Anti-Obesity Effect of Garlic-added Kochujang in 3T3-L1 Adipocytes

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Abstract

In order to develop a functionally improved kochujang with antiobesity effects, garlic-added kochujang was prepared with freeze-dried garlic powder and followed by fermentation for 60 days at 30°C. Antiobesity effect of the garlic-added kochujang was investigated by measuring the leptin secretions and mRNA expression levels of obesity-related gene such as TNFα, PPARγ, C/EBPα, and SREBP1c, in cultured 3T3-L1 adipocytes. Fermentation of garlic-added kochujang led to decreased levels of leptin secretions and reduced the mRNA expression levels of TNFα, PPARγ, C/EBPα, and SREBP1c in the 3T3-L1 adipocytes. Accordingly, these results suggest that the addition of garlic to kochujang has a potential as a valuable functional food for controlling obesity.

Key words: garlic, kochujang, 3T3-L1 adipocytes, anti-obesity, leptin

INTRODUCTION

Recently, the obesity rate has been increasing noticeably worldwide, so that obesity has been important health issue. Obesity is a major factor in increasing the risk of serious diseases such as heart disease, hypertension, stroke, cancer, diabetes, and osteoarthritis (1,2). That is why numerous studies have attempted to find functional foods or agents from such oriental foods or medicines, including Korean traditional fermented foods for weight control. The ob-protein, leptin, is secreted from adipose tissue and may be important in the development of obesity (3-5). Leptin concentration in the serum is directly related to the amount of body fat and the amount of energy stored in adipose tissue (6,7). Adipocyte reserve energy as well as the secretion of various transcription factors such as leptin, α2, TNFα, PPARγ, C/EBPα, and SREBP1c are major transcription factors for adipogenic response.

Kochujang, a fermented red pepper soybean paste, is one of the most famous traditional Korean fermented foods. Generally, traditional kochujang is prepared with glutinous rice, meju (fermented soybean blocks), red pepper powder and salt. The unique hot, sweet, salty and savory tastes as well as color and flavors of kochujang are produced by the actions of microorganisms such as koji mold, bacteria, and yeasts during the fermentation process (8,9).

Garlic (Allium sativum) is a member of lily family that has been cultivated by humans as a food plant for over 10,000 years. Since ancient times, numerous medicinal properties of garlic have been discovered. It has been reported that garlic and its associated sulfur compounds suppress weight gain, and TG and cholesterol concentrations. They also affect the normalization of plasma lipids, reduction of blood pressure and glucose, and inhibition of platelet aggregation (10-14). Sulfur compounds from garlic are known as alliin, allicin (diallyl thiosulfinate), diallyl sulfinate (DAS), diallyl disulfinate (DADS), etc.

In this study, in order to develop a functionally improved kochujang with antiobesity effects, garlic-added kochujang was made with freeze-dried garlic powder. Antiobesity effect of the garlic-added kochujang was investigated by measuring the leptin secretion levels and the mRNA expression levels of obesity-related genes such as TNFα, PPARγ, C/EBPα, and SREBP1c in cultured 3T3-L1 adipocytes.

MATERIALS AND METHODS

Ingredients and preparations of kochujang

Glutinous flour, malt flour, meju flour, and salt were purchased at a local market in Busan, Korea. Red pepper powder and garlic were purchased from Uiseong, Gyeongsangbuk-do, Korea. Kochujang was prepared by the standardized method (15) and fermented for 60 days at 30°C. Garlic was freeze-dried, powdered, and added in ratios of 0% and 3% during the preparation of kochujang and the prepared garlic-added kochujang was fer-
mented for 60 days at 30°C. The prepared kochujang was freeze-dried, powdered, and extracted 3 times with 20-fold methanol. The methanol extract was concentrated using a vacuum rotary evaporator and followed by dissolution in dimethylsulfoxide (DMSO).

**Cell culture and adipocyte differentiation**

3T3-L1 mouse cells were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Service Co. (USA). Methylisobutylxanthine (IBMX), dexamethasone (DEX), and insulin (INS) were purchased from sigma Chemicals Co. (USA). The mouse 3T3-L1 preadipocytes were grown to confluence in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2. At 1 day post-confluence (designated “day 0”), cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (5 μg/mL) in DMEM containing 10% FBS. On day 2 and day 4, the medium was replaced with DMEM containing 10% FBS and insulin (5 μg/mL) only. On day 6, thereafter the medium consisted of only DMEM plus 10% FBS, which was subsequently replaced every 2 days. The garlic-added kochujang extracts were used to treat adipocytes at the concentration of 1 mg/mL at day 8 after inducing differentiation. After the 24 hr, the medium was removed for analysis of leptin.

