Online Supplement

Activation of Renal (Pro)Renin Receptor Contributes to High Fuctose-Induced Salt Sensitivity

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Running title: (Pro)renin receptor in high-fructose-induced salt-sensitive hypertension

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Materials and Methods

Animal experiments

SD rats were fed a standard diet and randomized into 4 groups (n=5 / each group): Control, fructose (HF), fructose plus allopurinol (a xanthine oxidase inhibitor, Cat# A8003, Sigma, Louis, MO, USA) (HF + allopurinol), and fructose plus PRO20 (HF + PRO20). Fructose was given to the rats in the later two groups as a 20% solution in drinking water ad libitum for 12 weeks, whereas the control rats received normal drinking water. PRO20 was administered to the fructose plus PRO20 group at 700 μg/kg/day in 0.9% NaCl via subcutaneous injection three times a day (every 8 h) and the other three groups received a subcutaneous injection of 0.9% NaCl as a vehicle. Allopurinol was administered at 30 mg/kg/day in drinking water1. At week 12, rats were housed in metabolic cages to measure 24-h food and water intake. At the end of the experiment, all rats were sacrificed and blood and kidneys were harvested. After decapsulation, the kidneys were cut into cortex, outer medulla, and inner medulla. Urine and plasma electrolytes were determined with an automatic analyzer (9180 Electrolyte analyzer, Roche, Berlin, Germany). Urine and plasma uric acid (UA) was measured by using the QuantiChrom Uric Acid Assay Kit (Cat# DIUA-250, BioAssay Systems, Hayward, CA, USA) according to the manufacturer’s instructions.

Renin activity assay

Renin activity assay was performed as previously described2. Renin activity in plasma and urine was determined by using an ELISA kit and the delta value of Ang I generation after incubating the sample at 4 °C and 37 °C, respectively, for 1 h. Total renin content was measured with excess angiotensinogen (AGT) plus trypsinization and active renin content with excess AGT. Urine and plasma samples were spiked with the synthetic renin substrate tetradecapeptide (1 μM, Cat# R8129, Sigma-Aldrich, Saint Louis, MO, USA). Following incubation at 37 °C for 1 h, Ang I generation was assayed by using an Angiotensin-I EIA kit (Cat# S-1188, Peninsula Laboratories International, San Carlos, CA, USA) according to the manufacturer’s instructions. The values were expressed as nanograms per milliliter per hour of generated Ang I. For measurement of total renin content, trypsinization was performed to activate prorenin to renin. The samples were incubated with trypsin from bovine pancreas (100 g/L, Cat# T1426, Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C for 6 h. The reaction was then terminated with soybean trypsin inhibitor (100 g/L, Cat# T6522, Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C for 1 h.

qRT-PCR

Total RNA was isolated from snap-frozen renal samples by using TRIzol reagent (Cat# 15596018, Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Cat# 04379012001, Roche, Berlin, Germany) according to the manufacturer’s instructions. Total RNA concentrations were determined using NANODROP 2000 Spectrophotometer
(Thermo scientific) according to the manufacturer’s instructions. Quantitative real-time PCR was performed by using the ABI Prism StepOnePlus System (Applied Biosystems, Life Technologies, Singapore) and the FastStart Universal SYBR Green Master (ROX) (Cat# 04913914001, Roche, Berlin, Germany) according to the manufacturer’s instructions. Oligonucleotides were designed with Primer3 software (available at http://www.simgene.com/Primer3), their sequences are shown in Table S1. All reactions were run in duplicate. The data are shown as a relative value normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunoblot analysis

The kidney samples from each animal were lysed and subsequently sonicated in RIPA buffer (Biocolors, Shanghai, China) with protease inhibitor cocktail (Roche, Berlin, Germany). Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Cat# NCI3225CH, Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. Thirty micrograms of protein for each sample was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dry milk, followed by incubation with primary antibodies (PRR, HPA003156; Renin, AV41409; Na/K/2Cl cotransporter (NKCC2), SAB5200103; NHE3, SAB5200102; KHK, HPA007040; β-actin, A-2066; these primary antibodies were purchased from Sigma-Aldrich and were incubated for overnight at 4°C and then with goat anti-rabbit/mouse horseradish peroxidase-conjugated secondary antibody (Thermo Scientific) for 1 h at room temperature, and visualized with enhanced chemiluminescence (Thermo Scientific). They were then processed for signal detection by using a Tanon 5200 Luminescent Imaging Workstation (Tanon, Shanghai, China) and quantitated using Image-Pro Plus 6.0. The expression of protein was calculated in relation to β-actin.

