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Increased Lipolytic Activity by High-pungency Red Pepper Extract (var. Chungyang) in Rat Adipocytes *in vitro*

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Abstract

High-pungency red pepper and capsaicin modulate circulating hormone levels and induce lipolysis in adipose tissue *in vivo*. This study was designed to investigate the lipolytic activity of adipocytes by high-pungency red pepper extract *in vitro*. High-pungency red pepper (var. Chungyang) powder showed 126.1 mg% of capsaicinoid which was 3 x higher than low-pungency red pepper powder (var. Daemyung). To study the effects of high-pungency red pepper extract on lipolytic activity, preadipocytes were separated from the epidermal fat of 14 day-old rats, induced to differentiate into adipocytes and were treated with red pepper extracts. The amount of glycerol released from adipocytes into the culture medium was analysed to measure lipolytic activity. Glycerol release from adipocytes was increased in a dose-dependent manner with high-pungency red pepper extract treatment. However, there was no significant change in the glycerol release when adipocytes were treated with low-pungency red pepper extract. To investigate whether lipolysis by high-pungency red pepper extract is caused by capsaicin, glycerol release was detected after the treatment of adipocytes with capsaicin. Glycerol release was significantly increased by capsaicin. These results suggest that high-pungency red pepper extract might have a direct lipolytic activity in adipocytes that is mediated by capsaicin.

Key words: high-pungency red pepper extract, primary adipocyte culture, lipolysis, capsaicin

INTRODUCTION

Obesity is increasingly recognized as a serious public health threat, not only in the developed countries, but in developing countries as well. It is not clear that obesity is a direct health risk, but it unquestionably increases the risk of other diseases including coronary artery disease, diabetes, hypertention, certain cancers, and premature mortality. The incidence of obesity is increasing in Korea due to the economic growth and the westernized life style. According to the National Dietary Survey, the percentage of Koreans with a body mass index (BMI) greater than 25 has rapidly increased from 20% in 1995 to 26.3% of the population in 1998 (1,2). Dietary restriction and behavioral changes are keys to the control of obesity. Anti-obesity nutraceuticals that are safe and effective for the control and treatment of obesity are the subject of intense research throughout the world. Dietary components such as chitin-chitosan (3), dietary fiber (4,5), green tea (6), oolong tea (7), carnitine (8), red pepper (9,10), kochujang (11), and kimchi (12) are shown to be involved with the regulation of lipid metabolism and reduction of body weight, serum triglyceride, and cholesterol levels.

Capsicum species, hot pepper, are important culinary plants and have been used world wide as food, spices, and medicines. Red pepper has been shown to increase plasma catecholamine levels, induced lipolysis (13), and can reverse the effects of a high fat diet on body weight and blood and tissue lipids (10). Red pepper and capsaicin, (E)-N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide, have been reported to increase lipid oxidation in Japanese women (14). They also modulate total body fat and hepatic lipogenic enzyme activities in rats (15). Capsaicin activates the parasympathetic nervous system, which triggers the release of catecholamine (16,17), which then stimulates the β-adrenergic receptor. Energy metabolism triggered by the activation of β-adrenergic receptor results in decreased body fat mass (18). Capsaicin is also known to regulate the lipid concentrations in the blood (14). These studies suggest that capsaicin can be used for the treatment of obesity.

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Unfortunately, there has been no study to investigate the direct lipolytic effect of hot red pepper on primary rat adipocytes *in vitro*. In this study, the lipolytic effect of high-pungency red pepper extract on adipocytes was investigated and compared to that of low-pungency red pepper extract *in vitro*. Furthermore, we treated adipocytes with capsaicin to investigate whether capsaicin is a major inducer of lipolysis.

MATERIALS AND METHODS

Preparation of red pepper extract samples

Var. Chungyang and var. Daemyung red peppers were donated by the Jungyangjongmyo, Kyunggido Osan institute. Var. Chungyang is a native species of Korea and spicy but Var. Daemyung is not spicy, so we call them high-pungency red pepper and low-pungency red pepper, respectively. The peppers were freeze-dried, powdered, and extracted 3 times with 20 fold methanol. The methanol extracts were concentrated using a vacuum rotary evaporator and then dissolved in dimethylsulfoxide (DMSO).