**Measurement of leptin levels**

Measurement of leptin levels was performed with a sandwich enzyme-linked immunosorbent assay (ELISA). Anti-mouse leptin, recombinant mouse leptin, and biotinylated anti-mouse leptin antibodies were purchased from R&D Systems (MN, USA) (16).

**RNA isolation, RNA extraction and reverse transcription-polymerase chain reaction**

Total RNA was isolated from differentiated 3T3-L1 adipocytes using a Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). One μg of total RNA was used for first-strand cDNA synthesis using Superscript II reverse transcriptase (BD Bioscience, Palo Alto, CA). Reverse transcription was performed at 30°C for 10 min, 42°C for 30 min, and 99°C for 5 min to inactivate the avian myeloblastosis virus RTXL. Primers to specifically amplify the genes of interest are shown as Table 1. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with denaturing at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 30 sec for 25 cycles and finally 72°C for 7 min. The amplified PCR products were run in 1.0% agarose gels and stained with ethidium bromide (EtBr), and visualized under UV light. The intensities of the bands were estimated by densitometry (Multi Gauge V3.0 software, Fujifilm Life Science, Tokyo, Japan).

**Statistical analysis**

Data were expressed as mean±standard error values (n=3). Means with different letters are significantly different (p<0.05) by Duncan’s multiple range tests. Each experiment was replicated at least 3 times.

**RESULTS AND DISCUSSION**

**Effect of garlic-added kochujang on leptin secretions during fermentation**

In order to determine whether adding garlic affects the antiobesity properties of kochujang, garlic-added kochujang was prepared and fermented for 60 days. Since garlic includes a lot of active sulfur-compounds, one would expect that the addition of garlic powder to kochujang would induce a suppressive effect on lipid accumulation. The circulating leptin levels are correlated with adipose tissue mass (17,18). Therefore, the adipogenic response of garlic-added kochujang in differentiated adipocytes was determined by measuring the amount of leptin released in the medium by treatment

### Table 1. Gene-specific primers used for the RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Forward</td>
<td>5'-AGG CCT TGT GTT GTG TTT CCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGG GGG ACA GCT TCC TTC TT-3'</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Forward</td>
<td>5'-GAG ATG CCA TTC TGG CCC ACC AAC TCC GG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TAT CAT AAA TAA GCT TCA ATC GGA TGG TTC-3'</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Forward</td>
<td>5'-TGC TGG AGT TGA CCA GTG ACA A-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AAA CCA TCC TCT GGG TCT CC-3'</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>Forward</td>
<td>5'-ATC GGC GCG GAA GCT GTC GGG GTA GCG TC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACT GTC TGG GTT GTT GAT GAG CTG GAG CAT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5'-AGC CAT GTA CGT AGC CAT CC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCC TCT TCA GCT GTG GTG GTG AA-3'</td>
</tr>
</tbody>
</table>
with garlic-added kochujang (Fig. 1). Fermentation of the garlic-added kochujang reduced leptin secretion compared to that of the control adipocyte.

