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Suppression of adipocyte hypertrophy by polymethoxyflavonoids isolated from *Kaempferia parviflora*

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ABSTRACT

We previously demonstrated that ethyl acetate extracts of *Kaempferia parviflora* Wall. Ex Baker (KPE) improve insulin resistance in TSOD mice and showed that its components induce differentiation and adipogenesis in 3T3-L1 preadipocytes. The present study was undertaken to examine whether KPE and its isolated twelve components suppress further lipid accumulation in 3T3-L1 mature adipocytes. KPE reduced intracellular triglycerides in mature adipocytes, as did two of its components, 3,5,7,3′,4′-pentamethoxyflavone and 5,7,4′-trimethoxyflavone. Shrinkage of lipid droplets in mature adipocytes was observed, and mRNA expression levels of adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) were up-regulated by these two polymethoxyflavonoids (PMFs). Furthermore, the protein expression level of ATGL and the release level of glycerol into the cell culture medium increased. In contrast, the peroxisome proliferator-activated receptor γ (PPARγ) agonist, troglitazone, did not decrease intracellular triglycerides in mature adipocytes, and the mRNA expression level of PPARγ was not up-regulated in mature adipocytes treated with the two active PMFs. Therefore, suppression of lipid accumulation in mature adipocytes is unlikely to be enhanced by transcriptional activation of PPARγ. These results suggest that KPE and its active components enhance lipolysis in mature adipocytes by activation of ATGL and HSL independent of PPARγ transcription, thus preventing adipocyte hypertrophy. On the other hand, the full hydroxylated flavonoid quercetin did not show the suppressive effects of lipid accumulation in mature adipocyte in the same conditions. Consequently, methoxy groups in the flavones are important for the activity.

Introduction

Obesity or excess accumulation of visceral fat is often caused by excessive calorie intake and insufficient exercise, and is closely associated with insulin resistance. It is currently believed that insulin resistance plays a role in lifestyle diseases such as type-2 diabetes, hypertension and dyslipidemia, and represents an important risk factor for arteriosclerosis. Obesity-related insulin resistance is affected by an increase in the number and size of hypertrophied adipocytes. Adipose tissues of obese patients consist of hypertrophied adipocytes which are induced inflammation by macrophage infiltration. Inflammatory adipose tissue secretes insulin-resistance factors such as tumor necrosis factors (TNF)-α and interleukin (IL)-6 and insufficient insulin-sensitizing factors such as adiponectin and leptin. In contrast, mature adipose tissue (not be infiltrated by macrophages) secretes adequate insulin-sensitizing factors (Kadowaki and Yamauchi 2005; Weisberg et al. 2003). In addition, normal adipose tissues consist of small adipocytes which are differentiated from preadipocytes. Therefore, the regulation of preadipocyte differentiation and adipocyte hypertrophy are useful strategies for preventing obesity and insulin resistance.

Excessive calorie intake promotes the inhibition of lipolysis and the accumulation of triglycerides in adipocyte lipid droplets. Lipolysis in adipocytes is regulated in a step-wise fashion by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase. These lipases locate around the lipid droplet and HSL is activated by the adrenaline effect. Triglycerides are hydrolyzed by lipases into fatty acids and glycerol, and then these cleavage products are metabolized to produce energy. Thus,

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modulation of lipolysis plays an important role in energy homeostasis in living organisms and maintains the size of adipocytes.

Rhizomes of *Kaempferia parviflora* (KP) Wall. ex Baker (Zingiberaceae) have been used by the indigenous people of Thailand and Laos as a folk medicine to lower blood glucose levels, improve blood flow, and increase vitality. Extracts of KP rhizomes have been reported to have various pharmacological activities such as anti-gastric ulcer (Rujjanawate et al. 2005), anti-allergic (Tewtrakul and Subhadhirasakul 2007), anti-inflammatory (Sae-Wong et al. 2011) and vascular relaxant activities (Malakul et al. 2011). Such extracts also induce apoptosis in leukemic cells (Banjerdpongchai et al. 2008), modulate multidrug resistance in cancer cells (Patanasethanont et al. 2007), and, as we reported previously, exert preventive effects on obesity-related insulin resistance and its downstream symptoms (Akase et al. 2011). Compounds isolated from KP include polymethoxyflavonoids (PMFs) and phenolic glycosides, kaempferiaoside (Chaipech et al. 2011; Chaipech et al. 2012; Pattara et al. 2009; Sutthanut et al. 2007). Furthermore, HPLC analysis of ethyl acetate extracts of KP (KPE) revealed that KPE contains 12 predominant PMFs (Shimada et al. 2011). Of these, 3,5,7,4′-tetramethoxyflavone and 3,5,7,3′,4′-pentamethoxyflavone have an effect similar to nobiletin. Nobiletin strongly promotes differentiation of 3T3-L1 preadipocytes to mature adipocytes through the transcriptional activation of peroxisome proliferator-activated receptor γ (PPARγ), yet lacks ligand-binding activity (Horikawa et al. 2012; Saito et al. 2007). Therefore, these two PMFs in KPE are expected to affect adipose tissues and up-regulate the secretion of insulin-sensitizing factors by increasing the differentiation of mature adipocytes. However, the effects of KPE and its component PMFs on post-differentiated stages of adipocytes remain to be fully elucidated.

