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The Effect of *Monascus* Secondary Polyketide Metabolites, Monascin and Ankaflavin, on Adipogenesis and Lipolysis Activity in 3T3-L1

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The aim of the present work is to investigate the effects of *Monascus* secondary metabolites, monascin (MS) and ankaflavin (AK), on cell proliferation, adipogenesis, lipolysis and heparin-releasable lipoprotein lipase (HR-LPL) in 3T3-L1 preadipocyte. MS and AK inhibit the proliferation of 3T3-L1 cells in a dose-dependent fashion. At 8 μg/mL concentration MS inhibits proliferation for 80.5% after 48 h, whereas the value for AK is 69.2%. Adipogenesis is inhibited by MS and AK without dose-dependency. Triglyceride is decreased 37.1% and 41.1% respectively by treating 0.125 μg/mL MS and AK. Adipocyte-specific transcription factors peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein β (C/EBPβ), C/EBPδ and C/EBPα mRNA levels are measured by real-time polymerase chain reaction. The expression of the four transcriptional factors analyzed (PPARγ, C/EBPβ, C/EBPδ and C/EBPα) is reduced at the initial and the middle period. At the later period, there is no effect on the expression of PPARγ and C/EBPα by treating MS and AK. Furthermore, both MS and AK increase basal lipolysis of mature adipocytes by 113.2% and 278.3% upregulation, respectively. And both MS and AK reduce the activity of HR-LPL, by 45.3% and 58.1% reduction, respectively. This study reveals for the first time that *Monascus* secondary metabolites, MS and AK, can prevent the differentiatioin of preadipocyte and stimulate basal lipolysis of mature adipocytes, avoiding the accumulation of lipid.

KEYWORDS: 3T3-L1 preadipocyte; *Monascus* secondary metabolites; adipogenesis; differentiation; lipolysis

INTRODUCTION

Obesity is a complex, multifactorial, chronic disease that is considered to be a risk factor for the genesis or development of various diseases, including hypertension, type 2 diabetes, coronary heart disease, cancer, respiratory complications, and osteoarthritis (*1*). Obesity arises from an imbalance in energy intake and energy expenditure that leads to the pathological growth of adipocytes. Adipocytes are the major cellular component in fat tissue and excessive growth. Differentiation and hypertrophy of adipocytes are fundamental processes of obesity. 3T3-L1 cells facilitate the investigation of regulatory mechanisms of adipocyte differentiation (*2*). The differentiation of preadipocytes into adipocytes involves exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors. This cascade begins with the CCAAT/enhancer-binding protein (C/EBP)β and C/EBPδ, which finally induce the expression of C/EBPα and peroxisome proliferator-activated receptor (PPAR)γ (*3*). These transcription factors coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype.

The *Monascus* species have been used as a traditional food fungus and medicine in Eastern Asia for several centuries. The most famous secondary metabolite is monacolin K, which was proven as the inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in the cholesterol biosynthesis pathway (*4*). In addition to monacolin K, there are many types of polyketide secondary metabolites produced by *Monascus* spp. Monascin (MS) and ankaflavin (AK) are polyketide metabolites of yellow pigments from *Monascus*. The mechanism of formation of these two yellow pigments has not yet been elucidated (*5*). MS has been reported to have anti-inflammatory potential and antitumor-initiating effects (*6*, *7*). AK has been reported to have cytotoxic effect and induces cell death on Hep G2 cells (*8*). Some studies indicate that red mold dioscorea (RMD) has a greater antihypertensive bioavailability, hypolipidemic and antiatherosclerotic effect than red mold rice (RMR) and unfermented dioscorea because RMD contained a higher amount of anti-inflammatory yellow pigment, MS and AK (*9*, *10*).

Our previous study revealed that RMR can prevent body fat accumulation and improve dyslipidemia, and the extracts of RMR suppressed proliferation and differentiation in 3T3-L1 preadipocytes (*11*). This indicated that RMR has more than one bioactive component such as monacolin K. Another study demonstrated that the water extracts of RMR suppress adipogenesis in 3T3-L1 preadipocytes through the downregulated expression of adipogenic transcription factors and other specific genes, but results may not be due to monacolin K (*12*). On the basis of these

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findings, we suggest that yellow pigments, MS and AK, could suppress adipogenesis in 3T3-L1 preadipocytes.

In this study, we used the 3T3-L1 cell line to determine the effects of MS and AK on proliferation and differentiation in preadipocyte, lipolysis and the activity of heparin-releasable lipoprotein lipase (HR-LPL), a key enzyme responsible for lipogenesis, in mature adipocyte. Furthermore, we investigate the effects of MS and AK on the expression of C/EBPβ, C/EBPδ, PPARγ and C/EBPα genes.

