Polymethoxyflavonoids from Kaempferia parviflora Induce Adipogenesis on 3T3-L1 Preadipocytes by Regulating Transcription Factors at an Early Stage of Differentiation

Takumi Horikawa, a, b, h Tsutomu Shimada, c, # Yui Okabe, b, Kaoru Kinoshita, b, Kiyotaka Koyama, b Ken-ichi Miyamoto, a, Koji Ichinose, c, Kunio Takahashi, b and Masaki Aburada a, c

Regular Article

© 2012 The Pharmaceutical Society of Japan

Received November 10, 2011; accepted February 22, 2012; published online February 29, 2012

We previously reported that Kaempferia parviflora Wall. ex Baker (KP) and its ethyl acetate extract (KPE) improve various metabolic disorders in obesity-model mice. However the mechanism is not certain, and, in this study, in order to elucidate the mechanism of the suppressive effect of KP on fat accumulation, we focused on adipocytes, which are closely linked to metabolic diseases. The finding was that KPE and its components, 3,5,7,4-tetramethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone, strongly induced differentiation of 3T3-L1 preadipocytes to adipocytes. The above two polymethoxyflavonoids (PMFs) also induced adiponectin mRNA levels, and release of adiponectin into the medium. In addition, these PMFs enhanced the expression of peroxisome proliferator-activated receptor γ (PPARγ), but did not show PPARγ ligand activity. We then investigated the expression of the differentiation-regulator located upstream of PPARγ. Expression of CCAAT/enhancer-binding protein (C/EBP) β and -δ mRNA, a transcriptional regulator of PPARγ, was induced, and expression of GATA-2 mRNA, a down-regulator of adipogenesis, was suppressed by these PMFs. These functions of the KP PMFs that enhance adipogenesis and secretion of adiponectin are, to some extent at least, involved in the mechanisms of anti-metabolic disorders effects.

Key words Kaempferia parviflora; adipogenesis; polymethoxyflavonoid; adiponectin; 3T3-L1 cell

Obesity is a serious medical and social problem throughout the world, and is a causal factor for type-2 diabetes, hypertension, and hyperlipidemia. In obese people, adipose tissue forms hypertrophic adipocytes that synthesize inflammatory cytokines such as resistin, monocyte chemotactic protein-1 (MCP-1), and interleukin-6 (IL-6). These inflammatory adipocytokines induce insulin resistance and down-regulation of adiponectin. Adiponectin is expressed by small adipocytes, and improves insulin sensitivity. Therefore, an increased number of small adipocytes is important for maintaining metabolic homeostasis.

Small adipocytes are formed by differentiation from preadipocytes. Members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, C/EBPβ and -δ, and peroxisome proliferator-activated receptor γ (PPARγ) play critical roles in adipogenesis. During adipogenesis, C/EBPβ and -δ are rapidly induced by stimulation of hormonal signals, and function as transcriptional regulators of PPARγ. PPARγ acts as the master regulator in adipogenesis, and induces fatty acid-binding protein (FABP) 4, glucose transporter (GLUT) 4, and adiponectin expression. In contrast, factors that inhibit adipocyte differentiation at an early stage of differentiation include the GATA family, delta-interacting protein A (DIPA), and C/EBP homologous protein (CHOP10). In summary, differentiation of adipocytes is regulated by various transcriptional activators and suppressors.

Kaempferia parviflora Wall. ex Baker (KP) is a plant that belongs to the family zingiberaceae; is indigenous to Laos and Thailand; and has been used as a folk medicine to lower blood glucose levels, improve blood flow, and increase vitality. The ethanolic extract of KP has stomach-protecting, antioxidant, and antiadipogenic effects. KP contains polymethoxyflavonoid (PMF) and PMF glycosides and chalcone derivatives. Among the PMFs in KP, 5,7-dimethoxyflavone suppresses prostaglandin biosynthesis, and 5,7,4’-trimethoxyflavone and 5,7,3’,4’-tetramethoxyflavone have antiproliferative and antifungal effects. Furthermore, the chalcone derivatives (-)-hydroxypanduratin A and (-)-panduratin A show anti-inflammatory effects. Previously, we reported that KP and its ethyl acetate extract (KPE) have preventive effects on obesity and various related downstream metabolic disorders in Tsumura, Suzuki, obese, diabetes (TSOD) mice, this being a multifactorial genetic-disease animal model in which metabolic diseases develop spontaneously. To summarize, KP is a crude medicine with various potential therapeutic effects, but its anti-obesity mechanisms have not been fully elucidated. Furthermore, there have been no reports of the effects of KP on adipocytes, which are involved in metabolic diseases.

