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In vitro studies on anti-obesity activity of Korean Memilmuk through AMPK activation

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Abstract

The anti-obesity effect of Korean traditional food, Memilmuk, was examined through inhibition of differentiation of 3T3-L1preadipocytes by buckwheat flour extract. Oil-Red O staining showed that lipid accumulation in adipocytes was reduced upon adding buckwheat flour extract, indicating effective inhibition of adipocyte differentiation. Buckwheat flour extract also inhibited the expression of adipogenic transcription factor, peroxisome proliferator-activated receptor γ (PPARγ), and AMP-activated protein kinase (AMPK), an intracellular regulator of energy balance. Overall, the anti-obesity effect of Korean Memilmuk might be mediated through down-regulation of PPARγ expression via AMPK activation by buckwheat flour.

Key words

3T3-L1 cells, AMPK, Anti-obesity, Memilmuk

Introduction

Obesity, caused due to imbalance between energy intake and expenditure, has become a major global public health issue due to its association with many life-threatening diseases (Ahn *et al.,* 2008). It is an important risk factor for chronic metabolic diseases including type 2-diabetes, cardiovascular disease and specific cancers (Ahn *et al.,* 2008). The major cellular features of obesity are hypertrophy and hyperplasia, which lead to increases in the size and number of adipose tissue composed of differentiated adipocytes (Fernyhough *et al.,* 2005). Moreover, obesity is a state of chronic low-grade inflammation that is initiated by morphological changes in adipose tissue. Adipogenesis, by which preadipocytes become adipocytes, requires multiple signaling pathways for development of phenotypes for mature adipocytes. Two transcriptional factors governing the expression of adipogenesis-associated markers, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) are known to be crucial for adipogenesis (Gregoire, 2001). AMP-activated protein kinase (AMPK) is a master sensor of energy homeostasis that play a pivotal role in regulating food intake, body weight, glucose uptake and lipid metabolism (Park *et al.,* 2013). AMPK is activated in response to low energy status that depletes ATP supplies, including low glucose, hypoxia and ischemia. AMPK, as a heterotrimeric Ser/Thr kinase, is composed of a catalytic subunit and two regulatory subunits. Upon phosphorylating Thr within the catalytic subunit, AMPK stimulates ATP-producing catabolic pathways such as glucose and fatty acid oxidation, while simultaneously suppressing ATP-consuming anabolic pathways such as cholesterol, fatty acid and triacylglycerol biosynthesis (Ai *et al.,* 2002; Dagon *et al.*, 2006). Moreover, AMPK inhibits fatty acid biosynthesis through inhibiting acetyl-CoA carboxylase, an enzyme required for synthesis of malonyl CoA. AMPK-induced lipid regulation is also exerted by inhibiting glycerol-3-phosphate acyltransferase, an integral enzyme in triglyceride accumulation.

Mouse 3T3-L1 preadipocyte system has been widely used as an in vitro culture model for the study of adipocyte-

specific differentiation, due to its potential to differentiate between fibroblasts and adipocytes (Patel and Lane, 1991; Kim and Lee, 2012). The wide variety of natural products treated in the 3T3-L1 system revealed that AMPK activators from natural compounds inhibit adipocyte differentiation through blocking the expression of adipogenic transcription factors PPARγ, SREBP1c and C/EBPγ, induce apoptosis and promote glucose uptake (Daval *et al.,* 2006; Fang *et al.,* 2008). Moreover, AMPK has been considered a therapeutic target for the prevention and treatment of obesity. Coenzyme Q10 (Lee *et al.,* 2012), quercetin (Ahn *et al.,* 2008), flavonol glycoside (Ha do *et al.,* 2010) and (-)-epigallocatechin-3-gallate (EGCG) (Chan *et al.,* 2011) have been reported to inhibit adipogenesis *via* AMPK activation.