MATERIALS AND METHODS

Chemicals and Reagents. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT), crystal violet, dexamethasone, isobutylmethylxanthine, insulin, oil-red O, heparin, *p*-nitrophenyl butyrate, NaCl, $NaHCO_3$, KCl, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), trypsin and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD, USA). Ethanol (95%) was purchased from Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from HyClone Laboratories (Logan, UT, USA). All other chemicals were the highest purity commercially available.

Preparation of MS and AK. The desired compounds MS and AK were obtained as described with some modification (*13*). The effective compounds (>95% purity) were identified by Nuclear Magnetic Resonance (NMR, Varian Gemini, 200 MHz, FT-NMR, Varian Inc., Palo Alto, CA, USA) and electrospray ionization-mass spectrometry (ESI-MS, Finnigan MAT LCQ, Thermo Electron Co., Waltham, MA, USA) analysis.

Cell Culture. Mouse embryo 3T3-L1 cells (BCRC 60159) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). 3T3-L1 cells were cultured in basal medium (DMEM which containing 10% FBS and 1% antibiotic/antimitotic solution at 37 °C in 5% CO_2). To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone and 10 μg/mL insulin (MDI) added to basal medium. On day 3, the MDI medium was replaced with basal medium containing insulin only. On day 6 and thereafter, the cells were cultured in basal medium, which was freshly changed every 2 days until the cells were analyzed.

Proliferation Assay. Preconfluent 3T3-L1 preadipocytes were seeded in 24-well dishes at a density of 7.5×10^3 cells per 0.5 mL per well. After the cells adhered to the dishes, MS or AK was added to the culture medium at the indicated doses for 24 and 48 h. Viable cells at each dose and time point were stained for 10 min with 0.5% crystal violet, then dissolved in 2% SDS (wt/vol) after rinsed by H_2O, measured at A_{600}.

Cell Viability Assay. For mature adipocytes, cells were seeded in 24-well dishes at a density of 7.5×10^3 cells per 0.5 mL per well, grown to maturation. Adipocytes were incubated with MS or AK for 48 h. Viable cells at each dose and time point were evaluated by MTT colorimetric assay.

Differentiation Assay. MS or AK was added to the culture medium throughout the differentiation process. On day 9 after the initiation of differentiation, 3T3-L1 cells were scraped in 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1% Triton X-100 and then assayed to determine the triglyceride (TG) content using a commercial kit (TR213; Randox Inc., Antrim, U.K.). The results were normalized by using cellular protein (Protein Assay Kit; StrongBiotech Co., Taipei, Taiwan). For oil-red O staining, differentiated 3T3-L1 cells on day 9 were fixed with 10% formaldehyde and then stained with oil-red O. Pictures were taken using a microscope (ECLIPSE TS100; Nikon Co., Tokyo, Japan).

Cholesterol Accumulation. Preconfluent 3T3-L1 preadipocytes were seeded in 24-well dishes at a density of 1×10^4 cells per 0.5 mL per well. After the cells adhered to the dishes, MS or AK was added to the culture medium without FBS at the indicated doses for 24 h. 3T3-L1 cells were scraped in 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1% Triton X-100 and then assayed to determine the cholesterol content using a commercial kit (CH201; Randox Inc., Antrim, U.K.). The results were normalized by using cellular protein (Protein Assay Kit; StrongBiotech Co., Taipei, Taiwan).

