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Theobromine inhibits differentiation of 3T3-L1 cells during the early stage of adipogenesis via AMPK and MAPK signaling pathways

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Abstract

Obesity is characterized by hypertrophy and/or by the differentiation or adipogenesis of pre-existing adipocytes. In this study, we investigated the inhibitory effects of theobromine, a class of alkaloid in cocoa, on adipocyte differentiation of 3T3-L1 preadipocytes and its mechanisms of action. Theobromine inhibited the accumulation of lipid droplets, the expression of PPARγ and C/EBPα, and the mRNA expression of aP2 and leptin. The inhibition of adipogenic differentiation by theobromine occurred primarily in the early stages of differentiation. In addition, theobromine arrested the cell cycle at the G_0/G_1 phase and regulated the expressions of CDK2, p27, and p21. Theobromine treatment increased AMPK phosphorylation and knockdown of AMPKα1/α2 prevented the ability of theobromine to inhibit PPARγ expression in the differentiating 3T3-L1 cells. Theobromine reduced the phosphorylation of ERK and JNK. Moreover, the secretion and the mRNA level of TNF-α and IL-6 were inhibited by theobromine treatment. These data suggest that theobromine inhibits adipocyte differentiation during the early stage of adipogenesis by regulating the expression of PPARγ and C/EBPα through the AMPK and ERK/JNK signaling pathways in 3T3-L1 preadipocytes.

1. Introduction

Obesity is a complex disorder involving the abnormal accumulation of body fat as a result of energy imbalance and storage. In adults, obesity is associated with increased mortality and morbidity in a number of diseases, the most common being cardiovascular diseases and non-insulin-dependent diabetes mellitus[1]. Obesity occurs when adipose cells increase excessively in number (hyperplasia) and/or in size (hypertrophy)[2-4]. The concept of preventing obesity through stimulating the decomposition of accumulated fat or inhibiting the differentiation of adipocyte precursor cells has attracted a lot of research attention in recent years.

Adipogenesis is the process of adipocyte differentiation, whereby undifferentiated preadipocytes convert to differentiated adipocytes[5]. It is a complex process accompanied with changes in cell morphology and gene expression, as well as susceptibility to various hormonal changes. When induced to differentiate, growth-arrested preadipocytes initiate mitotic clonal expansion (MCE), which is known to cause reentry into the cell cycle for an additional two rounds of division. After the process of MCE, the cells initiate a series of transcriptional activation steps, with expression of adipogenic transcription factors such as CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ). The process of adipocyte differentiation is divided into the early, intermediate, and late stages. The early stage is mainly controlled by C/EBPβ and C/EBPδ, which are rapidly increased by inducers of cell division and hormonal stimulation[6]. Increases in the level of C/EBPβ and C/EBPδ trigger changes in the expression of PPARγ and C/EBPα, the master regulators of adipogenesis, either by acting alone or in cooperation with each other, after which the elevated levels are gradually decreased

in the late stage of adipocyte differentiation[7]. The late stage is regulated by PPARγ and C/EBPα, which interact with each other to have synergistic effects[8, 9]. In addition, the activation of PPARγ and C/EBPα regulates the expression of multiple genes including genes necessary for fat accumulation and insulin sensitivity[10].

In recent years, the use of nutraceuticals for the prevention and treatment of various chronic diseases or the improvement of health has received great amounts of interest[11]. Theobromine (3,7-dimethylxanthine), a caffeine derivative mainly found in cocoa bean and dark chocolate, belongs to a class of alkaloid molecules known as methylxanthines, which are related to caffeine and theophylline[12, 13]. In a previous report, methylxanthines were found to inhibit glucose transport in rat adipocytes by two independent mechanisms[14]. In addition, caffeine is also able to inhibit adipogenic differentiation of primary rat adipose-derived stem cells, a mouse bone marrow stromal cell line (M2-10B4) and 3T3-L1 cells through the suppression of adipogenesis-related factors[15]. Moreover, treatment with caffeine and its metabolites inhibits glucose uptake in differentiated adipocytes[16], while long-term feeding of cocoa prevents the development of obesity[17]. However, information regarding the biological activity of theobromine is limited, and the anti-adipogenic action of theobromine in preadipocytes has not yet been examined. Therefore, it would be worthwhile to examine the anti-adipogenic effects of theobromine. In this study, we validated the inhibitory effects and molecular mechanisms of action of theobromine on adipogenic differentiation using the 3T3-L1 adipocyte model in vitro.

2. Materials and Methods

2. 1. Reagents and Chemicals

Theobromine, isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, retinoic acid, oil red o, and isopropanol were purchased from Sigma-Aldrich Chemical (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was obtained from Corning Life Sciences (Tewksbury, MA). Fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Bovine calf serum (BCS) was obtained from Life Technologies (Grand Island, NY). Antibodies against phospho-p44/42 extracellular signal-regulated kinase (ERK), ERK, phospho-p38 MAPK, p38, phospho-SAPK/c-Jun N-terminal kinase (JNK), JNK, phospho-AMP-activated protein kinase α (AMPKα), AMPKα and β–actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against C/EBPβ, PPARγ, CDK2, p21 and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Formaldehyde and Isopropanol were purchased from Daejung Chemical & Metal Co., Ltd (Siheung, Gyeonggi). DC protein Assay Reagent was from Bio-Rad Laboratories (Hercules, CA). in-fect^{TM} in vitro Transfection Reagent was purchased from iNtRON Biotechnlogy, Inc (Sungnam, Korea).

2. 2. Cell Culture and Adipocyte Differentiation Induction

3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA). Mouse 3T3-L1 preadipocytes were cultured in DMEM containing 10% BCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

Cells were subcultured every 3–4 days at approximately 80% confluence. To induce adipocyte differentiation, 2 day post-confluent preadipocytes were exposed to differentiation medium (MDI) containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/ml insulin for 2 days. The differentiation medium was then exchanged for DMEM containing 10% FBS and 5 μg/ml insulin, and the cells were cultured for another 2 days. Finally, the cells were incubated in DMEM supplemented with 10% FBS for 2 more days. To evaluate the effects of theobromine on preadipocyte differentiation, the cells were cultured with differentiation medium in the presence or absence of various concentrations (50, 100, and 150 μg/ml) of theobromine at 2 day intervals when the medium was replenished. A range of concentrations of theobromine was prepared by serial dilution of a stock solution with 1.15 mM NaOH (Vehicle).

2. 3. Oil Red O staining and Lipid Quantification

Oil Red O staining was performed on Day 7 for staining of the accumulated lipid droplets in the differentiated adipocytes. After induction of adipocyte differentiation, the cells were washed with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde for 1 h at room temperature, and then rinsed with 60% isopropanol. 3T3-L1 cells were then stained with Oil Red O solution for 20 min at room temperature. After removing the staining solution, the stained cells were washed at least 3 times with distilled water and dried. The stained lipid droplets were visualized by light microscopy. The stained lipid was dissolved in 100% isopropanol and quantified with a ELISA reader (EMax Precision Microplate Reader; Molecular Devices, Sunnyvale, CA) at λ = 490 nm.

