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Methanolic Extract of Turmeric (*Curcuma longa* L.) Enhanced the Lipolysis by Up-regulation of Lipase mRNA Expression in Differentiated 3T3-L1 Adipocytes

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Abstract Effects of methanol extract from turmeric (*Curcuma longa* L.) (CME) on underlying mechanisms of lipolysis were investigated in 3T3-L1 adipocytes. Compared to the control, lipid accumulation with 72 hr treatment of CME at the concentration 20 μg/mL was significantly decreased by 19.9% as quantified by Oil red O dye. Intracellular triglyceride (TG) content was also lowered by 19.3%. To determine the mechanism for TG content reduction, glycerol release level was measured. Incubation of 3T3-L1 adipocytes with 15 and 20 μg/mL of CME significantly elevated the level of free glycerol released into the cultured medium by 20.4 and 28.6%, respectively. In subsequent measurements using quantitative real-time polymerase chain reaction (PCR), mRNA levels of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) were significantly increased by 21.2 and 24.9%, respectively, at the concentration 20 μg/mL. Results indicated that CME stimulated lipolysis through induction of HSL and ATGL mRNA expressions, resulting in increased glycerol release.

Keywords: *Curcuma longa* L., lipid accumulation, lipolysis, lipase, 3T3-L1

Introduction

The induction of obesity is mainly dependent on the regulation of lipid accumulation and energy storage (1). Excessive lipid accumulation in the adipocyte causes the pathological disorders such as type-2-diabetes, hypertension, and coronary heart diseases (2,3). Consequently, there has been the increased interest in preventing lipid accumulation or stimulating lipolysis of adipocyte triglyceride (TG). The lipid droplet in adipocytes is a major cellular compartment for lipid accumulation and plays crucial roles in lipid metabolism. Breakdown of TG in lipid droplet and the release of glycerol are important for the regulation of intracellular lipid accumulation (4). It has been documented that several factors such as lipases, tumor necrosis factor (TNF)-α, and perilipin A are mainly involved in the regulation of lipolysis pathway. Hormone-sensitive lipase (HSL) is the most important lipase that catalyses the process of lipolysis, and is subject to hormonal regulation like insulin and/or epinephrine (5). In the molecular basis, lipolysis is stimulated by protein kinase A (PKA) activation, which phosphorylates HSL, or by phosphorylation of HSL by G protein-coupled receptors and cyclic AMP-activated extracellular signal-regulated kinase (ERK) (6). The cytokine TNF-α has been also shown to increase the lipolysis rate in human and animal *in vivo* studies (7). Recently, adipose triglyceride lipase (ATGL) is identified as a TG-specific lipase that is considered the rate-limiting lipolytic enzyme in adipocyte (8). Otherwise, perilipin A, which incorporates into the compartment membrane of lipid droplets, inhibits the lipolysis by preventing both HSL and ATGL from TG access in the core of lipid droplets (9).

Turmeric (*Curcuma longa* L., Zingiberaceae family) is a perennial herb that grows mainly in the tropical regions of Asia and Africa, and has known to possess the various biological activities against carcinogenesis, biliary disorders, anorexia, inflammation, rheumatism, and bacterial infections (10-15). The major constituents of turmeric include the 3 curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) that have been attributed to many biological activities (16). However, the direct effect of turmeric on lipolysis in mature adipocytes remains largely unknown.

In the present study, the effect of mathanolic extract from turmeric (CME) on lipolysis was investigated in 3T3-L1 adipocytes. Furthermore, its mechanism of action relevant to lipolysis was determined.

Materials and Methods

Reagents and acquisition of CME Turmeric was generously gifted from Woori-Sool Co. (Gwangju, Korea). Oil red O stain, free glycerol detection reagents, 3-[4,5-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reagent, and 2-NBDG were purchased from Sigma-Aldrich (St. Louis, MO, USA). AdipoRed Assay reagent for cellular TG content was purchased from Cambrex BioSicence (Walkersville, MA, USA). RNeasy Mini kit and SyBr green Taqman real time polymerase chain reaction (PCR) kit was obtained from Qiagen (Valencia, CA, USA) and Applied Biosystems Inc. (Foster City, CA, USA).