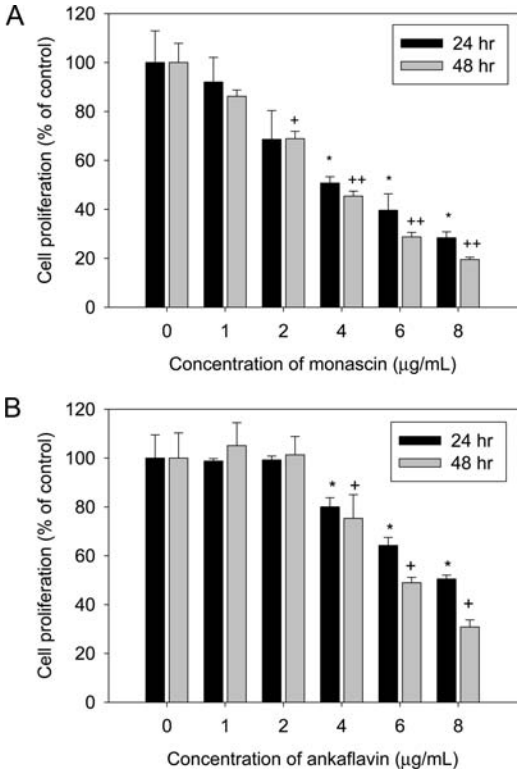

Figure 1. The effect of MS and AK on 3T3-L1 preadipoctye proliferation. MS and AK inhibited cell proliferation. 3T3-L1 preadipocytes were incubated with (**A**) MS and (**B**) AK at various concentrations (0, 1, 2, 4, 6, 8 μg/mL) for 24 or 48 h. All experiments were replicated in quadruplicate. (*) $p < 0.05$ versus the control group at 24 h. (+) $p < 0.05$, (++) $p < 0.01$ versus the control group at 48 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-Time PCR. Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA from 3 μg of RNA was generated using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer's instructions. The reverse-transcription product was diluted in water and a volume corresponding to 30 ng of original RNA was used for real-time PCR. Real-time PCR amplification and detection were performed using the SYBR Green qPCR SuperMix-UDG with ROX (Life Technologies) in a fluorescence thermal cycler (StepOne Real-Time PCR system, Life Technologies) according to the manufacturer's protocol. Gene expression was normalized using *β-actin* as a reference gene. Relative mRNA expression levels were calculated following the $\Delta\Delta C_t$ method. The primer: PPARγ sense, 5′-AAG ACC ACT CGC ATT CCT TTG ACA T-3′, antisense, 5′-TTG GGT CAG CTC TTG TGA ATG GAA T-3′; C/EBPβ sense, 5′-GCC AAG AAG ACG GTG GAC AAG CT-3′, antisense, 5′-ACC TTG TGC TGC GTC TCC AGG TT-3′; C/EBPδ sense, 5′-CTC CAC GAC TCC TGC CAT GTA CGA-3′, antisense, 5′-TGA AGA GGT CGG CGA AGA GTT CG-3′; C/EBPα sense, 5′-TGA AGA GGT CGG CGA AGA GTT CG-3′, antisense, 5′-GGC GGT CAT TGT CAC TGG TCA AC-3′; β-actin sense, 5′-ACA TCT GCT GGA AGG TGG AC-3′, antisense, 5′-GGT ACC ACC ATG TAC CCA GG-3′.

Lipolysis Assay. The fully differentiated 3T3-L1 adipocytes (days 8−12 after differentiation induction) were treated with MS or AK in Krebs Ringer bicarbonate (KRB) buffer (20 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 2% BSA; pH 7.4) for 24 h. Lipolysis activity was determined by measuring the amount of glycerol released into the incubation medium (GY105; Randox Inc.). The cells were scraped off for measurement of the TG content.

HR-LPL Activity Assay. The 3T3-L1 mature adipocytes were incubated with the experimental medium for 24 h. Subsequently, the medium

was discarded and the cells were rinsed with KRB buffer and then cultured in heparin-KRB buffer (10 U/mL heparin) at 37 °C for 1 h. The conditioned heparin-KRB was collected from each well for the assay of HR-LPL activity. LPL activity was measured on the basis of its esterase property using *p*-nitrophenyl butyrate as a substrate (*14*, *15*). The TG hydrolase activity of LPL with synthetic TG substrates is inhibited by molar sodium chloride (*16*), and this property has been used to distinguish LPL activity from the activities of other lipases in plasma (*17*, *18*). The HR-LPL activity was measured by the following equation: C (μM) = ($A_{400(0.15\text{MNaCl})} - A_{400(1\text{MNaCl})}$)/0.012, 0.012 is the micromolar extinction coefficient of *p*-nitrophenol.

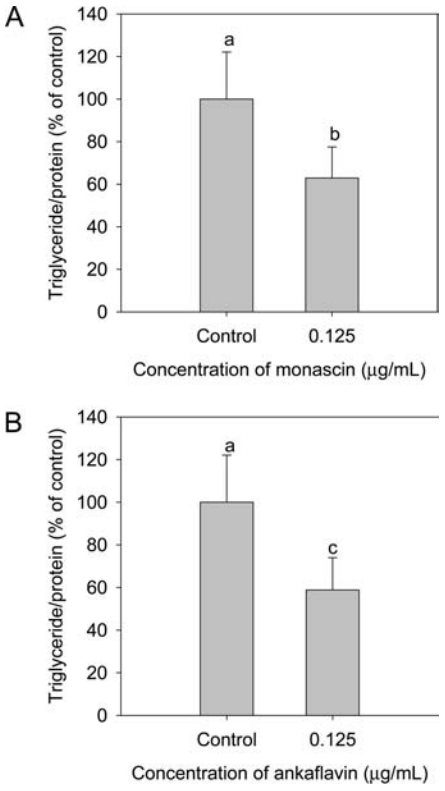

Figure 2. The effect of MS and AK on 3T3-L1 preadipocyte differentiation. (**A**) MS and (**B**) AK reduced TG content during preadipocyte differentiation. The differentiation of 9 day 3T3-L1 preadipocytes was induced by MDI medium to initiate adipogenesis as described in Materials and Methods in the absence or presence of MS or AK. Cells were incubated with MS (0, 0.125 μg/mL) or AK (0, 0.125 μg/mL). All assays were performed on four replicates for each treatment. a, b, and c: $p < 0.05$ versus the control group.