2. 4. Western Blot analysis

3T3–L1 cells were collected and suspended in homogenization buffer (50 mM Tris-Cl (pH 6.8), 1% glycerol, 2% SDS, and protease inhibitor cocktail. The total protein concentration of the lysates was then determined using DC protein Assay Reagent. The proteins in the lysates were separated electrophoretically on 8–15% sodium dodecyl sulfate polyacrylamide gels and then transferred to NC membranes (Merck Millipore, Billerica, MA). The membranes were blocked with 5% skim milk in tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 1 h at room temperature and then probed overnight at 4 °C with primary antibody diluted in TBS-T. After several washes with TBS-T, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBS-T at room temperature for 1 h. After another several washes with TBS-T, the immunocomplexes were developed using the Western blotting detection reagent (AbSignal; AbClon, Guro, Seoul). After measuring the intensity of each band by densitometry using the image processing software Image J, relative intensities were calculated by normalization to β–actin from the corresponding sample.

2. 5. Quantitative Real Time-PCR

Total RNA was extracted from the cells using Trizol Reagent, after which 1 μg/μl of the isolated RNA was subjected to reverse transcription using the cDNA synthesis kit Super Script II. Quantitative real time-PCR analysis for adipogenic genes was performed on a real time-PCR system (ABI Prism 7500; Applied Biosystems, Foster

City, CA). Gene expression was detected using SYBR Green, and the relative gene expression was determined by normalizing to the reference gene, GAPDH, with the relative quantitative method. The sequences of the primers corresponding to mouse adipogenic genes that was analyzed in this study were presented as follows: PPARγ (forward, 5′-GCC CTT TGG TGA CTT TAT GGA-3′; reverse, 5′-GCA GCA GGT TGT CTT GGA TG-3′); C/EBPα (forward, 5′-GCG AGC ACG AGA CGT CTA TAG A-3′; reverse, 5′-GCC AGG AAC TCG TCG TTG AA-3′); aP2 (forward, 5′-CCG CAG ACG ACA GGA AGG T-3′; reverse, 5′-AGG GCC CCG CCA TCT-3′); leptin (forward, 5′-TCG GTA TCC GCC AAG CA-3′; reverse, 5′-GGT GAA GCC CAG GAA TGA AG-3′); and GAPDH (forward, 5′-TGC ATC CTG CAC CAC CAA-3′; reverse, 5′-TCC ACG ATG CCA AAG TTG TC-3′).

2. 6. Cell Cycle analysis

Cell cycle analysis was conducted as described previously[18]. 3T3L-1 cells were treated with differentiation medium in the presence or absence of various concentrations (50-150 μg/ml) of theobromine for 24 h. Next, 10,000 cells from each experimental condition were fixed overnight with 70% ethanol at 4 °C, and then incubated with 10 μg/ml of RNase A and 50 μg/ml of propidium iodide for 30 min at room temperature in the dark. DNA content was measured with a Guava easyCyte™ Flow Cytometer (Merck Millipore, Billerica, MA), and analysis of the cell cycle was performed using the FCS Express 4 Flow Cytometry software (De Novo Software, Los Angeles, CA).

2. 7. RNA interference

To knockdown the endogenous AMPK, 3T3-L1 cells were transiently transfected with 75 nM mouse siRNAs targeting AMPKα1/α2 or non-silencing control siRNA using in-fect™ in vitro Transfection Reagent in culture medium without antibiotics according to the manufacturer's protocol. After 48 h, the medium was replaced with differentiation medium with or without theobromine.

2. 8. Statistical analysis

Each experiment was repeated at least three times, and the results of a representative experiment are shown. All values were expressed as the mean ± S.E.M. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Tukey's posttest. P value < 0.05 was considered to be statistically significant.

3. Results

3. 1. Theobromine inhibits the differentiation of 3T3-L1 preadipocytes

In this study, we investigated whether theobromine modulates adipocyte differentiation in 3T3-L1 preadipocytes. We first performed an MTT assay to examine whether theobromine has an influence on the cell proliferation of 3T3-L1 preadipocytes. The results revealed that theobromine and NaOH (solvent) had no cytotoxicity to the cells at the concentrations used in this experiment (Fig. 1A). Thus, concentrations of 50, 100, and 150 ug/ml were selected for all subsequent experiments. To examine the effects of theobromine on adipocyte differentiation in 3T3-L1 preadipocytes, post-confluent preadipocytes exposed to the differentiation medium (MDI) were treated with the indicated concentrations of theobromine for 7 days, after which the cells were stained with Oil Red O and visualized (Fig. 1B). Theobromine decreased the accumulation of lipid droplets in a concentration-dependent manner (Fig. 1C). It had the highest inhibitory effect on the formation of lipid droplets when treated at 150 μg/ml. These results suggest that theobromine inhibits adipocyte differentiation in 3T3-L1 preadipocytes.

3. 2. Theobromine inhibits the expression of adipogenic transcription factors and genes

Adipocyte differentiation involves various effectors, such as transcription factors and genes, including PPARγ, C/EBPα, aP2 and leptin[19]. Therefore, we next examined whether theobromine inhibits adipocyte differentiation through the

regulation of these effectors. Cells were treated with various concentrations of theobromine during the process of MDI-induced adipogenesis. At this time, the protein expression of PPARγ and the mRNA expressions of PPARγ and C/EBPα were found to be decreased by theobromine in a concentration-dependent manner (Fig. 2). Theobromine treatment also resulted in the inhibition of aP2 and leptin mRNA expression (Fig. 2B). Thus, these results demonstrated that theobromine inhibits the differentiation of 3T3-L1 preadipocytes through downregulation of PPARγ, C/EBPα, aP2 and leptin.

3. 3. Theobromine suppresses the early stage of adipogenesis

Since it is well known that the differentiation of preadipocytes into mature adipocyte takes place in several stages, from early (Day 0-2), to intermediate (Day 2-4) and terminal (Day 4-6) stages (Fig. 3A)[20], it was next examined which stage of adipocyte differentiation was critically affected by the theobromine treatment. To accomplish this, cells were treated with 150 μg/ml theobromine at various time points during adipogenesis, and the lipid accumulation was measured. As shown in Fig. 3B, the relative lipid contents of adipocytes treated with 150 μg/ml of theobromine during the entire period (Day 0-6) and at the early stages (Day 0-2) were decreased compared with the control cells, with similar inhibitory effects observed during the early adipocyte differentiation stage (Day 0–2) and the entire adipocyte differentiation stage (day 0–6). These results indicate that theobromine regulates the early stage of adipocyte differentiation.

We also examined the level of expression of C/EBPβ, which is an essential transcription factor expressed at the early stage of adipocyte differentiation[21, 22]. The

expression of C/EBPβ was significantly decreased by theobromine treatment in a concentration-dependent manner (Fig. 3C). Collectively, these results suggest that the inhibitory effects of theobromine occur during the early stage of adipocyte differentiation.

3. 4. Theobromine induces G0/G1 phase arrest through upregulation of p27 and p21

We next investigated the effects of theobromine on the cell cycle of adipocytes induced to differentiate from a resting state. Theobromine treatment was found to result in dose-dependent arrest of the cell cycle at the G0/G1 phase (Fig. 4A). Examination of the expression of cell cycle-regulatory proteins expressed during the early stage of adipogenesis revealed that theobromine inhibited the expression of CDK2, a cell cycle regulatory protein of the G0/G1 phase, while the expressions of p27 and p21, potent inhibitors of CDK which are involved in G1 arrest, were increased by theobromine (Fig. 4B). These results suggest that theobromine treatment induces G0/G1 cell cycle arrest by regulating the levels of CDK2, p21 and p27 expression.