In this study we examined whether KPE affects the process of adipocyte hypertrophy from differentiated small adipocytes and investigated its active constituents and mechanism of action.

Materials and methods

Materials

KP (rhizome) was purchased from LAO J.T.L. (Vientiane, Lao People's Democratic Republic, Laos) in 2005. A voucher specimen of the rhizome (No. MU01) was deposited at the Research Institute of Pharmaceutical Sciences, Musashino University (Tokyo, Japan). Extraction of KPE and the isolation of 12 flavonoids (Table 1) were performed as described previously (Shimada et al. 2011). Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) (Table 1) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Troglitazone was purchased from Sigma–Aldrich (MO, U.S.A.). These compounds were soluble in dimethyl sulfoxide (DMSO); the dissolved concentration of DMSO in the culture medium was less than 0.2%.

Cell culture

3T3-L1 murine preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2 until confluence. Two days after confluence, designated as day 0, the cells were switched to differentiation medium (DM) containing 1 μM insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone (DEX) in DMEM for another 2 days. Then, the cell culture medium was replaced with DMEM containing 1 μM insulin and incubation was continued for another 2 days. Next (on day 4), the cells were maintained in DMEM with medium changes every 2 days, after which mature adipocytes containing lipid droplets formed. On day 8, when differentiation was almost complete, the cells were treated with various concentrations of KPE, PMFs or vehicle for up to 4 days (until day 12). All media contained 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml).

Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. On day 12, the 3T3-L1 mature adipocytes treated with KPE, PMFs or vehicle were switched to a culture medium containing 10% phosphate-buffered saline (PBS)-buffered MTT (5 mg/ml) solution and incubated at 37 °C for an additional 4 h. Thereafter, the medium was removed and purple formazan crystals were dissolved in DMSO and read absorbance at 595 nm using a microplate reader (Bio-Rad Laboratories Inc., U.S.A.). Results were standardized using the vehicle group value.

Measurement of intracellular triglyceride

The 3T3-L1 mature adipocytes were washed with PBS and scraped into 1% triton-X in PBS. The cells were homogenized by sonication and the lysate was assayed for intracellular triglyceride levels using the Triglyceride E-Test Wako (Wako Pure Chemicals, Osaka, Japan) in accordance with the manufacturer's instructions. The protein concentrations of the cell lysates were determined by the Lowry method (Lowry et al. 1951). The results were expressed as the ratio of the triglyceride concentration to the protein concentration.

Quantitative real-time PCR (qRT-PCR)

Total RNA of 3T3-L1 mature adipocytes was extracted using a Tissue Total RNA Mini Kit (Favorgen Biotech Co., Ping-Tung, Taiwan) following the manufacturer's instructions. Reverse transcription (RT) was performed with a PrimeScript® RT reagent Kit (Takara, Shiga, Japan) using an equal amount of total RNA in each sample. After the RT procedure, the reaction mixture (cDNA) was used for SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR). The reaction and quantitative analysis were conducted using the SYBR® Premix Ex Taq™ (Takara) protocol using a MiniOpticon™ Real Time-PCR Detection System (Bio-Rad Laboratories Inc.,). The results were normalized to the expression of the housekeeping gene, β-actin. The primer sequences used in qRT-PCR were:

β-actin (NM_007393)
Forward: 5′-CCATCCTGCGTCTGGACCTG-3′
Reverse: 5′-TTCCCTCTCAGCTGTGGTGG-3′
Adiponectin (NM_009605)
Forward: 5′-GGCTCTGTGCTCCTCCATCT-3′
Reverse: 5′-AGAGTCGTTGACGTTATCTGCATAG-3′
ATGL (NM_001163689)
Forward: 5′-AACACCAGCATCCAGTTCAA-3′
Reverse: 5′-GGTTCAGTAGGCCATTCCTC-3′
HSL (NM_010719)
Forward: 5′-CCTACTGCTGGGCTGTCAA-3′
Reverse: 5′-CCATCTCGCACCCTCACT-3′
PPARγ (NM_011146)
Forward: 5′-TGAACGTGAAGCCCATCGAG-3′
Reverse: 5′-CTTGGCGAACAGCTGAGAGG-3′

Oil red O staining

On day 12, the 3T3-L1 mature adipocytes plated onto 6-well plates were washed twice with PBS, fixed with Mildform® (Wako Pure Chemicals) for 10 min, and then washed twice with PBS. After