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). The statistical significance in the biochemical effects was determined by one-way analysis of variance (ANOVA) using the general linear model procedure of SPSS software (SPSS Institute, Inc., Chicago, IL, USA), followed by ANOVA with the Duncan's test. All comparisons are made relative to controls, and the significant differences are indicated as */+, $p < 0.05$, **/++, $p < 0.01$, and ***/+++, $p < 0.001$, respectively.

RESULTS

Effect of MS and AK on Preadipocyte Proliferation. 3T3-L1 preadipocytes were treated with MS or AK at various doses for 24 and 48 h. As shown in **Figure 1**, both MS and AK caused a reduction in cell proliferation. MS showed stronger dose- and time-dependent inhibitory effects on the cell proliferation than AK. At 48 h, 2, 4, 6, and 8 μg/mL MS inhibited proliferation by nearly 31.1%, 54.6%, 71.3% and 80.5%, respectively ($p < 0.05$ or $p < 0.01$) (**Figure 1A**). At 4, 6, and 8 μg/mL AK, the inhibition of proliferation was nearly 24.7%, 51.1% and 69.2%, respectively ($p < 0.05$) (**Figure 1B**). These results indicate that both MS and AK can inhibit 3T3-L1 preadipocyte proliferation, and the effect of MS is better than the effect of AK.

Effect of MS and AK on Differentiation. Both MS and AK reduced TG accumulation as shown in **Figure 2**. MS significantly decreased intracellular TG accumulation at the 0.125 μg/mL concentration (nearly 37.1% decreased, $p < 0.05$) (**Figure 2A**). It was noted that the cells detached from the culture dishes in the presence of higher concentrations of MS. A similar change was observed in some previous studies. During the early phase (days 0−2) of adipogenesis, the cells changed in the presence of the

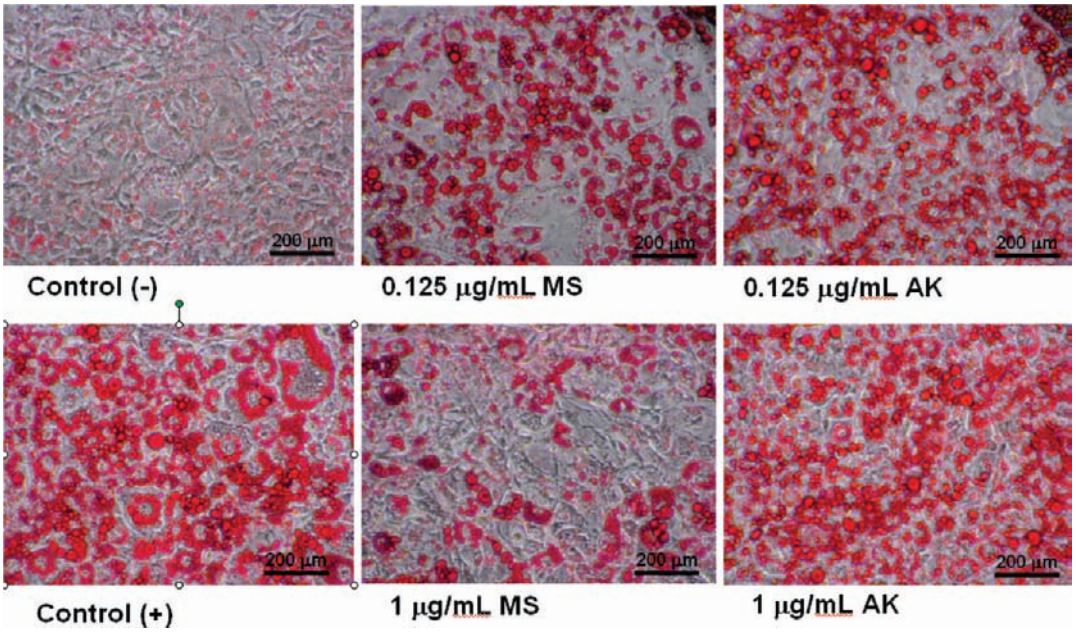

Figure 3. MS and AK suppress TG accumulation in maturing preadipocytes when added during induction of differentiation. MS and AK reduced TG accumulation in maturing preadipocytes. The differentiation of 9 day 3T3-L1 preadipocytes was induced by MDI medium to initiate adipogenesis as described in Materials and Methods in the absence or presence of MS or AK. Cells were incubated with MS or AK (0, 0.125, and 1 μg/mL). Cellular TG was stained with oil-red O. As the positive (+) and negative control (−), cells were incubated with/without MDI medium induction respectively.