Turmeric was extracted with 20 volumes of methanol, and it was filtered. The filtrate was dried with an evaporator under vacuum conditions, dried, and stored at –20°C until assays.

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Cell culture 3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC CL-173, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Waltham, MA, USA) containing 10% bovine calf serum (BCS) until confluent at 37°C in humidified 5% CO_2 atmosphere. At day 2 after confluences, the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS) and MDI solution [0.5 mM isobutylmethylxanthine (IBMX), 0.5 mM dexamethasone, and 10 mg/mL insulin] for 3 days. The cells then were maintained in 10% FBS and 10 mg/mL insulin in DMEM for another 3 days, followed by culturing with 10% FBS in DMEM for an additional 8 days, at which time over 90% of cells were mature adipocytes with accumulated lipid droplets. All media contained 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 10 mM glutamine (Hyclone) and were changed every 2 days thereafter until analysis.

Cell viability The number of viable cells was determined by the ability of mitochondria to convert MTT to formazan dye. The mature adipocytes with various concentrations of CME were incubated in 24-well plate for 72 hr. After adding 50 mg/mL of MTT in Hank's balanced salt solution (HBSS), the cells were further incubated for 4 hr at 37°C. Then, the supernatants were removed and resulting formazans were dissolved in 500 mL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm on a microplate reader (BioTek, Winooski, VT, USA) using a reference wavelength of 665 nm.

Oil red O staining and quantification of cellular TG content Cells were treated with CME in 60-mm culture dish for 48 and 72 hr, washed twice with phosphate buffered saline (PBS), and fixed with 4% formaldehyde containing 1%(v/v) calcium chloride for 30 min at room temperature. After air-flow dry, cells were stained with 0.4-mm filtered Oil red O in absolute alcohol for 40 min, and then washed 3 times with PBS to remove remains of Oil red O reagent. DMSO was treated to wash out the stained Oil red O and measured colorimetrically in the spectrophotometer (Applied Biosystems Inc.) at 560 nm. TG content was quantified using commercially available AdipoRed Assay reagent according to the manufacturer's instructions. AdipoRed, a solution of the hydrophilic stain Nile Red, is a reagent that enables the quantification of intracellular TG. Briefly, 3T3-L1 preadipocytes were plated in 24-well plates and differentiated into mature adipocytes as described above. Then, various concentrations of CME were treated for another 72 hr. After the cells were rinsed twice with PBS, wells were filled with 200 mL PBS, followed by the addition of 20 mL AdipoRed reagent. The incubation for 20 min was performed at room temperature and fluorescent signal was measured with an excitation at 485 nm and an emission at 572 nm.

Measurement of free glycerol for lipolysis assay Differentiated 3T3-L1 adipocytes were incubated with various concentrations of CME for 48 and 72 hr. The incubation medium was transferred to another set of tubes and heated at 70°C for 10 min to inactivate any enzymes released by the cells. Then, 50 mL of incubation medium was assayed for free glycerol using glycerol reagent (Sigma-Aldrich) in 1-mL disposable cuvette and absorption was measured at 540 nm. The protein content to compensate the cell number was determined using the Bradford method.

Quantitative real-time PCR Total RNA was isolated from cells using RNeasy Mini kit (Qiagen). One μg of total RNA obtained from the 3T3-L1 adipocytes was reverse-transcribed using an oligo(dT) 18 mer as a primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in order to produce the cDNAs. Real-time PCR was performed using the selective primer sets with Universal SYBR Green PCR Master Mix by manufactured instruction (Qiagen). The sequence of the sense and antisense primers used for amplification were as follows; GAPDH, 5'-CCATGAGAAGTATGACAACAGCC-3' and 5'-TGGCAGGTTTTTCTAGACGG-3'; HSL, 5'-ACCGAG ACAGGCCTCAGTGTG-3' and 5'-GAATCGGCCACCG GTAAAGAG-3'; ATGL, 5'-AACACCAGCATCCAGTTC AA-3' and 5'-GGTTCAGTAGGCCATTCCTC-3'; LPL, 5'-ACTCGCTCTCAGATGCCCTA-3' and 5'-TTGTGTTGC TTGCCATTCTC-3'. Data analyses are carried out using 7500 Systems SDS software version 1.3.1 (Applied Biosystems Inc.).

