EXPERIMENTAL STUDY

Effect of polypeptide 2B1 on condition of dampness pattern in rats in terms of Traditional Chinese Medicine

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Abstract

OBJECTIVE: This study investigated how polypeptide 2B1 is involved in regulating and governing dampness in rat models with dampness pattern defined in terms of Traditional Chinese Medicine.

METHODS: We randomly divided 48 SPF 10-week-old male Sprague-Dawley (SD) rats into a normal group, normal + Aristolochic acid I (AA-I) for 5 min group, normal + AA-I for 60 min group, dampness pattern group (DS-Group), dampness pattern + AA-I for 5 min group, and dampness pattern + AA-I for 60 min group. Groups were then treated accordingly. We took out the lung, stomach, liver, spleen, kidney, large intestine, and small intestine tissues to detect gene and protein expression of organic anion transporter polypeptide 2B1 (OATP2B1).

RESULTS: Gene expression of OATP2B1 in spleen, kidney, and small intestine of rats with dampness pattern was lower than that in normal rats (P<0.05). The gene expressions of OATP2B1 in liver, stomach, large intestine, and small intestine were lower than that in control rats at different time points after being stimulated by AA-I (P<0.05).

CONCLUSION: There is coordination among multiple viscera in handling the condition of dampness, and the mechanism underlying the action may rely on regulating the expression of OATP2B1.

INTRODUCTION

Water metabolism is mainly regulated and governed by lung, spleen, and kidney in terms of Traditional Chinese Medicine (TCM). Their dysfunction may induce a metabolic disorder called dampness pattern in TCM. Dampness may be produced in the body during metabolic processes. However, few studies have focused on how dampness is regulated and governed among multiple viscera in the body.
Modern studies have shown that organic anion transporter polypeptide (OATP) can transport a variety of compounds efficiently, and plays a role in dealing with dampness. Therefore, we focused on OATP2B1 gene and protein expression to investigate the biological basis and mechanism underpinning dampness management.

MATERIALS AND METHODS

Animals
Ten-week-old SPF healthy male Sprague-Dawley (SD) rats weighing 260-290 g were obtained from the Laboratory Animal Center of Sun Yat-Sen University (Production license number: SCXK [Cantonese] 2009-0011, use license number: SYXK [Cantonese] 2007-0081, experimental animal certification number: 0089588). The animal experiment procedure was reviewed and approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University (Approval ID: IACUC-2011-0701).

Medicine
Aristolochic acid I (AA-I, lot number: 20101126, 20 mg/piece, purity: 99%) was purchased from Delta Pharmaceutical Technology Co., Ltd. (Wuhu, China).

RT-PCR reagents and instruments
TRIzol extraction reagent (model number: 15596-018) was purchased from Invitrogen (Carlsbad, CA, USA); 5 × RT-buffer, 5 × quantitative PCR buffer, dNTPs, MMLV, Taq enzymes were purchased from Takara (Dalian, China). Chloroform, isopropanol, and absolute alcohol were purchased from Guangzhou Medium South Chemical Industry (Guangzhou, China).

Intelligent artificial climate box (model number: BD-PRX) was purchased from Nanjing Beidi Experimental Instrument Co. Ltd. (Nanjing, China). Tabletop high flux DNA synthesizer (model number: 3900) was purchased from ABI (Foster City, CA, USA). Qualitative PCR instrument (model number: C1000) was purchased from Bio-Rad (Hercules, CA, USA). Fluorescence quantitative PCR instrument (model number: 7500) was purchased from ABI. High-speed refrigerated tabletop centrifuge (-80 °C, model number: MDF-U4084S) was purchased from Sanyo (Tokyo, Japan).

Westernblot reagents and instruments
GAPDH (mouse) monoclonal antibody and primary antibody of OATPB (goat) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-goat IgG-HRP was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). PVDF membrane was purchased from Bio-Rad. Protein extraction liquid was purchased from Beyotime Institute of Biotechnology (Shanghai, China). 12 hydroxyl sulfate was purchased from Guangzhou Whiga Technology Company (Guangzhou, China). Dithiothreitol (DTT), acryl-amide, N,N'-methylenebisacrylamide, Tris alkali, and tween-20 were purchased from Sigma Company (St. Louis, MO, USA). Tetramethylethylenediamine was purchased from Shanghai Sangon (Shanghai, China). Ammonium persulfate, methanol, and glycerol were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China).

Ultrasonic cell disruption equipment (model number: JY96-IIN) was purchased from the Shanghai Touching Technology Co. Ltd. (China). Vertical electrophoresis and transfer system were purchased from Bio-Rad. Decolorization table was purchased from Rotomix Technology Company (Kansas City, MO, USA). An X-ray photography magazine (5 inch × 7 inch, glow cassette, Fuji X-ray films) was purchased from Yijia Biotechnology Co., Ltd. (Guangzhou, China).

Animal grouping
After purchase, rats were kept for 2 days until their weight increased to 270-300 g. They were grouped with a random number table into six groups (eight rats in each group). The groups were: normal group, normal + AA-I group for 5 min, normal + AA-I group for 60 min, dampness pattern group, dampness pattern + AA-I group for 5 min, and dampness pattern + AA-I group for 60 min.