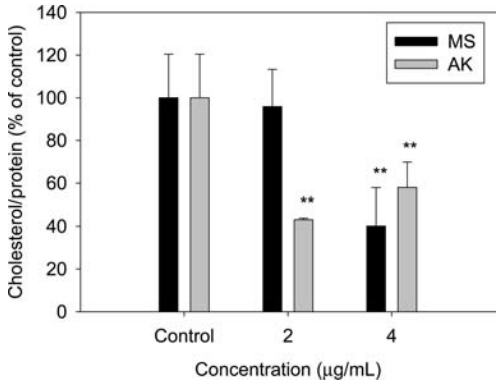

Figure 4. The effect of MS and AK on 3T3-L1 preadipocyte cholesterol accumulation. MS and AK decreased cholesterol accumulation. 3T3-L1 preadipocytes were treated with 2 or 4 μg/mL MS or AK with culture medium without FBS for 24 h. All assays were performed on four replicates for each treatment. (**) $p < 0.01$ versus the control group.

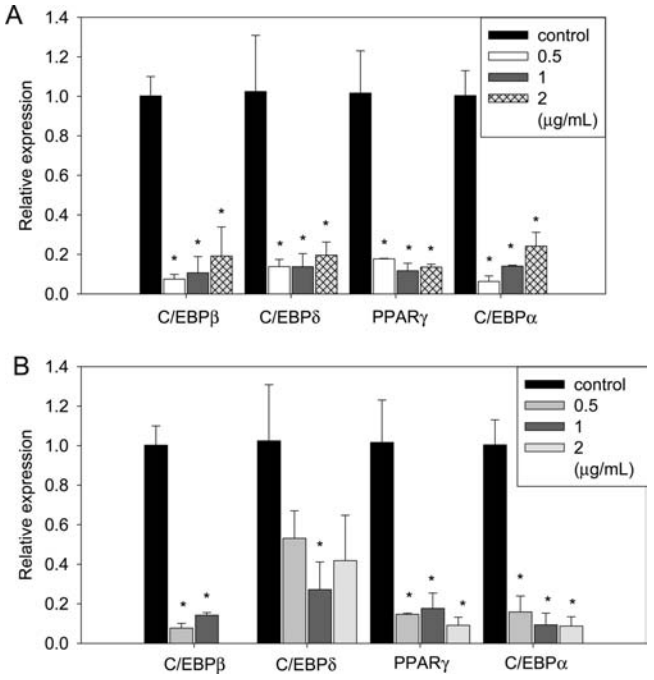

Figure 5. The relative expression of adipocyte-specific transcription factors after treating MS and AK for 2 days. (**A**) MS and (**B**) AK inhibited adipocyte-specific transcription factors expression during adipocyte differentiation. The differentiation of 2-day 3T3-L1 preadipocytes was induced by MDI medium to initiate adipogenesis as described in Materials and Methods in the absence or presence of 0.5, 1.0, and 2.0 μg/mL MS or AK. All gene expressions were normalized using β-*actin* as a reference gene. All experiments were performed in triplicate. (*) $p < 0.05$ versus the control group at each gene expression.

concentration of 10 μM simvastatin (*19*). Another study indicated that higher doses of red mold rice ethanol extracts (RMR-E) also caused this change but lovastatin did not. In addition, lovastatin at 10 μM only decreased TG accumulation by 11% (*11*).

AK inhibited the adipogenesis of 3T3-L1 preadipocytes. TG content decreased 41.1% by treatment with 0.125 μg/mL AK ($p < 0.05$) (**Figure 2B**). The lower concentration was more effective than the higher one.

Microscopic images showed the oil-red O staining, which demonstrated that both MS and AK attenuated lipid accumulation in 3T3-L1 preadipocytes (**Figure 3**).