3. 5. Theobromine regulates the phosphorylation of AMPK and MAPK in 3T3-L1 adipocytes

To determine whether the mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) signaling pathways that are associated with the early stage of adipocyte differentiation are involved in theobromine-inhibited adipogenesis, the levels of AMPK and MAPK phosphorylation were examined after

treating the cells with or without theobromine. As shown in Fig. 5A, theobromine treatment resulted in a significant increase in the phosphorylation of AMPK, which occurred in a concentration-dependent manner. To investigate whether theobromine-mediated AMPK activation is directly required for inhibition of adipocyte differentiation, we employed siRNA interference (Fig. 5B). Depletion of AMPKα1/α2 using siRNA significantly increased the expression of key adipogenic transcription factor PPARγ. Additionally, the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) displayed concentration-dependent attenuation upon treatment with theobromine (Fig. 5C). These results suggest that theobromine inhibits the early stage of adipogenesis through AMPK activation and suppression of the ERK and JNK signaling pathways in 3T3-L1 adipocytes.

3. 6. Theobromine inhibits the pro-inflammatory cytokine in 3T3-L1 adipocytes

Fat cells synthesize and secrete a variety of adipocytokines into circulation, including TNF-α and IL-6, angiogenic proteins, and metabolic regulators[23]. It is widely accepted that the levels of TNF-α and IL-6 are elevated in obesity, leading to modulate glucose homeostasis and lipid metabolism during adipogenesis[24]. To examine whether theobromine has an influence on the secretion of adipocytokines, the levels of TNF-α and IL-6 were measured using ELISA and Quantitative Real Time-PCR. As shown in Fig. 6, theobromine inhibited the production of both TNF-α and IL-6 in a concentration-dependent manner, implicating theobromine potentially has inhibitory effects on lipid metabolism.

4. Discussion

The regulation of adipogenesis, along with decrease in adipocyte numbers and the lipid content of adipocytes, could be a potential therapeutic strategy for treating obesity. Many natural compounds of plants and phytochemicals have been found to regulate adipogenesis without any side effects. Theobromine was previously reported to have antitumor effects against malignant glioblastoma and inhibitory effects on sensory nerve function and cough[25]. However, the regulation of adipocyte differentiation, adipogenic gene expression, signaling pathway and cytokine production in theobromine-treated adipocytes remains unknown.

In the present study, we determined that treatment with theobromine, a methylxanthine alkaloid derivative, leads to inhibition of the early stage of adipocyte differentiation via a signal transduction pathway involving AMPK activation and inhibition of the ERK and JNK pathways, indicating that theobromine has anti-adipogenic effects. The experimental results were consistent with the findings that the long-term feeding of cocoa is associated with inhibition of obesity[17].

Adipocyte differentiation is associated with multifunctional cellular pathways and requires the sequential regulation of adipogenic and lipogenic genes[5]. It is also well known that the critical adipogenic transcription factors, PPARγ and C/EBPα, act in cooperation to accelerate adipogenesis[26, 27]. In addition, the expression of these transcription factors is induced in the early phase of adipogenesis, at which time C/EBP factors bind to the PPARγ promoter. Theobromine was found to significantly suppress lipid accumulation in a concentration-dependent manner via the decreased expression of PPARγ and C/EBPα. Our results also demonstrated the inhibitory effect of theobromine on the accumulation of lipid droplets during early adipocyte

differentiation. Thus, theobromine was able to inhibit adipogenesis in 3T3-L1 preadipocytes at an early stage by regulating the expression of PPARγ and C/EBPα.

It was previously reported that mitotic clonal expansion (MCE) is required for adipocyte differentiation[28]. During the MCE phase, differentiating preadipocytes exit from the growth arrest signal that was initiated upon reaching cell confluence to reenter the cell cycle for a few rounds of cell division before expression of the adipogenic genes[28]. The differentiation inducer, MDI, causes the induction of C/EBPβ and C/EBPδ expressions during the early stage of adipogenesis, leading to increased expressions of C/EBPα and PPARγ, which terminate mitotic clonal expansion[21, 22]. In this study, theobromine exhibited blocking of the cell cycle at the G1/S transition induced by adipocyte differentiation, suggesting that theobromine affects MCE during adipocyte differentiation.

We next examined the expressions of cell cycle-regulatory proteins expressed during the early stage of adipogenesis in theobromine-treated cells. This is because the activation of CDK2-cyclin E/A and cyclin D1, as well as the turnover of p27kip1 are associated with MCE[29]. Cyclin-dependent kinases (CDKs) regulate the reentry of cells into the cell cycle. Formation of the Cyclin·CDK complex is regulated by two families of cyclin-dependent kinase inhibitors (CDKIs)[28]. Members of the CIP1/KIP1 family, such as p21, p27, and p57, inhibit CDKs by forming ternary complexes with various cyclin-CDKs, whereas the INK4 family, which includes p15, p16, p18 and p19, inhibits CDK activity through the formation of binary complexes with CDKs[30]. As a G_1/S checkpoint, Sulforaphane arrests the cell cycle at the G_0/G_1 phase through decreased expression of cyclin D1, CDK4, cyclin A, CDK2, and phosphorylated Rb protein, but not cyclin E[31]. Similarly, vitisin A, a resveratrol tetramer, inhibits adipogenesis through G_1 arrest by increased p21 expression, as well as decreased

expressions of cyclins A and B and CDK2, but not cyclin E, in 3T3–L1 cells[32]. The expressions of the CDK inhibitors p27 and p21 are downregulated during adipogenesis, but were restored herein by treatment with theobromine (Fig. 4B). These results demonstrate that theobromine induces cell cycle arrest at the G_0/G_1 phase through the upregulation of p27 and p21 in 3T3–L1 adipocytes.

A recent study demonstrated that the MAPK and AMPK pathways play an important role in adipocyte differentiation[33]. 5' AMP-activated protein kinase (AMPK), an intracellular energy sensor, is activated by many types of cellular stresses, either by decreasing catabolic generation of ATP such as hypoxia, ischemia, and glucose deprivation or increasing ATP consumption such as muscle contraction, leading an increase in cellular ADP:ATP and AMP:ATP ratios. Thus, AMPK regulates energy homeostasis in concert with other central signaling components in order to balance nutrient supply with energy demand[34]. AMPK has been suggested to play a role in the pathogenic development of metabolic diseases such as obesity, type 2 diabetes and cancer[35]. Interestingly, it is reported that knockout of AMPKα1 or AMPKα2 subunits cause insulin resistance and the development of obesity in vivo[36, 37]. The present data demonstrated that theobromine caused increased activation of the AMPK pathway. In addition, theobromine reduced the expression of key adipogenic transcription factor PPARγ and this effect was reversed by transfection of cells with AMPK-specific siRNA, suggesting that AMPK is directly involved in theobromine-mediated inhibition of adipocyte differentiation and activation of AMPK by theobromine could regulate energy balance during adipocyte differentiation. Mitogen-activated protein kinases (MAPKs) are widely related to many physiological processes[38]. Although it is also suggested that activation of ERK/MAPK pathway might have opposing effects in the process of adipogenesis[39], it is more widely

known that the MAPK pathways contribute to adipocyte growth and differentiation, and cause activation of various transcription factors. ERK is known to be required for the proliferation and differentiation of 3T3-L1 preadipocytes[40], and JNK is implicated in the development of obesity-related insulin resistance[41]. Hence, the MAPK and AMPK pathways are possible targets for treatment of obesity, because these pathways contribute to the expression or phosphorylation of the downstream proteins related to adipocyte differentiation. Consistent with the finding that ERK pathway is involved in induction of adipocyte differentiation[42], our observations showed that MDI treatment stimulated the ERK pathway and this effect was inhibited by theobromine.