Statistical analysis All data were expressed as mean± standard deviation (SD). The paired Student's *t*-test was used to assess significant difference among the treatment groups. The statistical significance was set at $p<0.05$.

Results and Discussion

Cytotoxic effect Turmeric has been extensively studied for its biological and pathological activities against growth of microbial organisms, inflammation, hypertension, cancer, arthritis, and diabetes (10-15). Interestingly, there are scanty reports to demonstrate the inhibitory effect of turmeric on obesity and lipid metabolism in adipocytes although a few were reported about the effects of curcumin that has known as a main active compound. In the present study, we examined the lipolytic effect and potential action mode of CME in differentiated 3T3-L1 adipocytes. The cytotoxic effects of CME on 3T3-L1 adipocytes were initially evaluated. The cells were treated for 72 hr with various concentrations ranging from 0 to 50 mg/mL of CME and were then subjected to the MTT assay. As shown in Fig. 1, CME had no effect on cell survival up to 25 mg/mL, and thus concentration of CME applied to 3T3-L1 adipocytes did not exceed 20 mg/mL for a safety purpose in all subsequent experiment.

Inhibition of lipid accumulation In the first place, lipid accumulation in 3T3-L1 adipocytes was determined by using both Oil red O staining and microscopic observation. Secondly, for more accurate quantification, intracellular TG content was fluorescently measured by AdipoRed reagent. Differentiated 3T3-L1 adipocytes were treated with either 0.2% DMSO or CME at various concentrations (0, 5, 10, 15, and 20 mg/mL) for 48 and 72 hr and intracellular lipid droplets were stained with Oil red O dye. Microscopic observations of Oil red O staining showed the gradual reduction of lipid droplets with increased concentration of

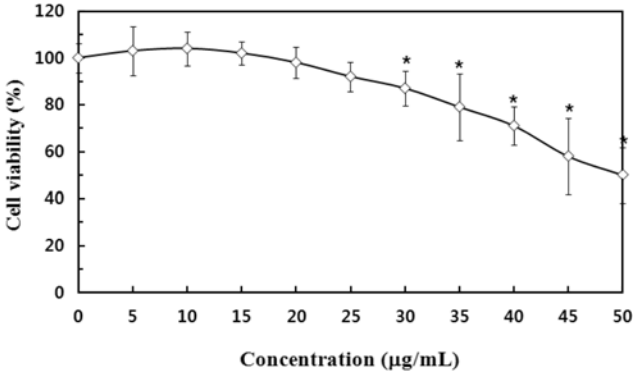

Fig. 1. Effects of methanol extract from turmeric (CME) on cell viability in differentiated 3T3-L1 adipocytes. Values are expressed as mean±SD of at least 3 independent experiments, each performed in triplicate (n=3). *Significantly different from that of the control treatment at p<0.05.

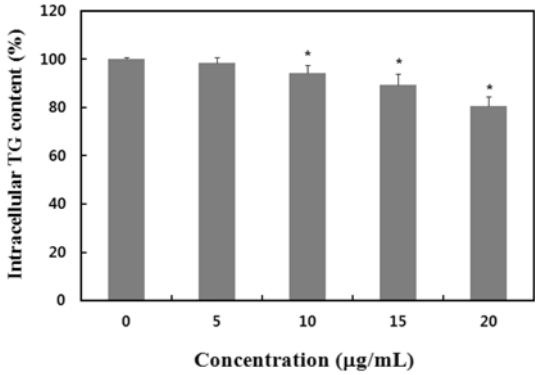

Fig. 3. Inhibitory effects of methanol extract from turmeric (CME) on the level of intracellular triglycerides (TG) in 3T3-L1 adipocytes. Values are expressed as mean±SD of at least 3 independent experiments, each performed in triplicate (n=3). *Significantly different from that of the control treatment at p<0.05.

CME in a dose-dependent manner (Fig. 2). However, there was no significant difference between 48 and 72 hr treatment at 15 and 20 mg/mL of CME. Similar effects on lipid accumulation with 72 hr treatment were observed by measuring intracellular TG content that was significantly reduced by 19.3% at 20 mg/mL of CME (Fig. 3). These results indicated that 20 mg/mL of CME treatment for 72 hr effectively induced the lipolysis in mature 3T3-L1 adipocytes.