In the present study, we investigated the effects of KPE on differentiation of adipocytes from 3T3-L1 preadipocytes. In addition, we clarified the active constituents, and the mechanisms of adipogenesis.

MATERIALS AND METHODS

Materials KP was purchased from LAO JTL (Vientiane, Laos People’s Democratic Republic). Trogilitazone was purchased from Sigma-Aldrich (MO, U.S.A.). Fetal bovine serum (FBS), 1% penicillin (10000 unit/mL)-streptomycin (10000 mg/mL) mixture, Dulbecco’s modified Eagle’s medium (DMEM), and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (CA, U.S.A.). Oil

* To whom correspondence should be addressed. e-mail: aburada@musashino-u.ac.jp
© 2012 The Pharmaceutical Society of Japan
Red O was purchased from Sigma-Aldrich. Insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). SYBR Green PCR Master Mix was purchased from QIAGEN (Dusseldorf, Germany).

**Methods. Preparation of KP Ethyl Acetate Extracts (KPE)** Extraction of 100 g of dried KP rhizomes was carried out five times by reflux for 1 h using 1 L of methanol. The solvent was removed under reduced pressure to obtain 12.56 g of methanol extract, which was then concentrated, and partitioned between ethyl acetate and water. Each partition fraction was then evaporated to dryness in vacuo, giving residues of 8.2 and 3.7 g from the ethyl acetate and water fractions, respectively.

**Isolation Procedure for KP PMFs** Chromatography of the KP methanol extract was carried out on silica gel, using hexane and 100% acetone in a 20:1 ratio, to give the following components, as shown in Table 1: 1: 5-hydroxy-3,7-dimethoxyflavone: 206.7 mg, 1.6%, w/w. 2: 3,5,7-trimethoxyflavone: 54.4 mg, 0.4%, w/w. 3: 3,5,7,4'-tetramethoxyflavone: 340.4 mg, 2.7%, w/w. 4: 5-hydroxy-3,7,4'-trimethoxyflavone: 135.2 mg, 1.1%, w/w. 5: 5,7-dimethoxyflavone: 163.3 mg, 0.3%, w/w. 6: 5-hydroxy-7,4'-dimethoxyflavone: 40.2 mg, 0.3%, w/w. 7: 5-hydroxy-3,7,3',4'-tetramethoxyflavone: 14.7 mg, 0.1%, w/w. 8: 3,5,7,3',4'-pentamethoxyflavone: 802.3 mg, 6.4%, w/w. 9: 5-hydroxy-7-methoxyflavone: 70.8 mg, 0.6%, w/w. 10: 5,7,4'-trimethoxyflavone: 396.5 mg, 3.2%, w/w. 11: 5,3'-dihydroxy-3,7,4'-trimethoxyflavone: 16.4 mg, 0.1%, w/w. 12: 5,4'-dihydroxy-7-methoxyflavone: 16.4 mg, 0.1%, w/w. The structures of Components 1 to 12 were determined by comparing the 1H- and 13C-NMR data with those in previous reports.

**Differentiation from 3T3-L1 Preadipocytes** The 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS and penicillin (100 unit/mL)–streptomycin (100 μg/mL). Preliminary experiments on differentiation agents indicated that KPE showed strongest differential effect when combing insulin and dexamethasone (DEX) as differentiation agents (data not shown). Therefore, preadipocytes were differentiated in the following way. After 2 d of confluence, differentiation was induced by addition of 0.25 μM DEX and 1 μg/mL insulin for 96 h, after which DMEM containing 10% FBS was added on alternate days as a nutrient supply. To examine the effects on differentiation, KPE and KP PMFs were dissolved in dimethyl sulfoxide (DMSO) and added to the medium for the first 4 d. Control groups were added DMSO only (final DMSO conc. 0.2%).