The MAPK and AMPK pathways have been suggested to play important roles in the production of proinflammatory cytokines and inflammation-related disorders[43]. In the current study, theobromine caused a significant increase in the phosphorylation of AMP-activated protein kinase α (AMPKα), and inhibited the phosphorylation of ERK and JNK. Theobromine treatment also resulted in the inhibition of mRNA level and secretion of TNF-α and IL-6. Recently, curcumin and resveratrol were demonstrated to reduce the levels of TNF-α, IL-1 and IL-6 in differentiated adipocytes via inactivation of NF-κB[44]. Based on these findings, it is plausible that theobromine could act through a similar mechanism. Taken together, these data suggest that theobromine suppressed the differentiation of preadipocytes via regulation of the ERK, JNK and AMPK signaling pathways.

In summary, our results demonstrated that theobromine suppresses adipogenesis in 3T3-L1 cells by regulating the expressions of PPARγ and C/EBPα, through modulation of the AMPK, ERK and JNK signaling pathways at the early stages of adipogenesis. The present data provide insight into the possible mechanisms of

theobromine-mediated inhibition of adipocytes, and suggest that theobromine could be useful to help prevent obesity and other inflammatory diseases. Further studies are needed to examine whether theobromine also has the same effects in vivo.

Fig. 1 - Inhibitory effects of theobromine on lipid accumulation in 3T3-L1 adipocytes

(A) 3T3-L1 preadipocytes were treated with the indicated concentrations of theobromine for 48 h. Cell proliferation was then determined by MTT assay. (B) 3T3–L1 preadipocytes were cultured in differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine or 10 μM retinoic acid (positive control) for 7 days. The cells were then stained with Oil Red O and photographed (× 100). (C) The cells stained with Oil red O were subject to quantitative analysis of the intracellular lipid accumulation. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. *$p < 0.05$ vs only MDI-treated cells.

Fig. 2 - Inhibitory effects of theobromine on the expression of adipogenic transcription factors and adipogenic genes in 3T3-L1 adipocytes

3T3–L1 preadipocytes were cultured in the differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine for 7 days. (A) The cells were harvested, and the lysates were subjected to Western blot analysis for PPARγ. (B) The cells were harvested, and the lysates were subjected to real time-PCR for adipogenic transcription factors and genes. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. The percentages of densitometry are relative to the theobromine-free control without inducers (100%). *$p < 0.05$ vs only MDI-treated cells.

Fig 3 - Inhibitory effects of theobromine on mitotic clonal expansion during the early stage of adipocyte differentiation in 3T3-L1 adipocytes

(A) 3T3–L1 preadipocytes were cultured in differentiation medium to which 150 μg/ml

theobromine was added for Days 0–2, 2–4, 4–6, and 0–6. (B) The cells were stained with Oil Red O and subjected to quantitative analysis of the intracellular lipid accumulation. (C) Adipocytes treated with 0, 50, 100, and 150 μg/ml theobromine for 4 h were harvested, and the lysates were subjected to Western blot analysis for C/EBPβ. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. The percentages of densitometry are relative to the theobromine-free control without inducers (100%). *p < 0.05 vs only MDI-treated cells.

Fig. 4 - Regulatory effects of theobromine on cell cycle progression in 3T3-L1 adipocytes

3T3–L1 preadipocytes were cultured in differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine for 24 h. (A) The cells were stained with a PI solution and analyzed by flow cytometry (upper panel). The percentage of the cell population at each stage of the cell cycle was determined using the FCS Express 4 Flow Cytometry software (lower panel). (B) The cells were harvested, and the lysates were subjected to Western blot analysis for CDK2, p27, and p21. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. The percentages of densitometry are relative to the theobromine-free control without inducers (100%). *p < 0.05 vs only MDI-treated cells.

Fig. 5 - Effects of theobromine on phosphorylation of AMPK and MAP Kinases in 3T3-L1 adipocytes

(A) 3T3–L1 preadipocytes were cultured in differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine for 1 h. The cells were lysed and phosphorylation

levels of AMPK were determined by western blotting. The intensity of the bands was quantitated by densitometry. *p < 0.05, significantly different from control (no treatment). (B) Cells were transfected with control siRNA or AMPKα1/α2 for 48 h followed by theobromine (50-150 μg/ml) treatment for a further 1 h or 7 days. The cells were lysed and PPARγ and AMPK proteins were then evaluated by western blotting. The results illustrated are from a single experiment, and are representative of three separate experiments. The β-actin protein level was considered as an internal control. The intensity of the bands was quantitated by densitometry. (C) 3T3–L1 preadipocytes were cultured in differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine for 2 h. The cells then were harvested, and the lysates were subjected to Western blot analysis for phosphorylated and total ERK, p38 and JNK. The intensity of the bands was quantitated by densitometry. *p < 0.05, significantly different from control (no treatment).

Fig. 6 - Inhibitory effects of theobromine on the secretion of pro-inflammatory cytokines in 3T3-L1 adipocytes

3T3–L1 preadipocytes were cultured in differentiation medium containing 0, 50, 100, and 150 μg/ml theobromine for 7 days. (A) The supernatants of the cells were then measured for IL-6 and TNF-α by ELISA. (B) Total RNA was extracted and the levels of IL-6 and TNF-α mRNA were determined by quantitative RT-PCR analysis. All values are presented as the mean ± S.E.M. of three experiments performed in triplicate. *p < 0.05 vs only MDI-treated cells.