Model establishment
Model rats were placed in an artificial climate box (18°C-25°C, relative humidity >90%) for 8 h daily. The cage bottoms had bedding 2 cm thick composed of wet sawdust (replaced daily). The rats were then placed in plastic tempered barrels with warm water (water temperature 25°C ± 1°C, depth 40 cm) and swim to their endurance limit daily (termination was initiated when the rats sank with continuous “bubbling” five times). The rats were fasted on odd days, and all other days the rats received intragastric administration of 4 mL lard along with a basic diet. The rats had access to water ad libitum. Modeling duration was 21 days.

Criterion of dampness pattern
Rats were considered to have dampness pattern when they were apathetic, drowsy, had weight loss, were emaciated, had loose stools, and had a lack of thirst and appetite.

Interventions
The second day after the end of the modeling cycle, the normal + AA-I group and dampness pattern + AA-I group were given AA-I by intragastric administration at 20 mg/kg. Then, tissue samples were removed after 5 min (normal + AA-I for 5 min group, N + AA-15M-Group; dampness pattern + AA-I for 5 min group, DS + AA-15M-Group) and 60 min (normal + AA-1 for 60 min group, N + AA-160M-Group; damp-
ness pattern + AA-I for 60 min group, DS + AA-I60M-Group). Six rats were killed in each group after anesthesia. The normal group (N-Group) and dampness pattern group (DS-Group) were intragastrically administered normal saline once with 4 mL in each animal and sampled after 6 h.

**Specimen collection**
After anesthetizing rats by intraperitoneal injection of 10% chloral, 0.3 mL/100 g dose, pieces (about 5 mm× 5 mm) of lung, stomach, liver, spleen, kidney, large intestine, and small intestine were removed. All specimens were placed into a refrigerator at -80°C within 15 min.

**OATP2B1 mRNA determination by RT-PCR**
For the extraction of total RNA, Different hydroxyl guanidine chloride-chloroform methods were used to measure OD values. Gene mRNA sequences were found in the GenBank and species-specific primers were designed in the coding sequence (CDS) area (Table 1). ABI Primer Express 2.0 software (Foster City, CA, USA) was used for designing primers and probes. Synthetic instrumentation was an ABI 3900 tabletop high flux DNA synthesizer.

**Reverse transcription reaction**
A total of 4 μL RNA template was used for reverse transcription in a Bio-Rad qualitative PCR instrument (Bio-Rad). Reaction system: 5 × RT-buffer 4 μL, reverse primer (10 pmol/μL) 0.5 μL, deoxyribonucleoside triphosphates (dNTPs) (10 mM) 0.5 μL, M-<span_	ext{m}^<\text{m}>_umire leukemia virus (MMLV) (200 U/μL) 0.5 μL, diethylpyrocarbonate (DEPC) 10.5 μL, RNA template 4 μL. Reaction conditions were: 37°C for 1 h, and 95°C for 3 min.

**Fluorescence quantitative PCR reaction**
The preparation of positive standard samples and gradient was done using 2% low melting point agarose gel electrophoresis (containing ethidium bromide, prepared with TAE buffer) to deal with PCR-amplified positive products of the preliminary test. The objective band was cut under ultraviolet light. After recovery and purification by QIAquick Gel Extraction Kit, the OD value was tested as 260/280>1.8, which proved the purity was acceptable. The concentration (copy/μL) quantitated by measurement of OD260 and fragment length was positive standard samples. A total of 5 μL positive standard samples were diluted 10 times (45 μL water were added). The gradient of positive standard samples was made through dilution. The samples to be detected and positive standard samples were as follows: 5×10 μL quantitative PCR buffer, forward primer (10 pmol/μL) 1 μL, reverse primer (10 pmol/μL) 1 μL, probe (5 pmol/μL) 1 μL, dNTPs (10 mM) 1 μL, Taq enzyme (3 U/μL) 1 μL, cDNA 5 μL, and ddH2O 30 μL. The reaction conditions were: 93°C for 2 min, then 93°C for 15 sec, 55°C for 25 sec, and 72°C for 25 sec, for a total of 40 cycles. The ABI7500 fluorescence quantitative PCR instrument was used (ABI), and data were analyzed by computer automatically after reactions. Results were calculated by 2^{ΔΔCt}, ΔΔCt=(Ct gene of interest – Ct internal control) sample A – (Ct gene of interest – Ct internal control) sample B. 14,17

**Determination of OATP2B1 protein expression with western blot**
Total protein of rat tissue was extracted according to the instructions of the kit (KGP total protein extraction kit). Tissue of lung, stomach, liver, spleen, kidney, large intestine, and small intestine were crushed and homogenized. Protein extraction was then obtained after ultracentrifugation (4°C, 10000 rpm, 5 min). Coomassie brilliant blue staining was used to detect the concentration of protein. Proteins were separated with SDS-PAGE. The membrane protein was transferred for 60 min, blocked, exposed to primary and secondary antibody, and finally ECL-chemiluminescence. GADPH (37 kDa) was used as a reference of protein expression to calculate the target protein expression levels.