Determinations of ascorbic acid, carotenoid, and capsaicinoid in the red pepper powders

The concentrations of total ascorbic acid, carotenoids, and capsaicinoid were analyzed in the high-pungency and low-pungency red pepper powders. Ascorbic acid was quantitated using the 2,4-dinitrophenyl hydrazine (DNP) method (19), the carotenoids were analysed according to the AOAC method (19). Capsaicinoid was extracted using the Hoffman method and analysed by high pressure liquid chromatography (HPLC) (20).

Rat preadipocytes primary culture and induction of differentiation

Fourteen day old Sprague-Dawley (SD) rats were purchased from Hyochang Science, Daegu, South Korea and kept in a cage with the mother rat. Fibroblastic preadipocytes were isolated from adipose tissue based on a previously reported method (21). The fat tissues were collected from epidermal fat pads of the 14 day-old Sprague-Dawley rats (10 rats per group) and digested in the buffer containing collagenase Type II (1 mg/mL; Sigma) to prepare preadipocytes. The 1.5×10^5 preadipocytes/mL were plated onto 12-well plates and grown to confluence in medium 199 (Gibco BRL) with 10% Fetal Bovine Serum. After the cells were grown to confluence, differentiation was induced by addition of medium 199 supplemented with isobutylmethylxanthine (0.5 mM; Sigma), dexamethasone (0.25 μM; Sigma) and insulin (5 μg/mL, Sigma). After 48 h, the induction medium was removed and replaced by medium 199 supplemented with insulin (5 μg/mL) only. This medium was replaced every 2 days until adipocytes were fully differentiated.

Treatment of adipocytes with red pepper extracts

The fully differentiated adipocytes were treated with red pepper extracts. Cells were treated with either 100 μg/mL, 10 μg/mL, or 1 μg/mL of high-pungency or low-pungency red pepper extract. Capsaicin was used at concentrations of 50 μg/mL and 250 μg/mL. After the 24 h incubation, medium was collected and stored at -80°C until analysis.

Glycerol assay

Lipolysis was estimated by measuring glycerol levels in the medium of red pepper extracts treated rat adipocytes. The glycerol level was measured by using the enzymatic reagent, GPO-TRINDER (Sigma). A 2.5 μL volume of each sample was added to 500 μL of prewarmed 37°C reagent. The 12.5 μg and 25 μg amounts of glycerol were used for calibration. After incubation at 37°C for 5 minutes, 200 μL of each sample was transferred to a 96 well plate (Nunc) and optical densities assessed at 540 nm using a plate spectrophotometer.

Oil red O staining

After adipocytes were treated with 100 μg/mL of high-pungency red pepper extract for 72 hours, lipid droplets from the adipocytes were stained as previously described (21). In short, adipocytes were fixed with 10% fresh Formalin (Sigma) and then stained with filtered Oil red O solution at 4°C for at least 1 hour.

Statistical analysis

The results were expressed as means ± SE. The significance of differences between groups was determined by carrying out ANOVA with Duncan's multiple range test or Student's t-test. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Ascorbic acid, carotenoids, and capsaicinoid concentrations in high-pungency and low-pungency red pepper powders

Active components of red pepper are known to include carotenoids, ascorbic acid, capsaicin, linoleic acid, etc. Choi (22) reported that the amount of capsaicin and other nutrients in pepper vary according to where it is grown. In this study, we compared nutrient contents of high-pungency red pepper with those of low-pungency red pepper. Total ascorbic acid in high-pungency red pepper powder was 201 ± 13 mg% and 168 ± 13 mg% for low-pungency red pepper powder (Table 1). Pigment of red pepper is mainly due to carotenoids, and the contents

Table 1. Concentrations of total ascorbic acid, carotenoids, and capsaicinoids in high-pungency red pepper powder (var. Chungyang) and low-pungency red pepper powder (var. Daemyung). (unit: mg%)