Immunofluorescence

The tissues were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. After deparaffinization, thin sections (4 μm) were processed for labeling with immunofluorescence. The slides were blocked in 1% BSA for 1 h and were then incubated with primary antibody (PRR, 1:200 dilution, ab40790, Abcam) or PRR antibody peptide (human ATP6IP2 peptide, 1:100 dilution, ab41522, Abcam) at 4°C for overnight. After washing off the primary antibody, sections were incubated for 1 h at room temperature with Donkey anti-Rabbit IgG- TRITC (1:100, Life Technologies). After washing off the secondary antibody, images were captured using a Leica DMI4000B fluorescence microscope (Wetzlar, Germany).

ELISA assays for sPRR and Ang II

sPRR levels, released into the culture medium, were determined by using a soluble (Pro)renin Receptor Assay kit (Cat# 27782, Immuno-Biological Laboratories, Gunma, Japan). Ang II levels in urine and plasma were determined by using the Ang II ELISA kit (Cat# ADI-900-204, Enzo Life Sciences Inc., Farmingdale, NY, USA)
according to the manufacturer’s instructions.

**In vivo NKCC2 activity**

To determine the effect of PRR on *in vivo* NKCC2 activity, we administered 0.8 mg/kg furosemide by the intraperitoneal route to all rats and removed food and water following this injection as previously described. One-hour urine samples were collected and urine electrolytes determined with an automatic analyzer.

**Blood pressure measurement**

After 10 weeks of treatment with Control, HF, HF + allopurinol, and HF + PRO20 as described above, the radiotelemetric device (Data Sciences Inc., St. Paul, MN, USA) was implanted via catheterization of carotid artery. Rats were allowed to recover for 1 week from surgery. The BP was then recorded for 4 hours from 9:30AM to 3:30PM before and after 1 week of a high salt diet (8% NaCl).

**Cell culture**

Human renal proximal tubule epithelial cells HK-2 were grown to 95% confluence in Dulbecco's Modified Eagle medium/Ham's F-12 medium supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin-streptomycin, 2 mM L-glutamine, insulin (10 mg/L), transferrin (5.5 mg/L), and sodium selenite (6.7 μg/L). To detect the direct effect of fructose on PRR expression, HK-2 cells were serum-starved for 12 h and then treated with 1, 3, 5, and 10 mM fructose for 72 h, or treated with 5 mM fructose for various time periods, or pretreated with allopurinol (100 μM) for 1 h and then treated with 5 mM fructose for 72 h. The cells were then harvested for PRR expression analysis, and the cell medium for sPRR ELISA assay.

To detect the direct effect of exogenous UA on PRR expression, HK-2 cells were serum-starved for 12 h and then treated with 10 mg/dl UA for various time periods, cells were then harvested for PRR expression analysis, and the cell medium for sPRR assay.

**References**

**Table S1.** Sequences of oligonucleotides used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>PubMed No.</th>
<th>Sequence (5'→3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| PRR  | NM_001007091.1 | (F)ATCCTTGAGACGAAACAAGA  
(R)AGCCAGTCATAATCCACAGT | 109 |
| Renin| NM_012642.4  | (F)GATCACCATGAAGGGGTCTCTGT  
(R)GTTCCTGAAGGGATTTTTTGCA | 274 |
| NKCC2| NM_001270618.1 | (F)CAGTGTTGAGCAGTCTTCCC  
(R)TGTTGTTGTGGCCAAGGTT | 272 |
| NHE3 | NM_012654.1  | (F)ACTGCTTAATGACGCGGTGACTGT  
(R)AAAGACGAAGCCAGCTCGATGAT | 223 |
| GAPDH| NM_017008.4  | (F)GTCTTCACATGGAGAGGAGG  
(R)TCATGGAGGACCTTGGCCAG | 197 |