**Measurement of Triglyceride (TG) Content** Eight days after induction of differentiation, the cells were washed with phosphate-buffered saline (PBS), harvested in 1% triton-X in PBS, and lysed by sonication. The TG levels in the lysates were quantified using the triglyceride E-test Wako kit (Wako), in accordance with the manufacturer’s instructions. The TG was normalized to the protein content determined by the Lowry method.

**Oil Red O Staining** Eight days after induction of differentiation, the cells were washed twice with PBS, fixed with 10% formalin for 10 min, and again washed twice with PBS. The cells were stained with Oil Red O for 20 min, washed twice with PBS, and then photographed.

**Adiponectin Concentration in the Medium** Eight days after induction of differentiation, the medium was collected. Adiponectin in the medium was measured using Mouse Adiponectin/Acrp30 (R&D Systems, U.S.A.).

**PPARγ Ligand Assay** The PPARγ ligand effects of KP PMFs were examined using an EnBio RCAS PPARγ Kit (En-Bio Tec Laboratories Co., Ltd., U.S.A.), in accordance with the manufacturer’s instructions.

**Quantitative Real-Time Polymerase Chain Reaction (PCR)** The total RNA from 3T3-L1 cells was isolated using an RNeasy Mini Kit (QIAGEN). Reverse transcription of 1 μg of total RNA was carried out using a Reverse Transcription Kit (QIAGEN), to obtain cDNA. SYBR Green-based quantitative real-time PCR was performed using a Mini Option Model CFD3121 (Bio-Rad Laboratories Inc., U.S.A.), in accordance with the manufacturer’s instructions. The primer sequences used in the real-time PCR were as follows: β-actin: Forward: 5'-CCA TCC TGC TGC TGG ACC TG-3'. Reverse: 5'-TTC

Table 1. The Structures of PMFs Isolated from KP

<table>
<thead>
<tr>
<th>Component</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comp. 1</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Comp. 2</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Comp. 3</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 4</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 5</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Comp. 6</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 7</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 8</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 9</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Comp. 10</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 11</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Comp. 12</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>
CCT CTC AGC TGT GGT GG-3', PPARγ: Forward: 5'-TGA ACG TGA AGC CCA TCG AG-3'. Reverse: 5'-CTG GGC GAA CAG CAG AGA GG-3'. Adiponectin: Forward: 5'-GGC TCT GTC CTC CTC CAT CT-3'. Reverse: 5'-GTA GTC GAC GAC CCA CAA CAT C-3'. C/EBPβ: Forward: 5'-GCC AAG ACC GTG ACC A-3'. Reverse: 5'-GCC CTC GAC CAC CTC-3'. C/EBPδ: Forward: 5'-GCC CGA GCG CAA CAA CAT C-3'. Reverse: 5'-CCA GGT CCC GCG TGA GCT-3'. GATA-2: Forward: 5'-GGA GAC GAT TGT GCT GAG TCA A-3'. Reverse: 5'-TTA GGC TGG TCT TCT GCA GAA ACT-3'. GATA-3: Forward: 5'-CCG GCG GTA CCA CCT TTC T-3'. Reverse: 5'-GCT GGT ACG CTG CTA CCT TCA T-3'.

**Immunoblotting**

Equal amounts of protein from 3T3-L1 cells were loaded on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in the presence of a protease-inhibitor cocktail, and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blot was incubated overnight at 4°C in a washing buffer [0.8% NaCl, 20 mM Tris–HCl (pH 7.5), 0.1% Tween 20] and primary antibody. The blots were then washed five times with the washing buffer, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive protein was visualized using an ECL-plus kit (GE Healthcare U.K., U.K.). The primary antibodies used were rabbit anti-PPARγ (Cayman, CO, U.S.A.) and mouse...
Fig. 3. Effects of Components 3 and 8 on Differentiation, Expression of PPARγ and Adiponectin in 3T3-L1 Cells

(A) Triglyceride concentrations, (B) expression of PPARγ mRNA, (C) PPARγ protein, (D) expression of adiponectin mRNA, and (E) release of adiponectin into media in 3T3-L1 cells 8d after induction of differentiation were analyzed as described in Materials and Methods. Abbreviation tro: troglitazone. Values represent means±S.D. (n=4). *p<0.05, **p<0.01, ***p<0.001: significantly different from the control group.