Measurement of glycerol release Lipid accumulation is controlled by balance between neutral lipid synthesis (lipogenesis) and degradation (lipolysis) (17,18). Lipogenesis encompasses the processes of fatty acid synthesis through *de novo* pathway where insulin-dependent glucose uptake plays a critical role as a source of TG synthesis (19). In contrast, lipolysis is accompanied by activation of HSL, resulting in release of glycerol (20). The amounts of free glycerol released from differentiated 3T3-L1 adipocytes into medium were measured to analyze the lipolytic effect of CME on the accumulated TG in adipocytes. The amounts of free glycerol in the medium after 72 hr treatment of CME were significantly increased compared to the control by 28.6% at the 20 mg/mL (Fig. 4). These results led to the conclusion that CME stimulated lipolysis in 3T3-L1 adipocytes as evidenced by the decreased lipid content in cells and the increased concentration of free glycerol in the

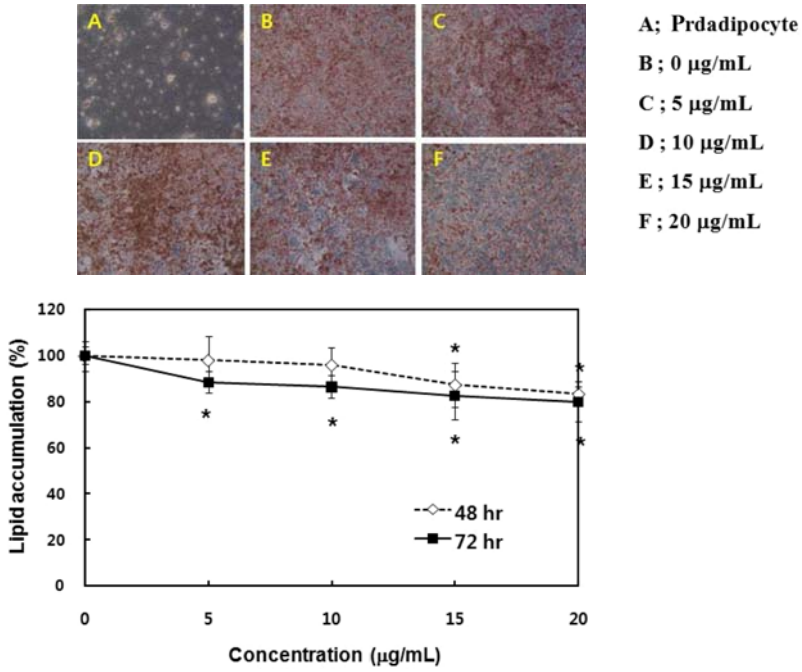

Fig. 2. Inhibitory effects of methanol extract from turmeric (CME) on the lipid accumulation in 3T3-L1 adipocytes. Values are expressed as mean±SD of at least 3 independent experiments, each performed in triplicate (n=3). *Significantly different from that of the control treatment at p<0.05.

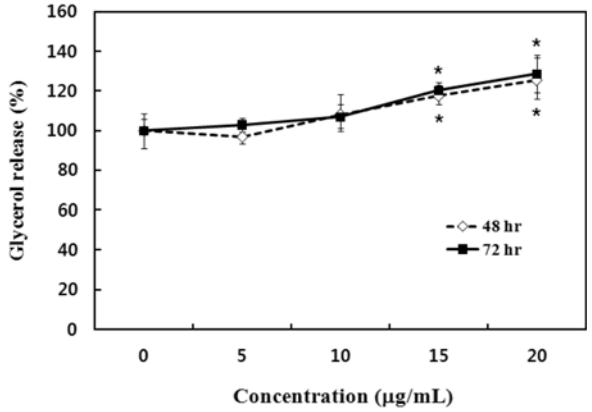

Fig. 4. Stimulatory effects of methanol extract from turmeric (CME) on glycerol release in 3T3-L1 adipocytes. Values are expressed as mean±SD of at least 3 independent experiments, each performed in triplicate (n=3). *Significantly different from that of the control treatment at p<0.05.