Memilmuk is a Korean traditional food made from Buckwheat (*Fagopyrum esculentum*) flour is mainly composed of starch. Buckwheat is highly nutritious pseudocereal known as a dietary source of starch, protein, vitamins, dietary fiber and essential minerals (Sedej *et al.,* 2012). Especially, phenolic compounds including six flavonoids; rutin, orientin, vitexin, quercetin, isovitexin and isoorientinare are found in abundance (Dietrych-Szostak and Oleszek, 1999; Zhang *et al.,* 2012). Moreover, buckwheat is also rich in many rare components, including flavones, phytosterols, D-chiro-inositol and myo-inositol (Zhang *et al.,* 2012). Buckwheat's significant health promoting activities include anti-oxidative, anti-inflammatory, anti-hypertensive as well as anti-adipogenic effects, and these activities are due to the presence of flavonoids including rutin (Qu *et al.,* 2013) and buckwheat proteins with high content of essential amino acids (Li and Zhang, 2001; Choi *et al.*, 2006; Tomotake *et al.,* 2006). Korean Memilmuk as a functional food has been expected to have anti-obesity activity, however, information on adipogenic inhibition by Memilmuk at the molecular and cellular level is not available. In the present study, anti-obesity activity of Memilmuk at molecular and cellular level in 3T3-L1 preadipocytes using buckwheat flour extract was examined.

Materials and Methods

Preparation of buckwheat flour extract: Buckwheat flour extract was prepared from commercial Korean buckwheat flour (Bong Pyeong Agricultural Union Corporation, Gangwon-do, Korea). Buckwheat flour was dissolved in 80% ethanol for 24 hrs for complete elucidation of active ingredients to dissolve in ethanol. The extract was then filtered and solvent from the extract was removed using rotary vacuum evaporator at 50ºC. Finally, buckwheat flour extract for Memilmuk (EM) was frozen dried and then used for *in vitro* study.

Cell culture and differentiation of 3T3-L1 cells: Mouse 3T3-L1 cells (American Type Culture Collection, Rockville, USA) were maintained in Dulbeco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 cell incubator. Preadipocyte 3T3-L1 cells were grown in 24-well plates until cells reached confluency. Confluent 3T3-L1 preadipocytes were induced to differentiate using medium containing 5 μg ml^{-1} insulin, 0.5 mM 1-methyl-3-isobuthyl xanthine, and 0.25 mM dexamethasone with 10% FBS in DMEM. After 48 hr, culture medium was changed to DMEM supplemented with 10% FBS and 5 μg ml^{-1} insulin for 4 days. Four days later, the medium was then replenished with DMEM and 10% FBS for 2 more days. To study the effects of EM, cell cultures were supplemented with EM at different concentrations during the time of stimulation of adipose conversion with differentiation mixture (day 4 to day 12) (Lee *et al.*, 2012).

Cell viability assay: To determine the effects of EM on 3T3-L1 cell viability, cell viability assay was performed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After exposure of EM, cells were washed and treated with MTT (5 mg ml^{-1}) to determine cell viability. After formazan formation by MTT, 100 μl dimethyl sulfoxide was added and absorbance was read at 570 nm (Denizot and Lang, 1986).

Oil-Red O staining : Cells on day 12 were washed twice with PBS, fixed with 4% formaldehyde for 10 min at room temperature, washed with PBS and dried completely. Fixed cells were then stained with 0.3% Oil-red O in working solution that made up isopropanol diluted (3:2) in distilled water for 1 hr at room temperature. Cells were then washed twice with PBS. Lipid droplets were stained and observed by light microscopy and photographed. Stained oil droplets were extracted with 1 ml isopropanol and absorbance was read at 510 nm (Park *et al.,* 2012).

Immunoblotting : Cells were washed twice with PBS, pH 7.4, and then scraped into lysis buffer (50 mMTris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_2VO_4, 1% NP40, 0.25% sodium deoxychloate and protease inhibitor cocktail). Lysates were clarified by centrifugation, and heated at 95°C for 3 min. Equal amount of proteins were separated by 10% SDS-PAGE gel, and then transferred onto polyvinylidenedifluoride (PVDF) membranes, blocked for 2 hr in blocking solution at room temperature. Proteins were immunoblotted with primary antibodies to peroxisome proliferator-activated receptor γ (PPARγ), phospho-AMP-activated protein kinase (p-AMPK) and AMP-activated protein kinase (AMPK). To detect antigen bound antibodies, blots were treated with secondary antibody conjugated with horse radish peroxidase coupled anti-IgG. Immunodetection was carried out by ECL Enhanced chemiluminescence western blotting detection reagent (Amersham Biosciences,

NJ, USA) (Ryu *et al.,* 2014).