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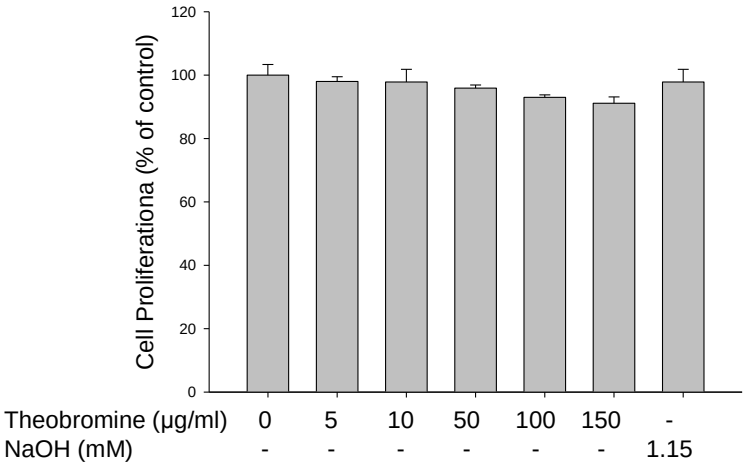
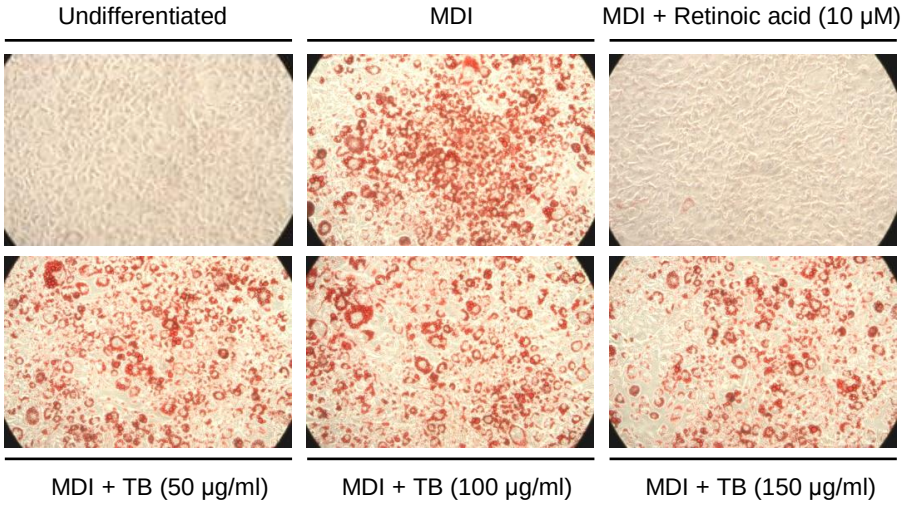
A


Cell Proliferationa (% of control)
120
100
80
60
40
20
0
Theobromine (μg/ml) 0 5 10 50 100 150 -
NaOH (mM) - - - - - - 1.15

B


Undifferentiated
MDI
MDI + Retinoic acid (10 μM)
MDI + TB (50 μg/ml)
MDI + TB (100 μg/ml)
MDI + TB (150 μg/ml)

C

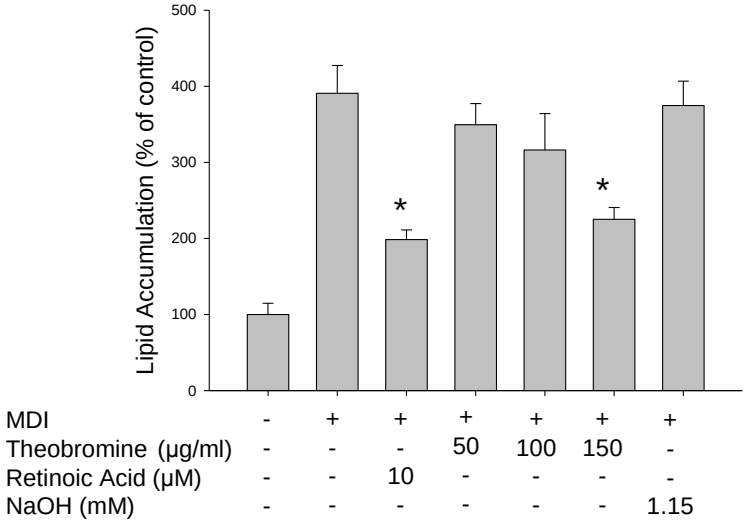
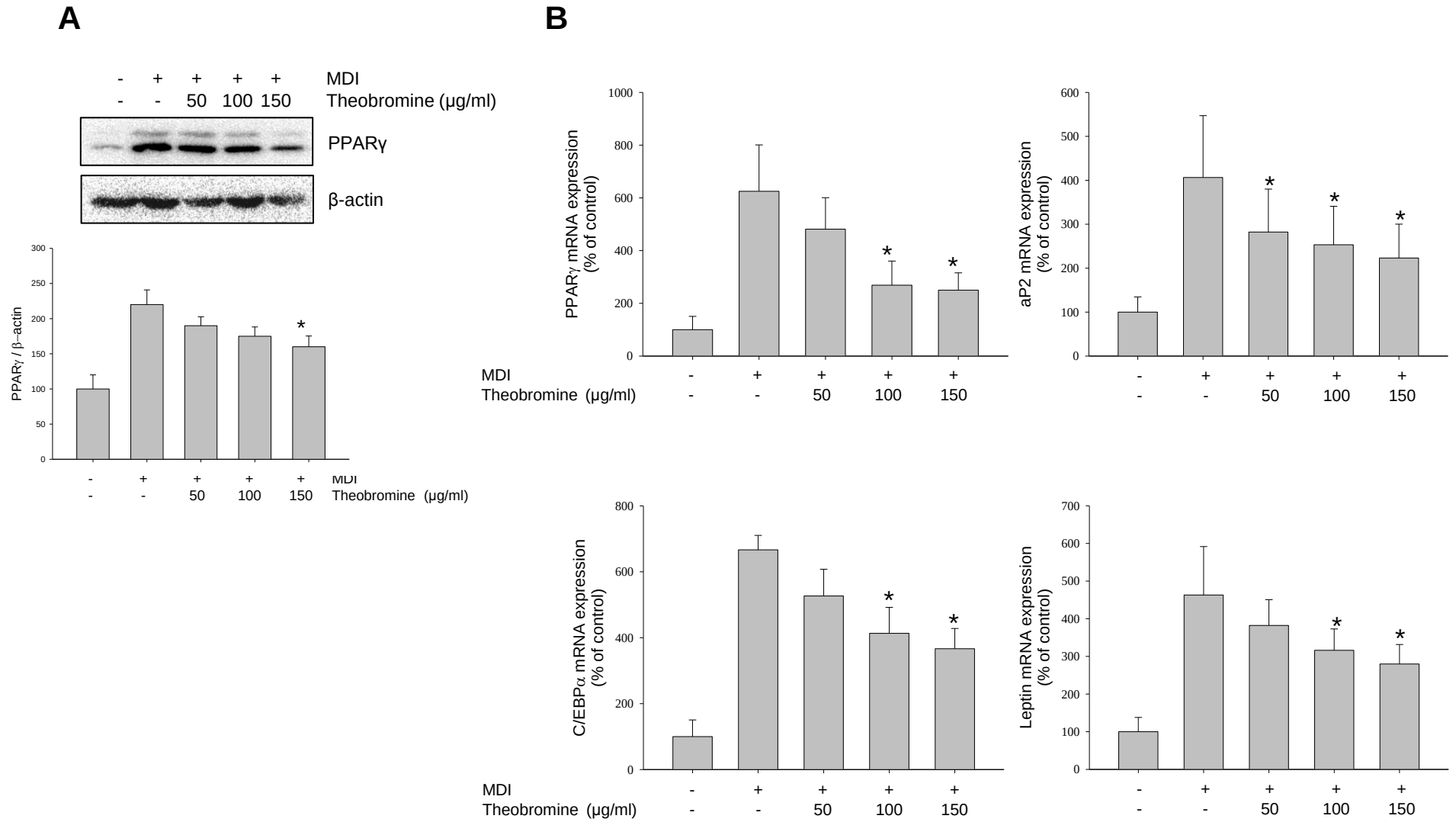
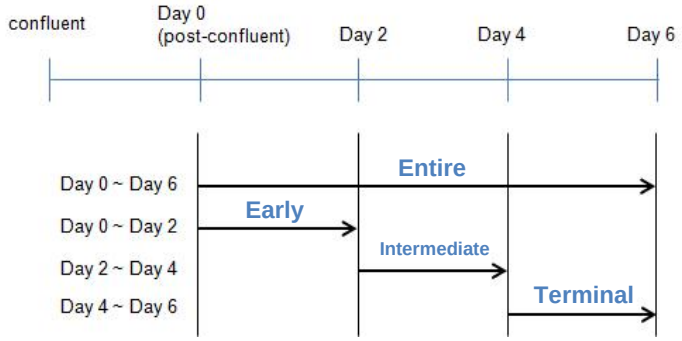