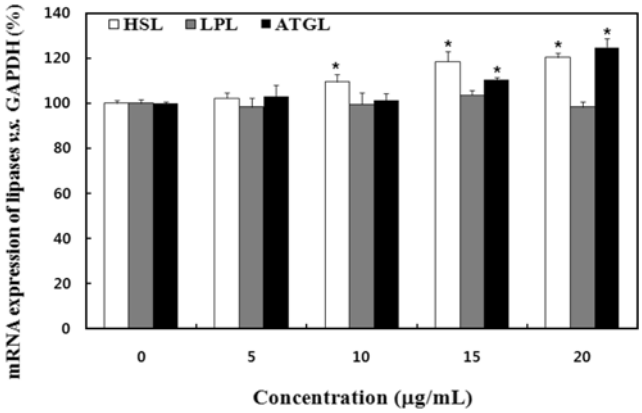

Fig. 5. Effects of methanol extract from turmeric (CME) on mRNA expression of hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), and adipose triglyceride lipase (ATGL) in 3T3-L1 adipocytes. Values are expressed as mean±SD of at least 3 independent experiments, each performed in triplicate (n=3). *Significantly different from that of the control treatment at p<0.05.

medium. Since lipases are major enzymes to break down TG in lipid droplet to glycerol and fatty acid, the mRNA expression of lipases after 72 hr treatment of CME was determined by real-time PCR.

mRNA expression of lipases The mRNA level of HSL was increased by treatment of CME at concentrations of 15 and 20 mg/mL by 18.5 and 20.4%, respectively (Fig. 5). In addition, mRNA level of ATGL was increased at the same concentrations by 10.5 and 24.9%, respectively. However, lipoprotein lipase (LPL) was not significantly affected by treatment of CME, compared to untreated control. It has been well known that HSL is instrumental in lipolysis and subjected to hormonal regulation. For example, the rate of lipolysis can be dramatically stimulated by adrenergic hormones by activation of PKA that phosphorylates HSL, a rate-limiting enzyme for lipolysis. In recent, ATGL has been found to be predominantly responsible for the first step of TG hydrolysis (21). ATGL first acts on TG to hydrolyze a fatty acyl chain (22). Then, HSL hydrolyzes a second acyl side chain of diacylglycerol. Finally, monoacylglycerol lipase hydrolyzes the last acyl side chain. Thus, the level of glycerol released from the adipocytes is mainly dependent on cellular expression of lipases such as HSL and ATGL. In contrast, LPL is the rate-limiting enzyme in the hydrolysis of serum TG derived from the TG-rich lipoprotein particles, very low density lipoprotein (VLDL) and chylomicrons. The TG liberated by LPL is used by adipocytes for storage as a form of lipid droplet during lipid accumulation. In some reports, however, overexpression of LPL in obese rodents prevented diet-induced obesity (23). Thus, although LPL plays a central role in lipogenesis on the cellular basis, the physiological consequences still remain unclear. In the present study, using quantitative real-time PCR analysis, treatment of CME at the 20 mg/mL significantly increased the mRNA expression of ATGL, followed by HSL, but not mRNA expression of LPL. These results are consistent with those of free glycerol released from mature 3T3-L1 adipocytes, suggesting that free glycerol detected in cultured media is liberated from TG in lipid droplet by enzymatic hydrolysis of HSL and ATGL. Because the over-released glycerol may, at least in part, influence the liver and renal function *in vivo* system (24,25), the regulation of plasma glycerol level could be another issue to be considered in lipid mobilization and utilization. In this study, there is a chance of which glycerol liberated from TG by the activities of HSL and ATGL could be recycled to regenerate the cellular lipid components, suggesting that reagents or natural phytochemicals acting for the activation of hormone-dependent lipases would be the good treatment to avoid the possible side effects.

In summary, we demonstrated that CME stimulated the lipolysis in mature 3T3-L1 adipocytes through up-regulating both HSL and ATGL. This study hardly implies that CME has a direct effect on obesity due to lack of animal and human study, but it could be considered as a possible candidate to regulate lipid accumulation in obesity.

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