Statistical analysis : Data from three independent experiments were expressed as mean ± SD. Statistical analysis was performed using SPSS 15.0 for Windows. Significant difference ($p<0.05$) between means were determined by Duncan's multiple range tests (Park *et al.,* 2013).

Results and Discussion

Obesity is currently viewed as a state of chronic inflammation elicited by secretory changes in adipose tissues and increased plasma level of pro-inflammatory proteins, whereas anti-inflammatory proteins are decreased during obesity. AMPK and PPARγ are major regulators of adipogenesis and accordingly have emerged as therapeutic targets for obesity (Ahn *et al.*, 2008). In the present study, the effect of Memilmuk on differentiation of 3T3-L1 preadipocytes was examined. 3T3-L1 cells were exposed to various concentrations of EM for 8 days, and then cell viability was determined by MTT assay. Fig. 1 indicates that the extracts from 0.018 to 0.157% EM did not result in cell death as compared with the control; thus, these concentrations were used for further *in vitro* study.

Mouse 3T3-L1 fibroblast cell system has widely been accepted as a model system for adipogenic differentiation *via* insulin stimulus. During differentiation of 3T3-L1 preadipocytes, cells undergo growth arrest and begin to differentiate simultaneously with transcriptional activation of adipose-specific genes and accumulation of lipid droplets (Kim *et al.,* 1991). To determine the inhibitory effects of buckwheat flour extract on adipocyte differentiation, 3T3-L1 preadipocytes were differentiated in the differentiation medium alone or differentiation medium with EM (0 - 0.157%) during adipocyte differentiation for 8 days. 3T3-L1 cells were fully differentiated by 8 days, and the accumulation of lipid droplets was visualized with a microscopic inspection and Oil-red O staining. EM treatment led to reduced in lipid droplets in a dose-dependent manner in 3T3-L1 adipocytes, as shown in Fig. 2. Cells treated with differentiation medium plus 0.036, 0.073, and 0.157% EM accumulated 82.9%, 80.6% and 75.5% lipid, respectively, as compared with control. These results indicated that buckwheat flour extract inhibited lipid accumulation without exerting cytotoxicity during 3T3-L1 differentiation in a dose dependent manner (Park *et al.,* 2012). These results also suggest that Memilmuk might effectively inhibit adipocyte differentiation in 3T3-L1 adipocytes.

Adipogenic differentiation is accompanied by a set of enhanced expression of transcription factors and adipogenic markers. Peroxisome proliferator-activated receptors

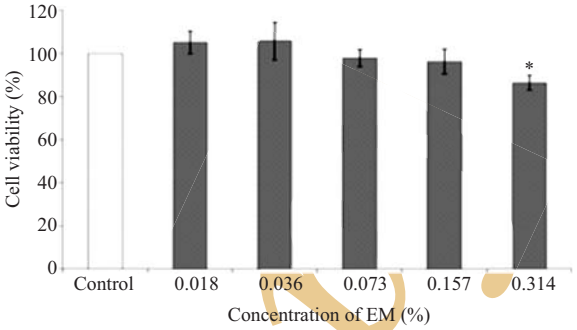

Fig. 1 : Effect of buckwheat flour extract for Memilmuk (EM) on viability of 3T3-L1 cells. Values are expressed as mean ± S.D. *$p<0.05$ vs. control

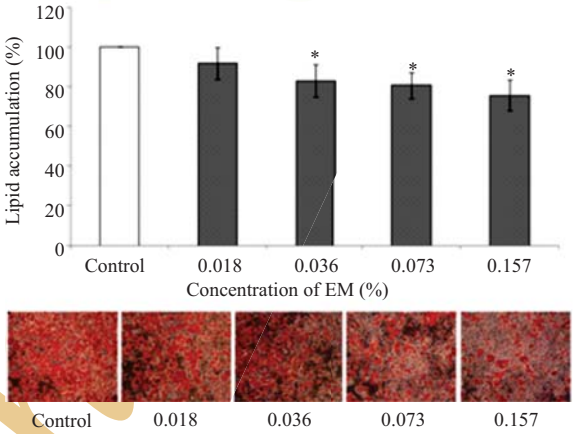

Fig. 2 : Effect of buckwheat flour extract for Memilmuk (EM) on lipid accumulation in 3T3-L1 cells. Values are expressed as mean ± S.D. of three different experiments done in triplicate. *p<0.05 vs. control