Lipid Accumulation (% of control)
500
400
300
200
100
0
*
*
MDI - + + + + + +
Theobromine (μg/ml) - - - 50 100 150 -
Retinoic Acid (μM) - - 10 - - - -
NaOH (mM) - - - - - - 1.15

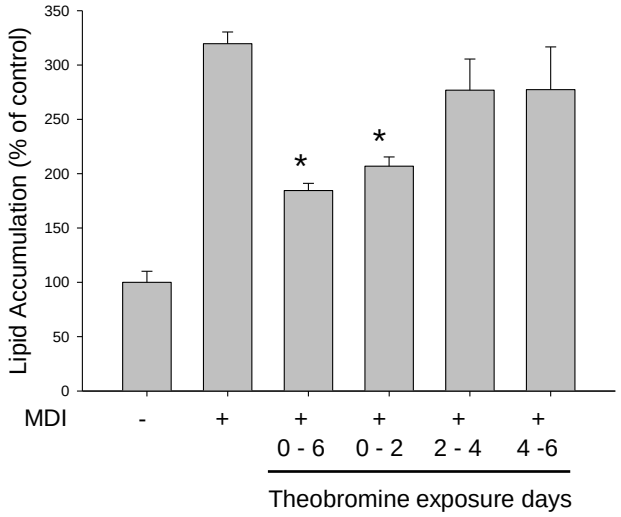
Fig. 2


A
MDI
Theobromine (μg/ml)
PPARγ
β-actin
PPARγ / β-actin
B
PPARγ mRNA expression (% of control)
aP2 mRNA expression (% of control)
C/EBPα mRNA expression (% of control)
Leptin mRNA expression (% of control)
0 50 100 150

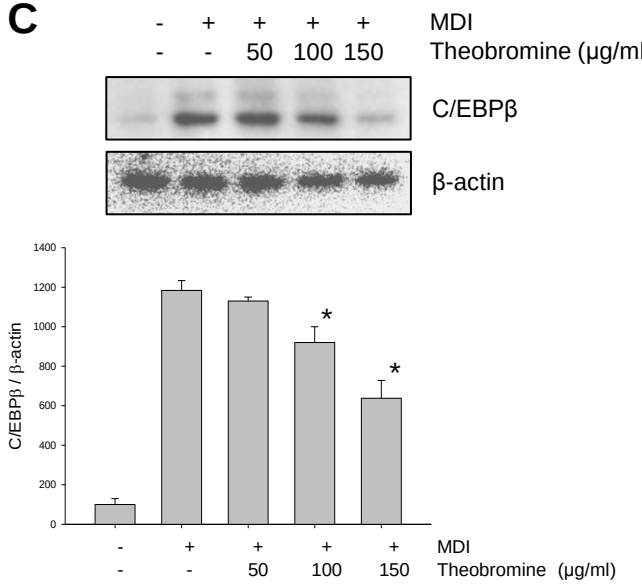
A


confluent
Day 0
(post-confluent)
Day 2
Day 4
Day 6
Day 0 ~ Day 6
Day 0 ~ Day 2
Day 2 ~ Day 4
Day 4 ~ Day 6
Entire
Early
Intermediate
Terminal

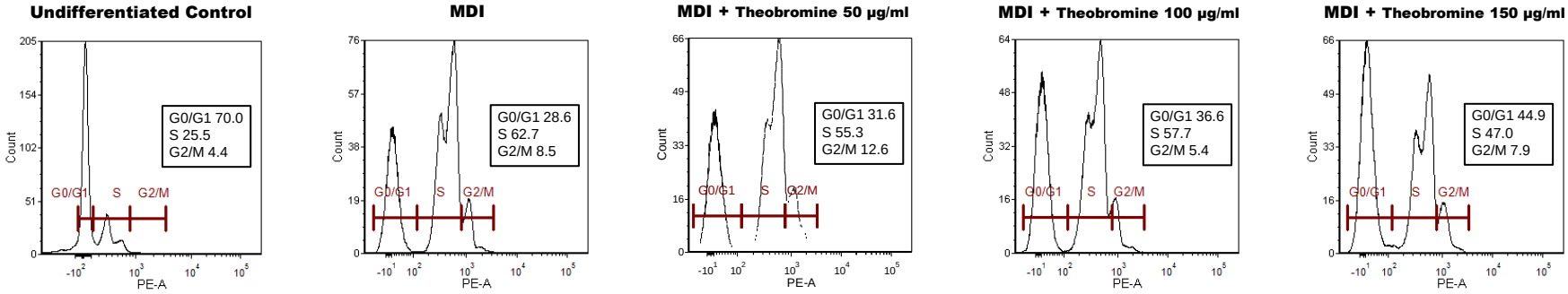
B

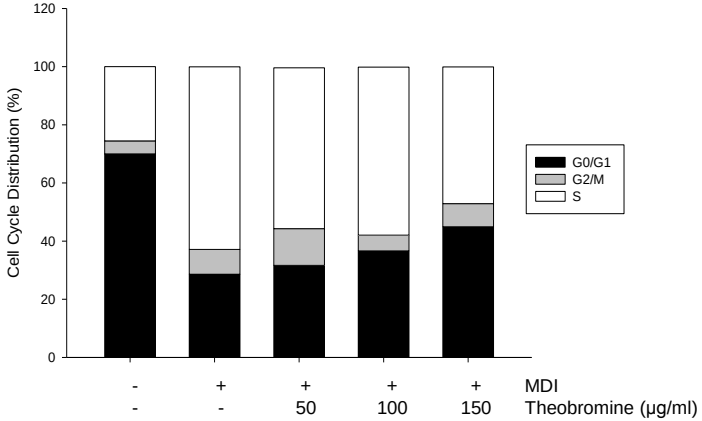

Lipid Accumulation (% of control)
350
300
250
200
150
100
50
0
MDI - + + + + +
0 - 6
0 - 2
2 - 4
4 -6
Theobromine exposure days
*
*

C

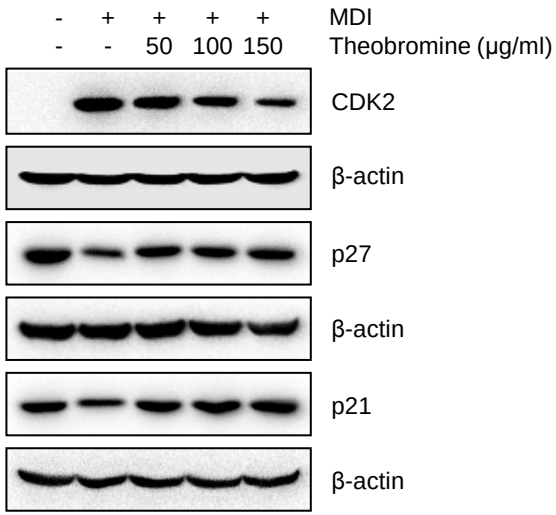

- + + + + MDI
- - 50 100 150 Theobromine (μg/ml)
C/EBPβ
β-actin
C/EBPβ / β-actin
1400
1200
1000
800
600
400
200
0
*
*
- + + + + MDI
- - 50 100 150 Theobromine (μg/ml)

