Boiogito Increases the Metabolism of Fatty Acids in Proximal Tubular Cells through Peroxisome Proliferators-Activated Receptor (PPAR) α Agonistic Activity

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The promotion of fatty acid metabolism, to which peroxisome proliferators-activated receptor (PPAR) α contributes, has been suggested to participate in maintaining the function of renal proximal tubular epithelial cells (PTECs). The loading of fatty acids to PTECs could result in cell inflammation and cell death. A “Kampo” medicine, Boiogito (BO), is used to treat overweight women exhibiting chronic fatigue and edema in the lower extremities or knees. BO improves renal function by reducing the portion of fatty acids, thereby preventing damage to PTECs. In this study, BO and Astragalus Root (AsR), a constituent crude drug of BO, were administered orally to intravenously bovine serum albumin (BSA)-administered mice to evaluate the PPARα–cAMP responsive element binding protein (CREB) binding protein (CBP) complex binding activity and/or mRNA expression of PPARα, as quantified by enzyme-linked immunosorbent assay (ELISA) and/or polymerase chain reaction (PCR). Increases in PPARα–CBP complex binding activity and the expression of PPARα mRNA were observed not only in BO-administered mice but also in AsR-administered mice, accompanied by a decrease in the amount of renal fatty acid.

Key words Boiogito; peroxisome proliferator-activated receptor α; fatty acid metabolism; Astragalus Root; proximal tubular cell

Obesity is a risk factor for incidence of albuminuria and chronic renal disease,1,2 and an accumulating visceral fat is involved in the development of primary stage of nephropathy3 and microalbuminuria. Fatty acid is major contributor to these renal disorders caused by obesity.4 Fatty acid bound to albumin generally presents in blood, incorporated into proximal tubular epithelial cells (PTECs) following glomerular filtration. A peroxisome proliferator-activated receptor (PPAR) α has been suggested that would regulate the metabolism of fatty acid. Since the glomerular filtration rate and the renal blood flow are elevated in overweight patients,5 a large quantity of free fatty acid is loaded into PTECs.

One of the “Kampo” medicines, Boiogito (BO) is used for the remedy of overweight woman exhibiting chronic fatigues as well as edema in the lower extremities or knees. BO is frequently used in Japan, however the mechanism of action for BO remains uncertain. BO is composed of six crude drugs: Astragalus Root (AsR), Atractylodes Rhizome, Ginger, Glycyrrhiza, Jujube, Sinomenium Stem and Rhizome. In this study, we focused on renal fatty acid metabolism through the PPARα activation, to clarify the therapeutic mechanisms of BO.

MATERIALS AND METHODS

Kampo Formulae All crude drug-pieces were defined by the Japanese Pharmacopoeia XVI for decoction were purchased from Tochimoto-tenkaido (Osaka, Japan). Boiogito (BO) was prepared according to the prescription for a 1-dose of 5 g: 5.0 g AsR (lot number; 001010001), 3.0 g Atractylodes Rhizome (lot number; 009315004), 1.0 g Ginger (lot number; 005811004), 1.5 g Glycyrrhiza (lot number; 007115012), 5.0 g Sinomenium Stem and Rhizome (lot number; 009713002).

Test Samples The prescription including six composed crude drugs of BO was decocted with 600 mL of water by boiling at 600 W until it was concentrated to 300 mL. This decoction was filtered through absorbent cotton and concentrated in decompression followed by freeze-dry. Each crude drug constituting BO was decocted and concentrated in the same way as the prescription. The yields of these extract were shown in Table 1.

PPARα–cAMP Responsive Element Binding Protein (CREB) Binding Protein (CBP) Complex Binding Assay by Enzyme-Linked Immunosorbent Assay (ELISA) PPARα–CREB binding protein (CBP) complex binding activity was measured by ELISA kit (Enbio RCAS for PPARα, FUJIKURA KASEI Co., Ltd., Japan). The binding activity (%) was calculated as A/B×100, where A is the absorbance of the sample and B is that of 0.25 mM bezafibrate, a PPARα activator.

Animals Six-week old female CBA/J mice were pur-

Table 1. The Yields of Extract Boiogito and Each Crude Drug Constituting Boiogito

<table>
<thead>
<tr>
<th>Extract / Crude Drug</th>
<th>Yield of extract (g / 300 mL)</th>
</tr>
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<tbody>
<tr>
<td>Boiogito</td>
<td>4.92±0.320</td>
</tr>
<tr>
<td>Astragalus Root</td>
<td>1.54±0.027</td>
</tr>
<tr>
<td>Atractylodes Rhizome</td>
<td>1.30±0.099</td>
</tr>
<tr>
<td>Ginger</td>
<td>0.24±0.015</td>
</tr>
<tr>
<td>Glycyrrhiza</td>
<td>0.46±0.031</td>
</tr>
<tr>
<td>Jujube</td>
<td>1.88±0.026</td>
</tr>
<tr>
<td>Sinomenium Stem and Rhizome</td>
<td>0.56±0.041</td>
</tr>
</tbody>
</table>

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chased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were singly housed in plastic cages with free access to food (CE-2, CLEA, Inc., Japan) and water and were kept in a room at 25±1°C, and 55±5% humidity with a 12-h light/dark cycle. After acclimation for 1 week, they were used for experiments. All experiments were approved by the animal experimental committee of the Tohoku Pharmaceutical University (Sendai, Japan) and experimental procedures were conducted in accordance with the ethical guidelines of the University.