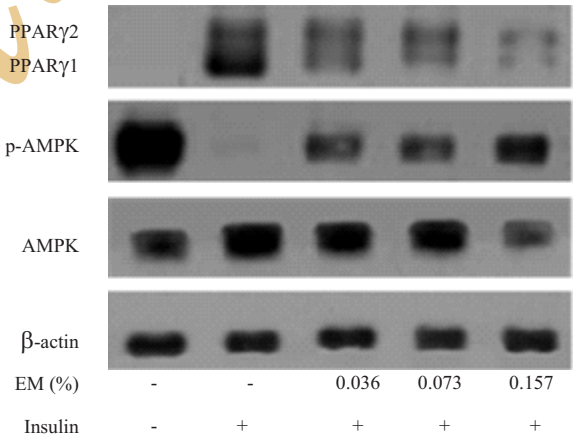

Fig. 3 : Western blot analysis of the effects of buckwheat flour extract for Memilmuk (EM) on the expression of PPARγ and AMPK in 3T3-L1 cells

(PPARs) govern the whole process of adipose tissue differentiation *via* regulating expression of specific target genes involved in inflammation, glucose and lipid metabolism (Kim *et al.,* 2012). Three isotypes of PPARs, PPARα, PPARβ and PPARγ were identified and exhibited tissue specific expression patterns. Their role in regulation of variety of genes associated with lipid metabolism, energy balance, and cellular differentiation is well known. Of these, PPARγ, predominantly expressed in adipose tissues, is a pivotal regulator of adipocyte differentiation. Thus, inhibition of PPARγ expression by specific ligands could trigger sequential anti-obesity signals. In this examination, PPARγ expression in 3T3-L1 cells upon EM treatment was measured by immunoblotting (Fig. 3). The differentiation medium including insulin markedly elevated PPARγ expression, whereas, EM addition notably reduced PPARγ expression during adipocyte differentiation. Decrease in the expression of PPARγ1 and PPARγ2 by EM occurred dose-dependently. Long-term AMPK activation regulates the expression of proteins involved in fatty acid synthesis, fatty acid oxidation and energy expenditure (Anavi *et al.,* 2010). The expression of PPARγ was reduced by AMPK activation, and AMPK activation resulted in inhibition of preadipocyte differentiation (Sung *et al.,* 2014). In Fig. 3, 3T3-L1 cells were treated with various concentrations of EM during differentiation, and the expression of AMPK phosphorylation (p-AMPK) was significantly reduced in the presence of differentiation medium including insulin. These results demonstrated that EM markedly induced AMPK activation *via* p-AMPK expression, resulting in the down-regulation of adipocyte differentiation.

Several studies have focused on the health benefits of buckwheat as a pseudocereal. The cholesterol-reducing and anti-obesity activity of buckwheat might be due to the presence of buckwheat proteins and rutin, as demonstrated in the feeding experiments (Zhang et al., 2012). However, little information is available on anti-adipogenic property of buckwheat food, Memilmuk, at molecular and cellular level. Thus, the present study focused on examining whether buckwheat flour inhibits 3T3-L1 adipocyte differentiation by modulating AMPK and PPARγ transcriptional factor. PPARγ plays a key role in initiation of adipocyte differentiation and induces synthesis of various adipogenic proteins. EM significantly inhibited expression of PPARγ, indicating that EM might inhibit 3T3-L1 differentiation and lipid accumulation via suppressing adipogenesis-related transcription factor expression. AMPK phosphorylates the transcriptional coactivator p300 and induces its interaction with PPARγ. EM significantly increased expression of phosphorylated AMPK in a dose-dependent manner in this study. These results suggest that EM might suppress PPARγ transcriptional factor through activating AMPK and phosphorylating transcriptional coactivators; hence, leading to the inhibition of adipocyte differentiation. It can be concluded that buckwheat flour extract inhibits 3T3-L1 adipocyte differentiation by modulating AMPK and PPARγ transcriptional factor in vitro. This study provides a solid evidence of anti-obesity effect of traditional Korean food, Memilmuk *in vitro*.

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