	Var. Chungyang	Var. Daemyung
Ascorbic acid	91	86
Dehydroascorbic acid	$125\pm14^{a1)}$	82 ± 8^{b}
Total ascorbic acid	201 ± 13^{a}	168 ± 13^{b}
Carotenoid	355 ± 23^{a}	286 ± 10^{b}
Capsaicin	86.2 ± 4.5^{a}	30.3 ± 1.2^{b}
Dihydrocapsaicin	39.9 ± 2.9^{a}	17.4 ± 0.7^{b}
Total Capsaicinoid	126.1	47.7

[1)]Means with the different letters in the same row are significantly different ($p<0.05$) by Duncan's multiple range test.

of carotenoid in the high-pungency red pepper powder and low-pungency red pepper powder were 355 ± 23 mg% and 286 ± 10 mg%, respectively. Furthermore, high-pungency red pepper powder contains 126.1 mg% of capsaicinoid and low-pungency red pepper powder contains 47.7 mg%. High-pungency red pepper powder contained 3 times more capsaicinoid than low-pungency red pepper powder. High-pungency red pepper powder also had higher concentrations of total ascorbic acid, carotenoid, and capsaicinoid. Especially, capsaicinoid concentrations were considerably higher than that of low-pungency red pepper powder. It has been previously reported that var. Daemyung contained the lowest level of capsaicinoid of major Korean red pepper varieties (var. Daemyung, var. Myungpum, var. Johong, var. Chungyang) and var. Chungyang contained the highest levels. Var. Chungyang was pungent and has been most often used for soup and preparing pungent foods (23).

The lipolytic effect of red pepper extracts on adipocytes *in vitro*

Fully differentiated adipocytes were treated with high-pungency red pepper extract and the amount of leptin released from adipocytes was measured. It has been reported that the size of adipocytes is highly correlated with leptin level *in vitro* (24). On the 8th day after induction of differentiation, the leptin level reached a maximum (21) and adipocytes were ready to be treated by red pepper extracts.

At first, glycerol concentration in the high-pungency red pepper extract was measured to facilitate determining whether glycerol release is due to lipolytic activity of high-pungency red pepper extract or is released from the high-pungency red pepper extract itself. Medium without adipocytes was treated with four different concentrations of high-pungency red pepper extract (1 μg/mL, 10 μg/mL and 100 μg/mL). Glycerol measurements revealed no significant changes (107% ~ 112% of the control) in glycerol release from the pepper extracts ($p>0.05$) (data not shown). This confirmed that the glycerol in the high-pungency red pepper extract itself does not affect the amount of glycerol released by lipolysis.

To determine the lipolytic activity of high-pungency red pepper extract, adipocytes were treated with the same doses as above (1 μg/mL, 10 μg/mL and 100 μg/mL). Glycerol release was markedly increased in a dose dependent manner. (Fig. 1) The amount of glycerol released increased 170%, 225%, and 273% above the control, respectively. This clearly suggests that the active components of high-pungency red pepper extract may have been taken up by the cells and stimulated lipolysis. Numerous studies (25,18) have suggested that spicy foods and capsaicin have antiobesity effects by stimulating energy metabolism. Salimath and Satyanarayana (26) reported that red pepper extract and capsaicin stimulated lipolytic activity by inhibiting cAMP phosphodiesterase activity in adipose tissue of rats. In that study only 50 μL of red pepper extract was required to inhibit cAMP phosphodiesterase by 80%.

Oil red O staining of adipocytes was performed to verify the effect of high-pungency red pepper extract on lipolysis in adipocytes. (Fig. 2) Oil red O specifically stains triglycerides and cholesteryl oleate, therefore, this technique is a valuable tool for quantitation of triglyceride accumulation in adipocytes (27).