**Effect of kochujang and garlic-added kochujang on leptin secretion**

To determine whether the addition of garlic affects the leptin secretion of kochujang in 3T3-L1 adipocytes, leptin level secreted in cultured media treated with garlic-added kochujang was compared to that of control kochujang (Fig. 2). Here, traditional kochujang prepared without garlic was used as a control kochujang. Also the control kochujang and garlic-added kochujang fermented 60 days were used for comparative analysis. Leptin secretions of control kochujang and garlic-added kochujang were decreased by 34% and 48%, respectively, compared to that of the control adipocytes. The addition of garlic powder into the kochujang reduced leptin secretion in the medium. This result demonstrated that making garlic-added kochujang has the potential to improve its antiobesity effects. It might be due to a synergistic effect between garlic and the products created by fermentation of kochujang. As possible active components responsible for the antiobesity effect of kochujang, capsaicin in red pepper powder, isoflavonoide produced from meju, and some glycoside products caused by fermentation could be included. Several studies have reported on antiobesity effects of fermented traditional kochujang as well as on the decrease of inbody weight, serum lipids, and body fat gain (19-22). Similar to traditional kochujang, commercial kochujang also decreased leptin secretion and adipocytes size in 3T3-L1 adipocytes by modulating adipogenesis and lipolysis (23). Also, various biological activities of garlic and its associated sulfur compounds such as alliin, allicin (diallyl thiosulfinate), DAS, DADS, have been reported (24-31). The effects of garlic powder on reducing total lipids, TG, and cholesterol contents were studied (11-14). In vivo, reducing effects of garlic on weight gain, TG and cholesterol contents, and lipid values of adipose tissue were reported (30).

**Effect of garlic-added kochujang on obesity related gene expressions**

TNFα is produced by adipocyte tissue and its mRNA level increases with the increasing adiposity. TNFα increases not only both leptin gene expression and leptin secretion in 3T3-L1 adipocytes but also lipolysis and released free fatty acid (32). Therefore, the mRNA expression levels of TNFα in the differentiated adipocytes treated with the control kochujang and garlic-added kochujang were compared (Fig. 3). Treatment with garlic-added kochujang in matured adipocytes markedly suppressed their TNFα expression compared with that of the control adipocytes. That is, adding of garlic during kochujang preparation might be associated with the reduced cellular lipid accumulation mediated by TNFα.

PPARγ is a member of the nuclear receptor superfamily of transcription factors and is predominantly expressed in adipose tissue. These transcription factors appear to function as dominant activators of adipocyte differentiation (33). PPARγ is a major coordinator of adipocyte gene expression and differentiation (34). PPARγ is induced prior to the transcriptional activation of most adipocyte-specific genes, and the expression of PPARγ

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**Fig. 1.** Changes in leptin secretion of garlic-added kochujang during fermentation. Adipocytes were treated for 24 hr at “day 8” after inducing differentiation with vehicle alone (control; 5 mM methylisobutylxanthine, 0.25 μM dexamethanesone, 10 μg/mL insulin) or 1 mg/mL of kochujang fermented for 60 days. Data are expressed as mean± standard error values (n=3). Means with different letters are significantly different (p<0.05) by Duncan’s multiple range test.

**Fig. 2.** Additional effect of garlic powder on leptin secretion of kochujang. Data are expressed as mean± standard error values (n=3). Means with different letters are significantly different (p<0.05) by Duncan’s multiple range test. K: control kochujang fermented for 60 days, GK: garlic-added kochujang (3%) fermented for 60 days.
is sufficient to induce growth arrest and to initiate adipogenesis in exponentially growing fibroblast cell lines (35). Effect of garlic-added kochujang on the mRNA expression level of PPARγ was evaluated by using RT-PCR analysis (Fig. 3). The mRNA expression of PPARγ of the control kochujang and garlic-added kochujang was decreased by 19% and 69%, respectively, compared to that of the control adipocytes.

PPARγ is expressed early in the differentiation of 3T3-L1 adipocytes and prior to C/EBPα (36). Overexpression of C/EBPα as well as PPARγ can induce adipocyte differentiation (37). In the present study, fermentation of garlic-added kochujang as well as control kochujang reduced the mRNA expression level of C/EBPα (Fig. 4). SREBP1c is also one of important transcription factors for adipogenesis. The expression of SREBP1c can induce endogenous PPARγ mRNA expression in 3T3-L1 adipocytes. Treatment with control kochujang and garlic-added kochujang led to down-regulation of SREBP1c mRNA (Fig. 4). However, there were a little significant difference between the adipocytes treated with control kochujang and garlic-added kochujang. In conclusion, fermentation of kochujang reduced expression levels of adipogenic genes such as PPARγ, C/EBPα and SREBP1c and theirs levels were more inhibited by garlic-added kochujang. Therefore, anti-obesity effect of garlic-added kochujang might be due to inhibiting regulation promoters of several adipogenic genes such as leptin though PPARγ, C/EBPα and SREBP1c transcription factors, resulting in inhibition of lipid accumulation by blocking adipogenesis. Further studies will be needed to explore the regulation and function at the in vivo level.

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