incubated overnight with the primary antibody (nNOS and eNOS: BD Transduction, Franklin Lakes, NJ; iNOS: Neo markers, Fremont, CA) at 4°C. The first antibody was localized by using the Dako EnVision-HRP Detection Kit (Dako, Kyoto, Japan) and color development with 3.3’-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with hematoxylin.

In the long-term experiment, the paraffin-embedded kidney samples (3 μm thick) were stained by periodic acid-Schiff and Masson’s trichrome method. The size of glomerulus and mesangial matrix index measured from digitized images using Image-J program (http://rsbweb.nih.gov/ij/) according to the method described previously [29]. Tubulointerstitial changes were assessed by the point-counting technique as described previously [28]. To analyze arterial thickening, the cross-sectional areas of the wall and lumen in the proximal interlobular arteries were measured using Image-J. The proximal interlobular arteries were defined as being within the inner cortex, branching for 500 μm from the arcuate arteries at the corticomedullary junction. The lumen-to-lumen and wall ratio was calculated by dividing the cross-sectional area of the lumen by that of the lumen and wall, as described previously [30].

Paraffin-embedded sections (3 μm thick) were also used for immunohistochemistry. Primary antibodies used were RXFP1 [31], Calbindin-D-28K (Sigma–Aldrich, St Louis, MO) as a marker of distal tubules [32], TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Smad2 (pSmad2; Cell Signaling Technology, Danvers, MA), α-smooth muscle actin (SMA) (Dako), and proliferating cell nuclear antigen (PCNA) (Oncogene Science, Uniondale, NY). Sections fixed in 4% paraformaldehyde were used for RXFP1, TGF-β1 and pSmad2, Calbindin-D-28K and

Fig. 2. In the short-term experiment, effect of RLX treatment on nNOS protein expression in DS and DR rats. Immunohistochemical staining for nNOS in the renal cortex from DR (A), DR + RLX (B), DS (C) and DS + RLX (D) rats. The nNOS-positive staining (brown) was identified in macula densa cells. (Magnification ×400; Bar = 25 μm.) (E) Upper panel shows representative western blotting; lower panel shows densitometry analysis of nNOS expressed as fold induction over DR control rats. The densitometric results were normalized to β-actin. At 1 week after RLX treatment, nNOS overexpression was noted in the kidney of rats treated with RLX compared to untreated rats. P = positive control. *: DS versus DS + RLX, P < 0.01; †: DR versus DR + RLX, P < 0.01.

Fig. 3. In the short-term experiment, effect of RLX treatment on eNOS protein expression in DS and DR rats. Immunohistochemical staining for eNOS in the renal cortex from DR (A), DR + RLX (B), DS (C) and DS + RLX (D) rats. The eNOS-positive staining (brown) was seen in glomerular endothelial cells and peritubular capillaries. (Magnification ×400; Bar = 25 μm.) (E) Upper panel shows representative western blotting; lower panel shows densitometric analysis of eNOS expressed as fold induction over DR control rats. The results of densitometry were normalized to β-actin. At 1 week after RLX treatment, the increased expression of eNOS was noted in the kidney of RLX treatment rats compared to untreated rats. P = positive control. *: DS versus DS + RLX, P < 0.05.
Table 1. Results of long-term experiment on body weight, kidney weight, SBP and laboratory data

<table>
<thead>
<tr>
<th>Group</th>
<th>DR (n = 3)</th>
<th>DS (n = 6)</th>
<th>DS + RLX (n = 6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>400 ± 6.6b</td>
<td>348 ± 8.1</td>
<td>367 ± 11.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Kidney weight (g/100 g BW)</td>
<td>0.57 ± 0.045c</td>
<td>0.83 ± 0.052</td>
<td>0.81 ± 0.053</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126.0 ± 1.5</td>
<td>227.0 ± 11.0</td>
<td>166.7 ± 10.2c</td>
<td>0.001</td>
</tr>
<tr>
<td>Urinary creatinine (mg/day)</td>
<td>21.9 ± 0.6d</td>
<td>17.9 ± 0.5</td>
<td>16.9 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine clearance (L/day)</td>
<td>5.67 ± 0.07</td>
<td>5.29 ± 0.15</td>
<td>4.73 ± 0.20c</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary creatinine (mg/day/100 g BW)</td>
<td>65.9 ± 15.1</td>
<td>228.0 ± 66.4</td>
<td>94.5 ± 22.1</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance (L/day)</td>
<td>1.93 ± 0.06</td>
<td>1.42 ± 0.16</td>
<td>1.99 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Renal renin content (ng/mg/h)</td>
<td>691.5 ± 253.1</td>
<td>236.7 ± 54.7</td>
<td>259.3 ± 41.7</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. ANOVA, analysis of variance, NS, not significant.