A

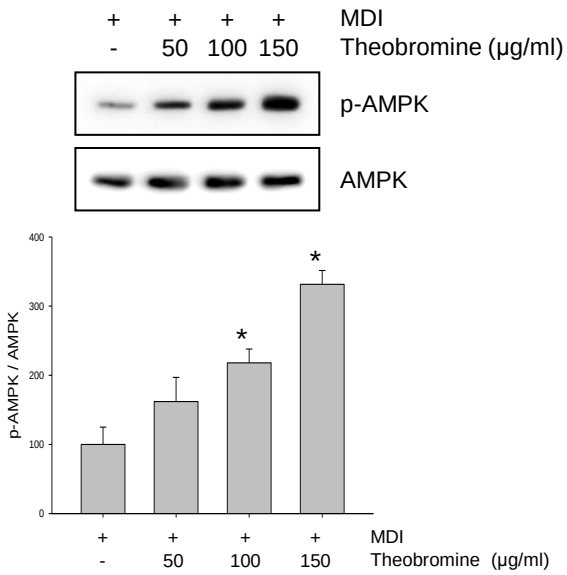

Undifferentiated Control
G0/G1 70.0
S 25.5
G2/M 4.4
MDI
G0/G1 28.6
S 62.7
G2/M 8.5
MDI + Theobromine 50 μg/ml
G0/G1 31.6
S 55.3
G2/M 12.6
MDI + Theobromine 100 μg/ml
G0/G1 36.6
S 57.7
G2/M 5.4
MDI + Theobromine 150 μg/ml
G0/G1 44.9
S 47.0
G2/M 7.9
Count
PE-A
G0/G1
S
G2/M


Cell Cycle Distribution (%)
G0/G1
G2/M
S
MDI
Theobromine (μg/ml)

B


MDI
Theobromine (μg/ml)
CDK2
β-actin
p27
β-actin
p21
β-actin

A


+ + + + MDI
- 50 100 150 Theobromine (μg/ml)
p-AMPK
AMPK
p-AMPK / AMPK
400
300
200
100
0
*
*
+ + + + MDI
- 50 100 150 Theobromine (μg/ml)

B

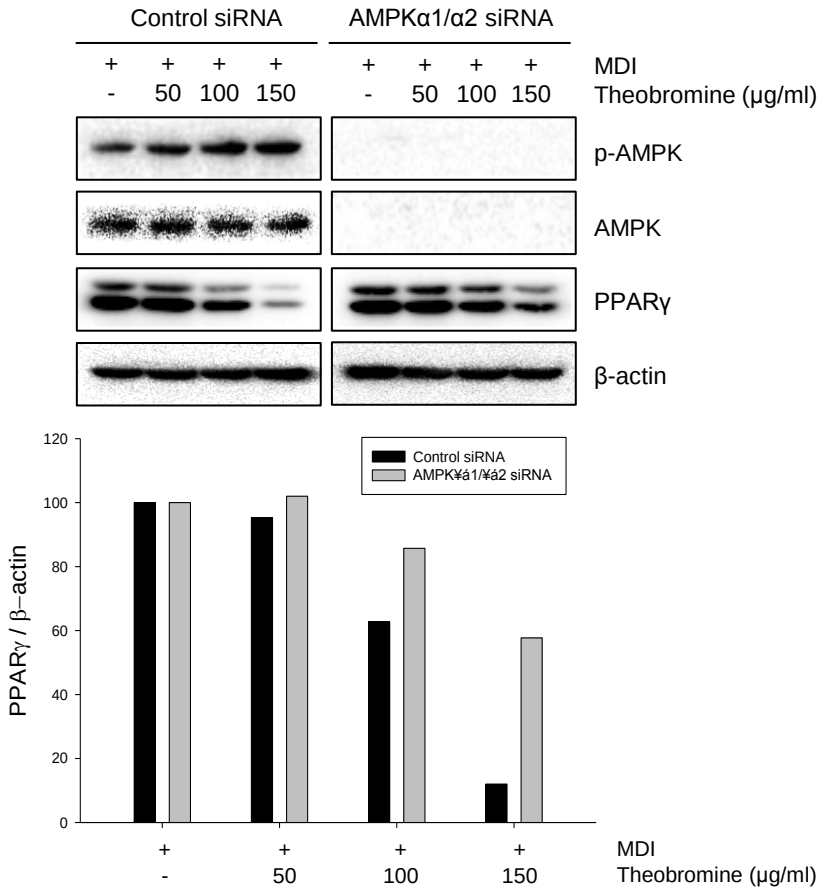
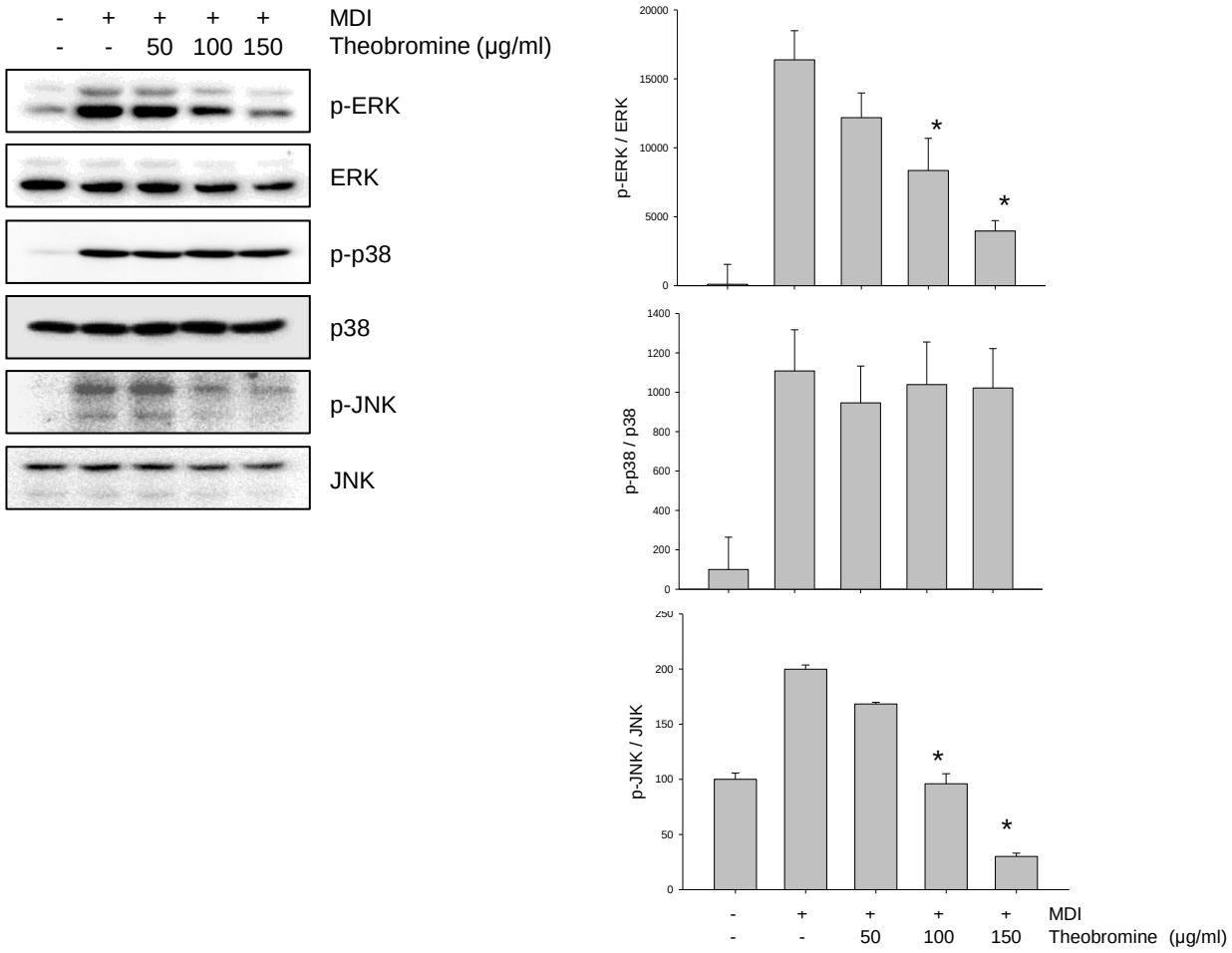
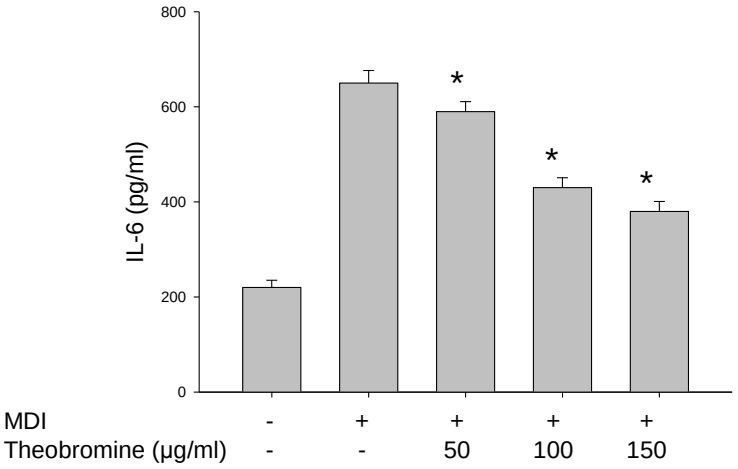