The adipocytes were treated with high-pungency red pepper extract for 72 hours and stained with Oil red O. Lipid droplets were revealed by the red color developed when stained with Oil red O. The degree of red color was markedly lower compared to the control (Fig. 1). This means that the lipid droplets were significantly

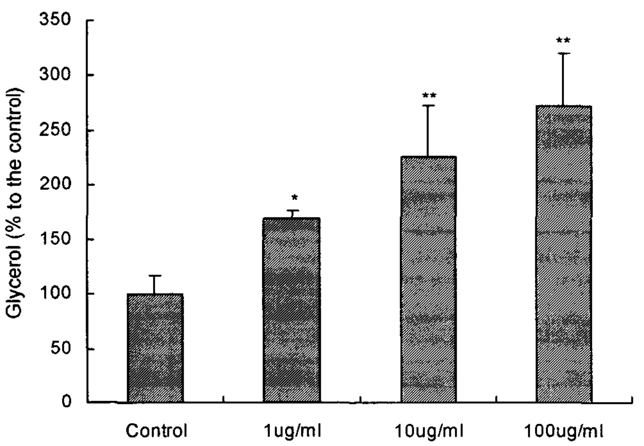

Fig. 1. Glycerol release after treatment with high-pungency red pepper extract. Primary adipocytes were treated with medium containing 1 μg/mL, 10 μg/mL and 100 μg/mL of high-pungency red pepper extract for 24 hours. Values are mean±SE (n=2~4). *$p<0.05$, **$p<0.01$.

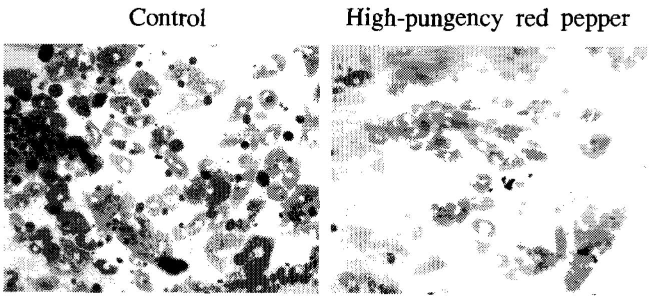

Fig. 2. Oil red O staining of rat primary adipocytes. Fully differentiated adipocytes were treated with 100 μg/mL of high-pungency red pepper extract for 72 hours.

lipolyzed into free fatty acid and glycerol by high-pungency red pepper extract, which shows that high-pungency red pepper extract has direct lipolytic effect on adipocytes, which is consistent with the previous result (Fig. 1).

In the next experiment, we investigated the lipolytic effect of low-pungency red pepper extract. Low-pungency red pepper extract was used to treat the adipocytes at concentrations of 10 μg/mL, 100 μg/mL, and 1 mg/mL, which were 10 times higher than those of high-pungency red pepper extract. The amount of glycerol released was not significantly different from that of the control, and there was no increased glycerol release even at the concentration of 1 mg/mL (Fig. 3). This result shows that low-pungency red pepper extract does not induce lipolysis in adipocytes even though 1 mg/mL of low-pungency red pepper extract contains more capsaicin than 100 μg/mL of high-pungency red pepper extract. This apparent inconsistency might be due to some masking components in low-pungency red pepper extract that blocked the effect of capsaicin. The concentration of the masking component may have been higher at the high level (1 mg/mL) of low-pungency red pepper extract than at the low level (10 μg/mL). Another possibility was provided by Watanabe et al. (28). They reported that the levels of adrenal catecholamine secretion by capsaicin in rats varied according to the structures of capsaicin analogs. In our study, the composition of capsaicin analogs in high-pungency red pepper may have been different from that of low-pungency red pepper. Therefore, further studies of capsicin analogs in red peppers will be needed to solve this problem.