**Experimental Protocol in Animals** Bovine serum albumin (BSA) (Sigma-Aldrich Co., Missouri, MO, U.S.A.) at 0.5 mg/kg was dissolved in saline and filtered through an aseptic 0.45 µm cellulose acetate filter (DISMIC-13CP, ADVANTEC, Tokyo, Japan). This solution was injected intraperitoneally once a day for 48 d to all groups (5 mice in each group). BO extract at 2000 mg/kg and AsR extract at 500 mg/kg were dissolved in distilled water and injected orally once a day for 48 d. A control group was injected with distilled water.

Tail-vein blood was collected from the mice on days 0 and 49 with capillary tubes and centrifuged (6000 r.p.m., 10 min) to obtain serum. On day 49, the mice were killed under ether anesthesia and subcutaneous and visceral adipose tissue and kidney were collected. The weights of these organs were measured, and the kidney was prepared for each measurement listed below. The mice were fasted for 15 h in advance to collect blood and organ samples.

**Miscellaneous Experiments on Animals** Amount of renal fatty acid was assessed using a Free Fatty Acid Quantification Kit (Abcam, Cambridge, U.K.). Kidney (30 mg) was extracted by homogenization with 1% TritonX-chloroform solution, and the extract was centrifuged (4000 r.p.m., 4°C, 5 min). The organic phase was dried in decompression at 35°C to quantify the amount of fatty acid.

Amount of renal collagen I was measured by ELISA. Kidney (30 mg) was extracted by homogenization with 0.5% acetic acid solution, and the extract was centrifuged (4000 r.p.m., 4°C, 10 min). The supernatant was used for this measurement as well as anti-collagen Type I antibody (MILLIPORE, Darmstadt, Germany), goat anti-rabbit immunoglobulin G (IgG) antibody conjugated alkaline phosphatase (BIO RAD, CA, U.S.A.) and p-nitrophenyl phosphate (Sigma-Aldrich Co.). Amount of renal collagen I was calculated with a calibration curve using collagen I (MyBioSource, CA, U.S.A.).

Blood urea nitrogen concentration was measured with a Urea Nitrogen (BUN) Colorimetric Detection Kit (Arbor Assays, MI, U.S.A.).

**RNA Extraction and Real Time Polymerase Chain Reaction (PCR)** Using Nucleo Spin RNA kit (MACHEREY-NAGEL, Düren, Germany), total RNA was extracted from 30 mg of kidney. Total RNA was treated with an Oligotex-dT30 <Super> mRNA Purification Kit (TaKaRa Bio Inc., Shiga, Japan) to extract mRNA. mRNA was reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bio Inc.). To quantify the expression of mRNA expression for PPARα, we performed real-time PCR using Premix Ex Taq (TaKaRa Bio Inc.) and TaqMan Gene Expression Assays (Applied Biosystems, CA, U.S.A.) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The reverse-transcription and real-time PCR were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The expression of mRNA for PPARα represents the 2^−ΔΔCt method, where Ct is the threshold cycle.

**Statistics** Statistical analysis was performed using Dunnett’s multiple test after one-way ANOVA. Values with a p<0.05 were regarded as significant.

**RESULTS**

PPARα–CBP complex binding activity was measured by ELISA. CBP is involved in transcriptional coactivation of PPARα. As can be seen in Fig. 1, BO concentration-dependently potentiated the binding activity. The binding activity of each crude drug constituting BO, was measured. AsR contributed substantially to the activity (Fig. 2). Therefore, AsR is crucial for the binding activity shown by BO.

We used albumin-induced nephropathy model mouse whereby fatty acid is overloaded to PTECs by administering BSA to investigate whether BO and AsR could be up-regulated fatty acid metabolism through PPARα activation. BO and AsR solutions were administered orally to the model mice once a day for 48 d. At day 49, the amount of renal fatty acid in BO-loaded mice decreased significantly as well as AsR-
loaded mice (Fig. 3A). PCR was conducted to compare the expression of mRNA for renal PPARα (Fig. 3B). As expected, the expression of mRNA for renal PPARα induced significantly in BO-loaded mice.