Control siRNA
AMPKα1/α2 siRNA
+ + + + + + + + MDI
- 50 100 150 - 50 100 150 Theobromine (μg/ml)
p-AMPK
AMPK
PPARγ
β-actin
Control siRNA
AMPK¥á1/¥á2 siRNA
PPARγ / β-actin
120
100
80
60
40
20
0
+ + + + MDI
- 50 100 150 Theobromine (μg/ml)

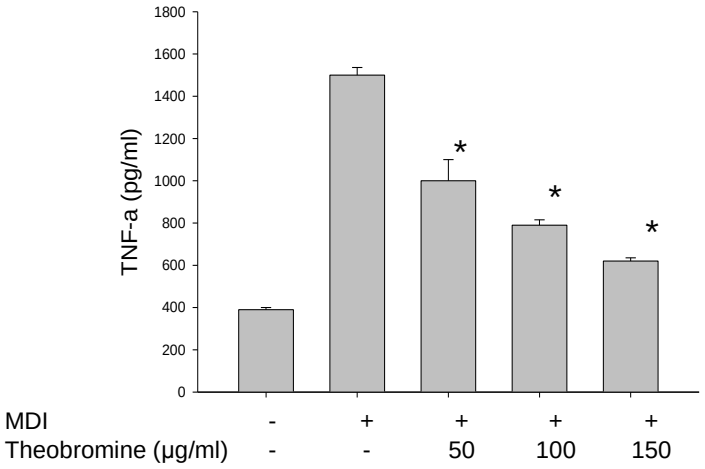
Fig. 5 (continued)

C

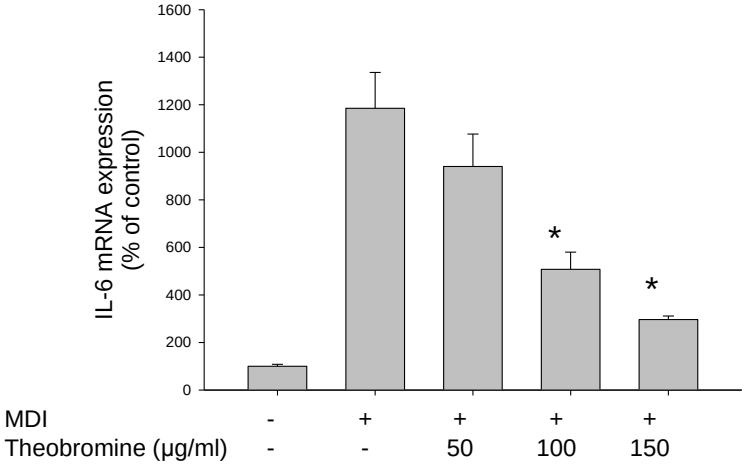


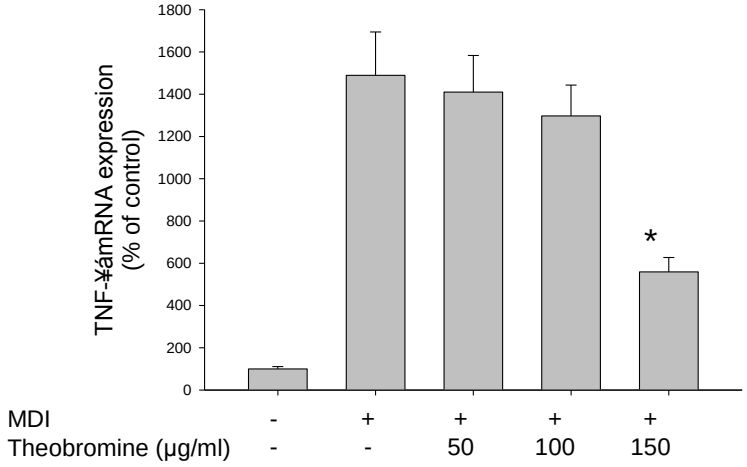
A


IL-6 (pg/ml)
800
600
400
200
0
*
*
*
MDI - + + + +
Theobromine (μg/ml) - - 50 100 150


TNF-a (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
MDI - + + + +
Theobromine (μg/ml) - - 50 100 150

B


IL-6 mRNA expression
(% of control)
1600
1400
1200
1000
800
600
400
200
0
*
*
MDI - + + + +
Theobromine (μg/ml) - - 50 100 150


TNF-¥ámRNA expression
(% of control)
1800
1600
1400
1200
1000
800
600
400
200
0
*
MDI - + + + +
Theobromine (μg/ml) - - 50 100 150