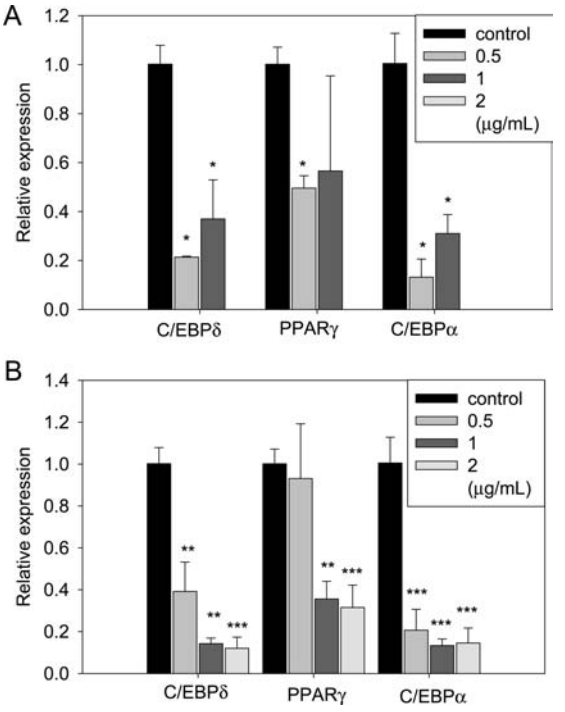

Figure 6. The relative expression of adipocyte-specific transcription factors after treating MS and AK for 4 days. (**A**) MS and (**B**) AK inhibited adipocyte-specific transcription factor expression during adipocyte differentiation. The differentiation of 4-day 3T3-L1 maturing preadipocytes was induced by MDI medium to initiate adipogenesis as described in Material and Methods in the absence or presence of 0.5, 1.0, and 2.0 μg/mL MS or AK. All gene expressions were normalized using β-*actin* as a reference gene. All experiments were performed in triplicate. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ versus the control group at each gene expression.

Effect of MS and AK on Cholesterol Accumulation. Since the adipogenesis result showed that MS caused cell detachment from the culture dishes, we predicted that the change was related to cholesterol accumulation. Both MS and AK suppressed cholesterol accumulation as shown in **Figure 4**. Cholesterol content decreased 4.2% and 59.9% by treatment with 2 and 4 μg/mL MS, respectively; while the decrease after treatment with 2 and 4 μg/mL AK was 57.0% and 42.1%, respectively.

Effect of MS and AK on Adipocyte-Specific Transcription Factors Gene Expression during Adipocyte Differentiation. Since the adipogenesis data showed that MS and AK decreased TG accumulation, we selected the time points of day 2, day 4 and day 6 to investigate the gene expression of adipocyte-specific transcription factors, C/EBPβ, C/EBPδ, PPARγ and C/EBPα. At day 2, both MS and AK significantly reduced the expression of C/EBPβ, C/EBPδ, PPARγ and C/EBPα (**Figure 5**). However, 0.5−2.0 μg/mL MS seemed to have similar reduced effects on the expression in 3T3-L1 preadipocytes, because the expression decreased approximately by 80%−90% with controls (**Figure 5A**). AK reduced the expression without obvious dose dependency at day 2.

C/EBPβ was not detected at day 4. The expression of C/EBPδ, PPARγ and C/EBPα were significantly reduced by MS and AK, and were not detected by 2.0 μg/mL MS or AK treatment (**Figure 6**).

Only PPARγ and C/EBPα could be detected at day 6, but were not detected by 1.0−2.0 μg/mL MS or AK treatment. After the treatment with 0.5 μg/mL MS, the expression of PPARγ was nearly 205% greater than control, but the concentration of MS had no significant effect on the expression statistically (**Figure 7A**). The expression of C/EBPα was reduced by the

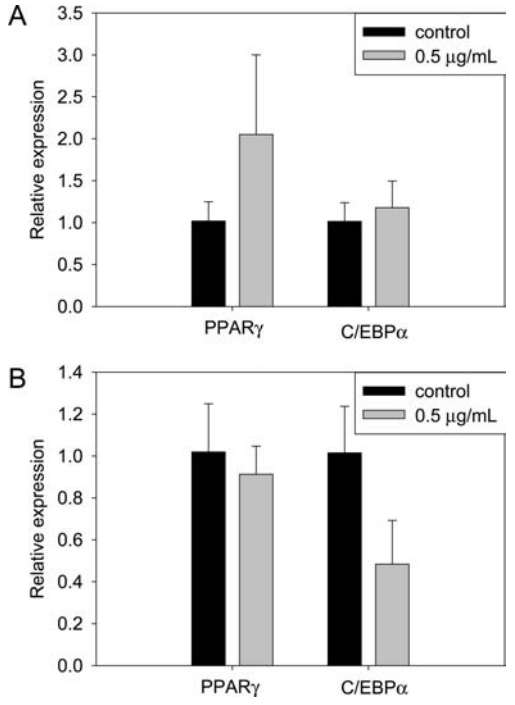

Figure 7. The relative expression of adipocyte-specific transcription factors after treating MS and AK for 6 days. (**A**) MS and (**B**) AK inhibited adipocyte-specific transcription factor expression during adipocyte differentiation. The differentiation of 6-day 3T3-L1 maturing preadipocytes was induced by MDI medium to initiate adipogenesis as described in Material and Methods in the absence or presence of 0.5 μg/mL MS or AK. All gene expressions were normalized using β-*actin* as a reference gene. All experiments were performed in triplicate.