STATISTICAL ANALYSIS Results were expressed as means± standard deviations (S.D.). Statistical analyses of these data were carried out using one-way analysis of variance (ANOVA), followed by Dunnet’s test. All statistical analyses were performed using PRISM software (version 5.0).

RESULTS

KPE Induces Differentiation of 3T3-L1 Preadipocytes to Adipocytes The effects of KPE on adipocyte differentiation from 3T3-L1 preadipocytes were monitored by measuring intracellular TG accumulation, and staining lipid droplets using Oil Red O. Figure 1A shows the results for the accumulated TG contents. 3T3-L1 adipocytes treated with 1 or 3 μg/mL KPE showed significant, dose-dependent increases in accumulation of TG and lipid droplets (Fig. 1B).

Components 3 and 8 Are Active Constituents of KPE with Respect to Effects on Differentiation of 3T3-L1 Preadipocytes To identify the active constituents of KPE, we investigated the effects of KP PMFs on differentiation of 3T3-L1 cells. Figure 2A shows that treatment with 30 μM Component 2, 3 or 8 markedly increased intracellular TG accumulation in comparison with the control. Since Components 3 and 8 are main constituents of KPE,21) we suggest that Components 3 and 8 are active constituents with respect to differentiation of 3T3-L1 preadipocytes. The numbers of lipid droplets in the cells detected using Oil Red O staining were increased by treatment with Components 3 or 8, in comparison with the control (Fig. 2B). In addition, Components 3 and 8 increased intracellular TG in a dose-dependent manner (Fig. 3A), and the genetic expression of PPARγ and its target, adiponectin, were increased in a dose-dependent manner (Figs. 3B, C). Furthermore, the expression of PPARγ proteins by the cells, and the presence of adiponectin in the medium, were increased in a dose-dependent manner.

KP PMFs Are Not PPARγ Ligands We examined whether KP PMFs act as ligands for PPARγ, using an Enbio RCAS PPARγ kit. A known specific PPARγ ligand, troglitazone, used as a positive control, showed strongly, dose-dependent activation (Fig. 4). In contrast, none of the KP PMFs showed activation, at any dose.

Components 3 and 8 Regulate Early-Stage Differentiation Signals We investigated the expression of C/EBPβ and -δ, which is positioned upstream of PPARγ and enhances the expression of PPARγ. Treatment with 30 μM Components 3 and 8 significantly increased the expression of C/EBPβ and -δ mRNA in comparison with the control (Fig. 5). In addition, we investigated the expression of GATA-2/3, which are transcription factors that repress adipocyte differentiation, and found that treatment of cells with Component 8 markedly reduces expression of GATA-2 mRNA to less than 60% of the control, and slightly reduces expression of GATA-3 mRNA (Fig. 6). In contrast, Component 3 did not affect the expression of GATA-2/3 mRNA.

DISCUSSION

In this study, we demonstrated that KPE and KP PMFs induce adipocyte differentiation from 3T3-L1 preadipocytes similarly to troglitazone, which is an antidiabetic agent.

To investigate the effects of differentiation induction on 3T3-L1 preadipocytes, we monitored them by measuring TG accumulation and Oil Red O-staining lipid droplets. KPE showed significant, dose-dependent increases in accumulation of TG. The results suggested that KPE induces adipogenesis. Next, in order to identify the KPE constituents that are active with respect to adipocyte differentiation, we assessed the effects of KP PMFs on differentiation of 3T3-L1 cells. Lipid droplets were significantly increased by treatment with Components 2, 3 and 8, in comparison with the control. The common structural elements of the three active PMFs, the 3,5,7-trimethoxy substitutions, would be an important factor on a differentiation-inducing activity. Although much flavonoids have been reported as natural compounds, 5-methoxy flavone showing differentiation-inducing activity in this paper is characteristic structure as KP PMFs in natural sources. Since Components 3 and 8 are mainly isolated from KPE,21) we suggest that Components 3 and 8 are active constituents with respect to differentiation of 3T3-L1 preadipocytes. In addition, Components 3, 8 significantly increased PPARγ expression and adiponectin levels. These results suggest that KP PMFs, especially Components 3 and 8, strongly induce adipogenesis, and secretion of adiponectin, and are of similar effects to troglitazone.