Fig. 4. In the long-term experiment, serial changes in SBP in the three rat groups. SBP increased significantly in a time-dependent manner in DS rats fed high-salt diet compared to DR rats. SBP in the DS + RLX group was significantly lower than in DS rats. Data are mean ± SEM of three to six rats in each group. *P < 0.01 versus other groups. **P < 0.05 versus DR group.

Results

Effects of short-term RLX treatment

Figure 1A shows changes in systolic blood pressure (SBP) after RLX treatment. There was no difference in SBP between DS and DR rats before the high-salt diet. After 1-week high-salt diet, SBP increased in DS rats but not in DR rats. Furthermore, RLX completely blocked the increase of SBP in DS rats and had no effect on DR rats.

Effects of specific NOS inhibition

As stated above, SBP increased significantly in DS rats after 5-day high-salt diet and treatment with RLX ameliorated such
increase, resulting in normalization of SBP (Figure 1A). Treatment with each selective NOS inhibitor inhibited the anti-hypertensive effect of RLX, resulting in increase in SBP (Figure 1B).

Effects of long-term high-salt diet and long-term RLX treatment

Table 1 and Figure 4 provide details on the basic parameters measured after RLX treatment. SBP was persistently higher on DS rats fed high-salt diet compared with DR rats. Furthermore, RLX treatment of DS rats resulted in a significant reduction of SBP. Dietary intake was not different among the DR, treated and untreated DS rats. Creatinine clearance at 8 weeks was not significantly different among three groups. Macroscopically, the kidneys were larger in DS rats compared to DR rats, although this tended to improve after RLX treatment albeit insignificantly. Renal renin contents were similar in DS rats between with and without RLX treatment (Table 1).

Table 2 lists the results of histological analysis. In DS rats, the renal vessels showed intimal thickening of small arteries and arterioles, together with deposition of fibrinoid material (Figure 5B and E). In contrast, DR rats showed almost no pathological changes (Figure 5A and D). Treatment of DS rats with RLX significantly lessened the above vascular changes (Figure 5C and F).

Glomerular hypertrophy was noted in DS rats but it was significantly attenuated after long-term RLX treatment. Mesangial matrix index was significantly higher in DS than in DR rats, and RLX treatment slightly decreased mesangial matrix but without significance in DS rats (Table 2, Figure 5).

In DS rats, the tubulointerstitium showed patchy changes with tubular casts and dilatation, round cell infiltration and fibrosis around the sclerosed glomeruli and arterioles (Figure 5E). RLX markedly attenuated such lesions in

<table>
<thead>
<tr>
<th>Group</th>
<th>DR (n = 3)</th>
<th>DS (n = 6)</th>
<th>DS+RLX (n = 6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular area, μm² × 1000</td>
<td>12.6 ± 0.8</td>
<td>17.2 ± 0.6b</td>
<td>13.7 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mesangial matrix index (%)</td>
<td>21.7 ± 1.06d,e</td>
<td>31.4 ± 1.16</td>
<td>28.0 ± 1.53</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Arterial narrowing</td>
<td>0.279 ± 0.228</td>
<td>0.150 ± 0.008b</td>
<td>0.298 ± 0.013</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tubulointerstitial injury index (%)</td>
<td>31.4 ± 1.61d</td>
<td>49.1 ± 3.83</td>
<td>38.2 ± 2.26b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>α-SMA- and PCNA-positive cells in glomeruli (n)</td>
<td>14.3 ± 2.73b</td>
<td>65.5 ± 9.33</td>
<td>35.8 ± 7.57b</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TGF-β1 (score)</td>
<td>1.40 ± 0.06</td>
<td>2.69 ± 0.13b</td>
<td>1.66 ± 0.22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phosphorylated Smad2 (positive cells per field)</td>
<td>17.77 ± 6.52</td>
<td>49.41 ± 4.25b</td>
<td>14.36 ± 4.76</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2. Results of long-term experiment

**Effects of RLX treatment on renal histology and immunohistochemistry**

Table 2 lists the results of histological analysis. In DS rats, the renal vessels showed intimal thickening of small arteries and arterioles, together with deposition of fibrinoid material (Figure 5B and E). In contrast, DR rats showed almost no pathological changes (Figure 5A and D). Treatment of DS rats with RLX significantly lessened the above vascular changes (Figure 5C and F).

Glomerular hypertrophy was noted in DS rats but it was significantly attenuated after long-term RLX treatment. Mesangial matrix index was significantly higher in DS than in DR rats, and RLX treatment slightly decreased mesangial matrix but without significance in DS rats (Table 2, Figure 5).

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Fig. 5. In the long-term experiment, effects of RLX treatment on renal injury in DS and DR rats. Representative kidney tissue sections stained with Masson’s trichrome staining. DR rats showed almost no pathological lesions (A and D). DS rats showed glomerular hypertrophy, protein casts and intimal thickening of small arteries (B and E). Treatment with RLX protected against these histological changes (C and F). A–C: Bar = 200 μm. D–F: Bar = 50 μm.
DS rats. Quantitative analysis showed a significant increase in tubulointerstitial injury index in DS rats compared to DR rats (Table 2, Figure 5E). DS rats with RLX treatment showed significantly lower tubulointerstitial injury compared to untreated DS rats (Table 2, Figure 5F).