Continuous uptake of albumin into PTECs by endocytosis is known to be involved in the development of renal fibrosis. Amount of renal collagen I, marker of renal fibrosis, did not differ significantly as compared with control, but tended to decrease in BO and AsR-loaded mice. Blood urine nitrogen assay also performed to examine the renal disorder by continuous uptake of albumin. The concentration of blood urine nitrogen decreased significantly in AsR-loaded mice (Fig. 4B).

Cumulative food intake (Fig. 5A) and body weight (Fig. 5B) gain have no significant effect on BO and AsR-loaded, but AsR-loaded mice tended to increase in cumulative food intake. BO-loaded mice tended to reduce the amount of subcutaneous and visceral adipose tissue, but the accumulation of visceral adipose tissue in AsR-loaded mice appeared to increase (Fig. 6).

**DISCUSSION**

PPARα, a nuclear hormone receptor belonging to the steroid receptor superfamily, expresses in the liver, heart, kidneys (proximal tubules), small intestinal mucosa, testicles, brown adipocytes, and vascular walls. PPARα is activated by fatty acids which are endogenous ligands, and peroxisome proliferators such as fibrate formulations which are chemosynthetic ligands. PPARα stimulates β-oxidation in mitochondria and peroxisomes, induction of fatty acid transporter protein. It has been reported that the promotion of fatty acid metabolism, to which PPARα contributes, is essential to maintain proximal tubular epithelial cells (PTECs) function in kidney. Fatty acid bond to albumin to reabsorb glomerular filtrate from the proximal tubule, the energy for the reabsorption is supplied by albumin endocytosis. But excess load of fatty acid to PTECs is major cause of the cell dysfunction or the cell death.

One of the “Kampo” medicines, BO is used for the remedy of overweight women exhibiting chronic fatigues as well as edema in the lower extremities or knees in Japan. To investigate whether BO has a potential of chemosynthetic ligands to prevent renal damage or to improve renal function by reduc-
ing the amount of fatty acids in PTECs, the expression levels of mRNA for renal PPARα and the amount of renal fatty acid were examined in BSA-overloaded mice.

In vitro study, a concentration-dependent activation of PPARα–CBP complex was observed at BO, and AsR exhibited the strongest binding activity in crude drugs constituting BO. In order to investigate whether BO and AsR activate PPARα in vivo, renal fatty acid and PPARα expression of the mice, which were overloaded fatty acid in PTECs by intraperitoneal administration of BSA bound to fatty acid, were measured. The mouse, which was created by heterologous-protein overload such as BSA, is an established model used for investigating tubulointerstitial damage.4) The amount of renal fatty acid was decreased significantly after the administration of BO or AsR. Interestingly, BO-loaded mice increased significantly in the expression of mRNA for renal PPARα. Despite the kidney damage by fatty acid-free BSA are weak, fatty acid-binding BSA-treatment leads to severe proximal tubular injury, suggesting that tubular injury by fatty acid-binding BSA is associated with the breakdown of fatty acid homeostasis in proximal tubular epithelial cells.4) In addition, significant expression of mRNA for renal PPARα was observed with PPARα activator-loaded mouse.9) Our findings indicate that BO promotes not only as a ligand for PPARα in PTECs but also the expression of mRNA for PPARα, implying that BO suppress the renal dysfunction caused by fatty acid, and that AsR should play central crude drug for PPARα activator in BO.

The complex signal transduction cascades in PTECs are initiated by albumin uptake into PTECs. In the process of these cascades, the expression of transforming growth factor-β type II receptor (TGF-β II R) is upregulated.5) It’s already known that TGF-β II R is involved in collagen synthesis which causes renal fibrosis, but PPAR agonists contribute to preventive effect of renal fibrosis.10) In our experiment, BO and AsR inhibited renal fibrosis synthesis compared with the absence of PPARα agonist. In addition, AsR reduced the concentration of blood urea nitrogen, suggesting that AsR may prevent renal dysfunction induced by BSA-overload.

BO suppresses significantly the increase in weight, visceral fat mass, and expansion of adipocytes in Sprague-Dawley (SD) rats.11) In our study, BO tended to decrease in visceral fat weight, whereas AsR increased the accumulation of amount of visceral fat regardless of a decrease in renal fatty acid. AsR contains ‘phytoestrogens’ whose structure is similar to gonadal hormones, known that its phytoestrogens show either estrogenic or anti-estrogenic effects in estrogen receptor (ER)-α and ER-β.12) It has been suggested that AsR would induce the accumulation of visceral fat in response to ER-β stimulation.

In conclusion, BO and AsR improve fatty acid metabolism in PTECs through activation of PPARα–CBP complex and the increased expression of PPARα, thus these results raise the possibility that BO may prevent the renal dysfunction by fatty acid toxicity associated with obesity.

Conflict of Interest The authors declare no conflict of interest.

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