Change in glycerol release caused by capsaicin

To investigate whether the active component of high-pungency red pepper extract that induces lipolysis is capsaicin, adipocytes were treated with two different concentrations of capsaicin (50 and 250 μg/mL). The amount of glycerol release was more than 3 times as much as the control (Fig. 4). It suggests that capsaicin has high liploytic activity and the lypolytic effect of high-pungency red pepper extract might be induced by capsaicin in it. In many studies, it has been reported that capsaicin has antiobesity effects as below. Capsaicin decreased total serum cholesterol and lipid content in turkeys fed a high cholesterol diet (29). Capsaicinoid also increased the breakdown of fat in adipose tissues and increased the release of free fatty acid (13). Choo and Shin (18) reported that capsaicin possesses potent body fat suppressive effects in rats that are mediated through β-adrenergic stimulation. Capsaicin is also reported to elevate hormone sensitive lipase and lead to lipolysis in rats (27). Capsaicin is metabolized rapidly and produced vanillylamine, vanillin, vanyllyl alcohol, and vanillic acid and finally excreted through urea in rats (30). It will be useful to test the lipolytic activities of these metabolites to understand the antiobesity effect of capsaicin. However, to full verify that capsaicin is the active component, study blocking the effect of capsaicin

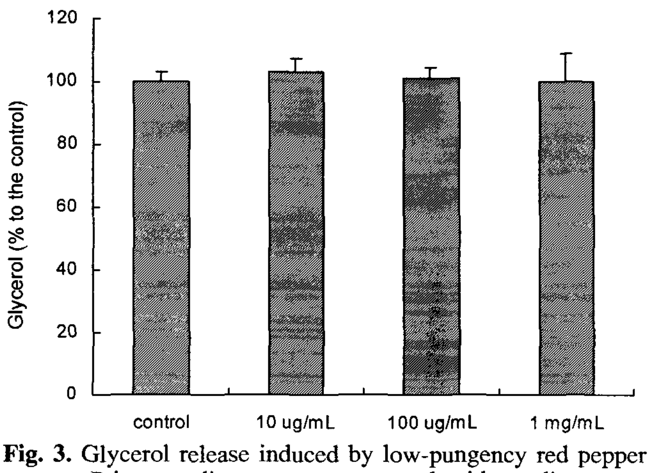

Fig. 3. Glycerol release induced by low-pungency red pepper extract. Primary adipocytes were treated with medium containing 10 μg/mL, 100 μg/mL, and 1 mg/mL of low-pungency red pepper extract for 24 hours. Values are mean±SE (n=3).

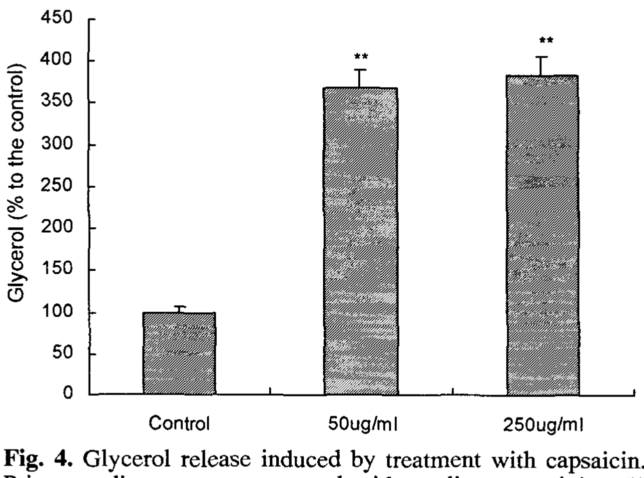

Fig. 4. Glycerol release induced by treatment with capsaicin. Primary adipocytes were treated with medium containing 50 μg/mL and 250 μg/mL of capsicin. Values are mean±SE (n=4). $^{**}p<0.01$.

or high-pungency red pepper extract through the use of capsazepin, a capsaicin inhibitor, is needed to verify that capsaicin is the cause of glycerol release.

In conclusion, we investigated the lipolytic activity of high-pungency red pepper extract (var. Chunyang) *in vitro* by measuring the glycerol release from adipocytes.

High-pungency red pepper extract stimulated glycerol release from adipocytes and capsaicin seemed to be the active component in lipolysis because glycerol release was considerably increased by treating adipocytes with high-pungency red pepper extract, which contained higher level of capsaicin. On the contrary, low-pungency red pepper extract, which contained low level of capsaicin, did not stimulate glycerol release and did not cause any significant lipolytic activity, even though it was treated at a higher concentration than high-pungency red pepper extract. Therefore, taking small amounts of high-pungency red pepper should be more effective than taking large amounts of low-pungency red pepper for the treatment of obesity.

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