Immunohistochemical analysis showed a significant increase in the density of both α-SMA- and PCNA-positive cells in the glomeruli of DS rats compared to DR rats. Furthermore, RLX significantly decreased these immunopositive cells compared to untreated rats, suggesting that RLX attenuates mesangial activation and proliferation.

Immunohistochemistry also showed higher expression of both TGF-β1 and pSmad2 in the glomeruli, tubules and interstitial tissues of DS rats (Figure 6B and E) compared to DR rats (Figure 6A and D). However, DS rats treated with RLX showed decreased immunopositivity for TGF-β1 and pSmad2 (Figure 6C, F and G).

RLX receptor expression in the kidney

The distribution and colocalization of RXFP1 with calbindin-D-28K were examined immunohistochemically in serial sections. RXFP1 (Figure 7A and B) was identified in calbindin-D-positive distal tubular cells (Figure 7C and D). Figure 7F shows RXFP1 protein expression levels in both DS and DR rats after 8-week high-salt diet. The RXFP1 protein expression in the renal cortex was significantly higher in DS rats compared with DR rats (DR: 1.00 ± 0.14, DS: 2.28 ± 0.34).

Discussion

To our knowledge, this is the first study to investigate the effect of RLX in experimental salt-sensitive hypertension. The major new findings of the study are: (i) short-term RLX treatment decreased BP by increasing NOS expression while inhibition of the NOS isoforms blocked anti-hypertensive effect of RLX and (ii) long-term treatment with RLX significantly lowered SBP and improved renal histological changes in salt-sensitive hypertension as well as decreased TGF-β1 activation. These results suggest that RLX can act in the kidney to convert salt sensitivity to salt resistance.

Firstly, we evaluated the short-term effects of RLX treatment on the involvement of NO in salt-sensitive
The results showed that RLX significantly attenuated the long-term treatment with RLX in salt-sensitive hypertension. It mainly reflects the activation of RLX signaling as a vasodilatory hormone in response to high-salt diet. However, since BP increased in DS rats despite increased RXFP1, intrinsic RLX probably does not play a major role in the development of salt-sensitive hypertension.

Our group investigated previously the effects of RLX in anti-thymocyte nephritis, a well-studied model characterized by a transient increase in TGF-β1 and extracellular matrix accumulation [22]. RLX treatment significantly decreased pSmad2 and extracellular matrix accumulation without changes in BP. These results suggest that RLX may act directly to decrease TGF-β1 activation in diseased kidneys partially independent of its anti-hypertensive effects. The anti-fibrotic effect of RLX in the kidney has been demonstrated in several in vivo models such as the bromoethylamine model [38], renal mass reduction model [39] and aging Munich–Wistar rats [40]. In addition, RLX knockout mice developed renal fibrosis with aging that was reversed by exogenous RLX treatment [41].

**Fig. 7.** In the long-term experiment, RXFP1 expression in the kidneys of DS and DR rats. Co-localization of RXFP1 (A and B) and calbindin-D-28K (C and D) was shown in serial sections by immunohistochemistry. RXFP1 expression was identified in calbindin-D-positive distal tubular epithelium of DR (A and C) and DS (B and D) rats. Bar = 50 μm. (E) Representative immunoblots showing RXFP1 protein levels in the renal cortex at 8 weeks after high-salt diet. (F) Quantitative data of RXFP1 expression in the distal tubules. Immunoblot demonstrated that salt loading resulted in a significant increase in RXFP1 expression in the renal cortex of DS rats, relative to the baseline, but not in DR rats. RLX protein could not be detected by our ELISA system (data not shown). To date, very few studies have examined the regulation of RXFP1 messenger RNA expression in disease models, but in one study, RXFP1 messenger RNA expression in the heart was reduced in a rat model of cardiac failure induced by myocardial infarction [37]. Therefore, it is possible that increased RXFP1 expression reflects the activation of RLX signaling as a vasodilatory hormone in response to high-salt diet. However, since BP increased in DS rats despite increased RXFP1, intrinsic RLX probably does not play a major role in the development of salt-sensitive hypertension.
RLX ameliorates salt-sensitive hypertension

In conclusion, we demonstrated the presence of a local RLX receptor in the kidney and that RLX attenuated salt-sensitive hypertension with NOS up-regulation and TGF-β1 down-regulation. Although further experimental and clinical trial studies are necessary to clarify the direct renal effects of RLX, we consider RLX a promising therapeutic agent for renal fibrotic diseases such as salt-sensitive hypertension.

Conflict of interest statement. None declared.

References


Received for publication: 8.2.11; Accepted in revised form: 24.9.11