**Statistical methods**
Data were analyzed with SPSS 13.0 (IBM, Armonk, NY, USA). Wilcoxon rank sum test and t-test were performed. P<0.05 was considered significant.

**RESULTS**
The rats with dampness pattern ate and drank less
from the seventh day during the course of establishing the dampness pattern model (Figure 1B, 1C). Ten days later, they had loose stools, and 14 days later, they began to lose weight significantly (Figure 1A). Rats appeared apathetic and sleepy, and had dark and withered fur.

The differences from pre-modeling to post-modeling in weight, food intake, and drinking quantity of the rats with dampness pattern were lower than those in the normal group ($P<0.05$, Table 2).

**OATP2B1 gene expression**

Compared with the N-Group, all the gene expressive quantities of OATP2B1 in spleen ($P<0.05$), kidney ($P<0.05$) and small intestine ($P<0.05$) in the dampness DS-Group were significantly lower (Figure 2A). All the gene expressive quantities of OATP2B1 in liver ($P<0.05$) and large intestine ($P=0.005$) in the DS + AA-I5M-Group were significantly lower than those in the N+AA-I5M-Group (Figure 2B). The gene expressive quantity of OATP2B1 in stomach ($P=0.003$), liver ($P<0.05$), large intestine ($P<0.05$), and small intestine ($P<0.05$) in the DS+AA-I60M-Group were significantly lower than those in the N+AA-I60M-Group (Figure 2C). There was no statistical significance between the two groups that were treated with AA-I ($P>0.05$) (Figure 3). The gene expressive quantities of OATP2B1 in large intestine ($P<0.05$) in the DS + AA-I5M-Group and those in lung ($P<0.05$), stomach ($P<0.05$), kidney ($P<0.05$), and small intestine ($P<0.05$) in the DS + AA-I60M-Group were lower compared with the N-Group (Figure 4).

**OATP2B1 protein expression**

The changing trend in protein expression was similar to that of the gene expression (Figure 5).

**DISCUSSION**

In the present study, a dampness pattern model was successfully established with a high-humidity environment, excessive fatty food, and overstrain. Rats presented symptoms that are analogous to humans, such as apathy, drowsiness, weight loss, emaciation, loose stool, and lack of thirst.

Our study found that the gene and protein expression level of spleen and kidney OATP2B1 was lower in rats with dampness pattern than those in normal rats. The gene and protein expression level of OATP2B1 in small intestine was also lower, which indicated less absorption of dampness. The mechanism for this is unknown, but this might suggest that small intestine responds to the decrease in transportation of turbid dampness in spleen and kidney had protective effects on the body.

AA-I is a toxic and carcinogenic substance that causes potential harm to the kidney, inducing gene mutation. The toxic substance is similar to exogenous dampness in terms of TCM theory. After the rats were given AA-I for 5 min, the gene and protein expression of OATP2B1 in the large intestine was lower than that in control mice. This indicated that the absorption of water dampness was limited to prevent the accumulation of dampness in the body. After the rats were given AA-I for 60 min, the gene and protein expression levels of OATP2B1 in the kidney and small intestine were lower as well. The gene and protein expression levels of OATP2B1 in lung and stom-
ach also appeared to be lower while the dampness increased gradually in rats. After AA-I intervention for 5 min, the gene and protein expression levels of OATP2B1 in liver and large intestine of rats with dampness pattern were lower than those in the AA-I rats. The gene and protein expression levels of OATP2B1 in stomach and small intestine also appeared to decrease following the decreased expression of OATP2B1 in liver and large intestine at 60 min. This indicated that the regulating and dredging function of the liver was damaged first. The introversive transportation of dampness was limited through the large intestine at the same time, and was regulated through stomach and small intestine to adjust the relative balance of water in the body.

In conclusion, our study found that there was a close relationship between the gene and protein expression levels of OATP2B1 and regulation and governing of dampness in viscera and tissues. This suggests that OATP2B1 might be one of the basic factors in removing dampness.

REFERENCES

Table 2 Weight, food intake, and drinking quantity in normal and dampness pattern groups (x ± s)

<table>
<thead>
<tr>
<th>Index</th>
<th>Normal (n=24)</th>
<th>Dampness pattern (n=24)</th>
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Notes: compared with pre-modeling, P<0.01. “Normal” includes normal and normal + AA-I groups (both 5 and 60 min); “Dampness pattern” includes dampness pattern and dampness pattern + AA-I groups (both 5 and 60 min).


Figure 5 Expression of OATP2B1 protein of viscera tissue in each group
A: normal group; B: normal + AA-I for 5 min group; C: normal + AA-I for 60 min group; D: dampness pattern group; E: dampness pattern + AA-I for 5 min group; F: dampness pattern + AA-I for 60 min group. OATP2B1: Organic anion transporter polypeptide 2B1; 1: lung; 2: stomach; 3: liver; 4: spleen; 5: kidney; 6: large intestine; 7: small intestine. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.