To investigate the molecular mechanisms of the differentiation-inducing effects of KP PMFs, we examined whether KP PMFs act as ligands for PPARγ. In the PPARγ ligand assay, KP PMFs showed no PPARγ ligand activity, even at high doses, despite troglitazone showing PPARγ ligand activity. However, as KP PMFs induced PPARγ expression, we investigated whether KP PMFs induce the expression of transcription factors upstream of PPARγ. The expression of PPARγ was found to be increased by C/EBPβ and -δ, which are positioned upstream of PPARγ.25) In this study, Components 3 and 8 increased the expression of C/EBPβ and -δ mRNA levels during early-stage differentiation, to significant degree, respectively.

**Fig. 4. PMFs Isolated from KP Are Not PPARγ Ligands**

The PPARγ ligand activities of PMFs isolated from KP were measured using a nuclear receptor cofactor assay system, as described in Materials and Methods. Abbreviation troc: troglitazone.
Nobiletin, a polymethoxylated flavone found in citrus fruits such as *Citrus reticulata* and *C. depressa*, has been reported to induce differentiation in connection with adipogenesis, by induction of C/EBPβ expression, with no function as a PPARγ ligand. The structure of nobiletin closely resembles the KP PMFs, especially Components 3 and 8. Therefore, these results showed that induction of C/EBPβ and -δ by Components 3 and 8 leads to enhanced adipogenesis at an early stage of differentiation.

In preadipocytes, there are certain transcriptional factors that suppress differentiation of adipocytes at an early stage, GATA-2 and GATA-3, are known as transcription factors that suppress adipogenesis by direct binding to the C/EBPβ protein and to the GATA-binding site on the PPARγ-promoter. Therefore, decrease in expression of GATA-2 and/or GATA-3 is linked to induction of adipogenesis, so we examined whether KP PMFs affect the expression of GATA-2 and/or GATA-3. The finding was that Components 8 and 3 reduced the expression of GATA-2 mRNA by significant and slight degrees, respectively. Sakuranetin (5,4′-dihydroxy-7-methoxyflavone), which is extracted from the bark of the cherry tree, has been reported to induce differentiation of 3T3-L1 preadipocytes by reducing the expression of GATA-2/3. In this study, Component 8, which has a flavonoid molecular skeleton similar to that of sakuranetin, was found to suppress GATA-2 expression. Components 3 and 8 increased the expression of C/EBPβ and -δ mRNA levels, and decreased that of GATA-2/3 mRNA levels. These results suggest that KEPE PMFs regulated these transcription factors or their upstream factors. Previous studies have indicated that C/EBPβ is potentially required for mitotic clonal expansion (MCE), which is the necessary step for the terminal differentiation of 3T3-L1 preadipocytes. In this study, since Components 3 and 8 increased the expression of C/EBPβ mRNA levels at an early stage of differentiation, further studies will be required to research for MCE because these components may regulate several rounds of division.

In the present study, we demonstrated that KP PMFs strongly induce differentiation of 3T3-L1 cells. KP PMFs induced expression of PPARγ without PPARγ ligand activity. The expression of PPARγ was induced, at least in part, by activation of induction pathway C/EBPβ and -δ, and by inhibition of suppressive pathways of the GATA family, especially GATA-2. The various functions of the KP PMFs that enhance adipogenesis and secretion of adiponectin are, to some degree at least, linked to the mechanisms of anti-metabolic disorder effects.

REFERENCES

3) Suganami T, Nishida J, Ogawa Y. A paracrine loop between...