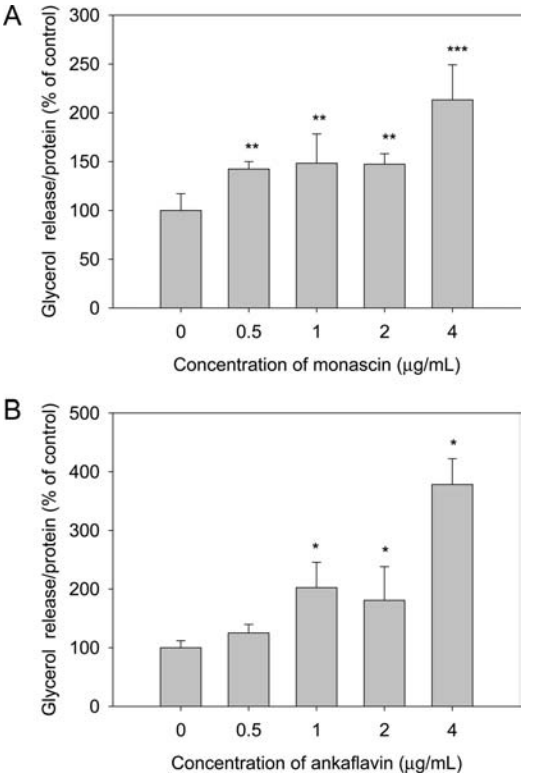

Figure 8. The effect of MS and AK on lipolysis activity in 3T3-L1 adipocytes. (**A**) MS and (**B**) AK increased lipolysis. 3T3-L1 mature adipocytes were incubated with MS and AK at various concentrations (0, 0.5, 1, 2, 4 μg/mL) for 24 h. All experiments were performed on four replicates for each treatment. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ versus the control group.

treatment with 0.5 μg/mL AK, but there was no significant effect compared with the control statistically (**Figure 7B**). Furthermore, the expression of PPARγ and C/EBPα was not detected by treatment with 1.0 and 2.0 μg/mL MS or AK.

Our results showed that the expressions of the four transcriptional factors (PPARγ, C/EBPβ, C/EBPδ and C/EBPα) were reduced at the initial and the middle period. At the later period, there were no effects on the expression of PPARγ and C/EBPα by treating MS and AK.

Effect of MS and AK on Mature Adipocyte Viability. 3T3-L1 mature adipocytes were treated with MS or AK at various doses for 48 h. After treatment, the number of live cells was determined by the MTT assay. Both MS and AK had no significant effect on cell viability (data not shown).

Effect of MS and AK on Lipolysis in Mature Adipocytes. To determine whether MS and AK exert lipolytic effects in 3T3-L1 mature adipocytes, we treated cells (days 8−12 after differentiation induction) with various doses for 24 h. In the treatment with 4 μg/mL, AK (278.3% increase) was more effective than MS (113.2% increase). Treatment of mature adipocytes with 0.5−2.0 μg/mL MS or 1.0−2.0 μg/mL AK increased the release of glycerol but not in a significant dose-dependent manner (**Figure 8**).

Effect of MS and AK on HR-LPL Activity in Mature Adipocytes. To examine lipid accumulation decreased by MS and AK, we measured the HR-LPL activity, the critical enzyme for lipogenesis in adipocytes. We treated cells (days 8−12 after differentiation induction) with various doses for 24 h. As shown in **Figure 9**, treatment of mature adipocytes with MS or AK suppressed HR-LPL activity in a dose-dependent manner. After a 24 h incubation of mature adipocytes with 4.0 μg/mL MS, HR-LPL activity was nearly 45.3% lower than control ($p < 0.01$) (**Figure 9A**), while the decrease after treatment with AK was nearly 58.1% lower than control ($p < 0.001$) (**Figure 9B**).

DISCUSSION

Adipocytes have been studied with increasing intensity as a result of the emergence of obesity as a serious public health problem and the realization that adipose tissue serves as an integrator of various physiological pathways (*20*). The process of adipogenesis includes alteration of cell shape, growth arrest and clonal expansion, a complex sequence of changes in gene expression and storage of lipid (*21*). In this study, we showed that either MS or AK had inhibitory effects on proliferation and adipogenesis, and enhanced the effect on lipolysis in 3T3-L1 preadipocytes. The treatment of 3T3-L1 preadipocytes with MS and AK suppressed cell proliferation in a dose-dependent trend, and the effect of MS was stronger than AK.

The presence of two compounds decreased TG accumulation during differentiation, and the higher concentration of MS caused the cells to become detached from culture dishes. Some studies indicated similar changes caused by simvastatin (*19*) and RMR-E (*11*). Statins are a family of drugs widely used in the treatment of hypercholesterolemia. They are competitive inhibitors of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the enzyme that converts HMG-CoA to mevalonate (*22*, *23*). Mevalonate is the precursor of farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, the isoprenoid lipids involved in posttranslational modification of a number of proteins, including most G-proteins (*24*, *25*). It is now well-known that Rho GTPases regulate the actin cytoskeletion and cell adhesion, as well as activities such as cell cycle progression, differentiation, and gene