**Table S2.** General physiological data in rats.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>HF</th>
<th>HF + Allopurinol</th>
<th>HF + PRO20</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔBody weight (g)</td>
<td>372.0±10.2</td>
<td>329.4±6.1*</td>
<td>311.34±7.40*</td>
<td>314.8±8.7*</td>
</tr>
<tr>
<td>KW/BW (g/kg)</td>
<td>5.04±0.18</td>
<td>5.56±0.12*</td>
<td>6.12±0.23 ‡</td>
<td></td>
</tr>
<tr>
<td>Food intake (g/24h)</td>
<td>16.48±1.02</td>
<td>11.05±0.71†</td>
<td>11.50±1.63†</td>
<td>12.33±0.99†</td>
</tr>
<tr>
<td>Na⁺ intake (mmol/24h)</td>
<td>2.15±0.13</td>
<td>1.44±0.09‡</td>
<td>1.50±0.21*</td>
<td>1.61±0.13*</td>
</tr>
<tr>
<td>Water intake (ml/24h)</td>
<td>22.95±1.31</td>
<td>46.84±4.04‡</td>
<td>71.08±3.75‡¶</td>
<td>45.05±4.66‡</td>
</tr>
<tr>
<td>Total energy intake (kJ/24h)</td>
<td>214.20±13.27</td>
<td>300.21±17.64‡</td>
<td>387.19±11.34‡</td>
<td></td>
</tr>
<tr>
<td>Food-derived energy (kJ/24h)</td>
<td>214.20±13.27</td>
<td>143.59±9.16‡</td>
<td>149.50±21.22*</td>
<td>160.23±12.92*</td>
</tr>
<tr>
<td>Drinking-derived energy (kJ/24h)</td>
<td>0</td>
<td>156.62±13.52‡</td>
<td>237.69±12.53‡</td>
<td></td>
</tr>
<tr>
<td>Urine volume (ml/24h)</td>
<td>12.62±1.22</td>
<td>27.84±3.05‡</td>
<td>48.38±4.47‡¶</td>
<td>26.03±3.38*</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>3.63±0.16</td>
<td>5.49±0.07*</td>
<td>4.50±0.26*§</td>
<td>6.47±0.22§</td>
</tr>
<tr>
<td>Plasma insulin (pg/ml)</td>
<td>0.72±0.17</td>
<td>1.22±0.12*</td>
<td>0.71±0.15†</td>
<td>0.79±0.10†</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.50±0.01</td>
<td>0.48±0.01</td>
<td>0.49±0.01</td>
<td>0.47±0.01</td>
</tr>
</tbody>
</table>

ΔBody weight: change in body weight, KW/BW: Kidney Weight / Body Weight, *p < 0.05, †p < 0.01, and ‡p < 0.001 vs. Control; §p < 0.05, †p < 0.01, and ‡p < 0.001 vs. HF.

Figure S1
Figure S1. Effect of allopurinol on UA production in HF-fed normal rats. Analysis of urinary (A) and plasma (E) UA. KHK protein expression of kidney cortex (B) and outer medulla (C) was analyzed by immunoblotting and densitometric analysis (D). N = 5 per group. Data are mean ± SEM. ***p < 0.001 vs. control, ###p < 0.001 vs. HF.
Figure S2. Effect of allopurinol and PRO20 on plasma renin and Ang II levels in HF-fed SD rats. SD rats were randomly divided into the following 4 groups: Control, HF, HF + allopurinol, or HF + PRO20. The plasma samples were assayed for renin and AngII. (A) Plasma renin activity. (B) Plasma active renin concentration. (C) Plasma total renin concentration. (D) Plasma prorenin concentration. (E) Plasma Ang II concentration. N = 5-10 per group. Data are mean ± SEM. **p < 0.01 and ***p < 0.001 vs. control, ##p < 0.01 vs. HF.
Figure S3. Effect of fructose and UA on PRR expression in HK-2 cells. (A) Dose-response studies of fructose regulation of PRR expression. Cells were exposed to fructose at various concentrations for 48 h, followed by immunoblotting and densitometric analysis of PRR protein expression. The expression was normalized by β-actin. N = 5 per group. Data are mean ± SEM. *p < 0.05 and **p < 0.01 vs. control or 1 mM, †p < 0.05 vs. 3 mM. (B) Time-course studies of fructose regulation of PRR expression. Cells were exposed to 5 mM fructose for the indicated time periods and PRR protein expression was analyzed by immunoblotting and densitometric analysis. The expression was normalized by β-actin. N = 5 per group. Data are mean ± SEM. *p < 0.05 vs. control or 12 h. (C and D) Effect of allopurinol on HF-induced PRR expression and medium sPRR secretion in HK-2 cells. Cells were pretreated with 100 μM allopurinol and then treated with 5 mM fructose for 72 h. PRR protein expression was analyzed by immunoblotting and densitometric analysis (C) and medium sPRR was measured by ELISA and expressed as pg/mg of total cellular proteins. N = 5 per group. Data are mean ± SEM. *p < 0.05 and **p < 0.01 vs. control, †p < 0.05 and ‡‡p < 0.01 vs. HF. (E, F) Time-course studies of UA regulation of PRR expression in HK-2 cells. Cells were exposed to 10 mg/dl UA for the indicated time periods and PRR protein expression analyzed by immunoblotting and densitometric analysis (E). The expression was normalized by β-actin. (F) ELISA analysis of medium sPRR and expressed as pg/mg of total cellular proteins. The cells were exposed to 10 mg/dl UA for 24 h. N = 6 per group. Data are mean ± SEM. **p < 0.01 vs. control or 6 h, ***p < 0.001 vs. control or 6h or 12 h.