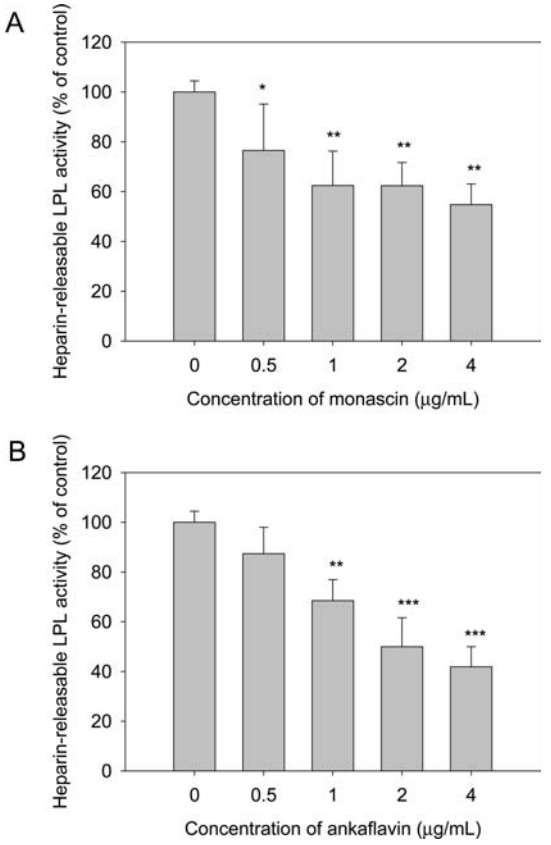

Figure 9. The effect of MS and AK on HR-LPL activity in 3T3-L1 adipocytes. (**A**) MS and (**B**) AK decreased HR-LPL activity. 3T3-L1 mature adipocytes were incubated with MS and AK at various concentrations (0, 0.5, 1, 2, 4 μg/mL) for 24 h. All experiments were performed on four replicates for each treatment. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ versus the control group.

transcription (*26*, *27*). Onset of adipogenesis is usually defined by extracellular matrix remodeling, characterized by the conversion from fibronectin-rich stromal matrix to the laminin-rich basement membrane (*28*, *29*). Although the interplay between the changing extracellular matrix, cytoskeleton, and cell shape and gene expression programs during adipocyte differentiation is not yet understood, previous study showed that the relationship between Rho family proteins and integrins is correlated with 3T3-L1 adipocyte differentiation (*30*). Therefore, statins cause the depletion of mevalonate, which is known to alter the expression of key proteins involved in isoprene metabolism. When the expression of these key proteins like Rho family proteins is altered, the differentiation of preadipocyte is going to be suppressed.

The previous studies concerned with the effect of RMR on adipogenesis indicated that the antiadipogenic activity of RMR may be due to other components (*11*, *12*). In this study, we reported that both MS and AK attenuated the cholesterol accumulation in 3T3-L1 cells. It is the first to demonstrate that both MS and AK are antiadipogenic compounds in *Monascus*-fermented product.

At the molecular level, the adipocyte differentiation process is regulated by the sequential expression of transcriptional activators, mainly PPAR and C/EBP families, and they are major coordinators of adipocyte gene expression and differentiation (*31*, *32*). Our results indicated that MS and AK decreased the gene expression of C/EBPβ, C/EBPδ, PPARγ and C/EBPα thereby suppressing the differentiation of preadipocytes to adipocytes at the initial and the middle period. At the later period, there was no statisitically signifcant effect on the expression of PPARγ and C/EBPα by treating a lower concentration of MS and AK, but when treating a higher concentration, the expression of these two genes could not be detected. Our data showed that lower MS upregulated the expression of PPARγ and lower AK downregulated the expression of C/EBPα. Besides, higher MS and AK could inhibit the expression of PPARγ and C/EBPα. The previous finding showed that adipocytes failed to develop in the absence of PPARγ (*33*). Another finding presented PPARγ's ability to increase the number of small adipocytes by stimulating adipogenesis and decrease the number of large adipocytes (*34*). We found that MS and AK stimulated lipolysis when added to mature adipocytes by glycerol release and downregulation of HR-LPL activity, and the effects of AK are stronger than those of MS. The result could be related to the downregulation of C/EBPα expression by AK. MS also showed a stimulatory effect on mature adipocyte lipolysis, suggesting that some other mechanism besides PPARγ and C/EBPα may create lipolysis activity as well.

In conclusion, these data demonstrated that MS and AK acted on 3T3-L1 cells primarily by reducing preadipocyte proliferation and differentiation by decreasing TG accumulation and suppressing the expression of adipocyte specific transcription factors, C/EBPβ, C/EBPδ, PPARγ and C/EBPα. MS and AK also promoted mature adipocyte delipidation by glycerol release and downregulation of HR-LPL activity. This is the first time that antiobesity effects by inhibiting differentiation of preadipocytes, and by promoting lipolysis of mature adipocytes of MS and AK, the yellow pigments of *Monascus*, are reported.

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