# Anti-adipogenic effect and underlying mechanism of lignan-enriched nutmeg extract on 3T3-L1 preadipocytes

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**Abstract.** Nutmeg is the seed derived from *Myristica fragrans*. Nutmeg seeds contain alkylbenzene derivatives such as myristicin, which are toxic to the human organism, and lignan compounds such as nectandrin B, which possess anti-aging and anti-diabetic properties. However, the anti-adipogenic, prolipolytic and anti-inflammatory effects of lignan-enriched nutmeg extract (LNX) on preadipocytes remain unclear. In the present study, the effects of LNX on lipid accumulation, glycerol release and inflammatory cyclooxygenase-2 (COX-2) expression in differentiated 3T3-L1 preadipocytes were investigated. Oil red O staining demonstrated that treatment with LNX resulted in a concentration-dependent reduction in lipid accumulation in differentiating 3T3-L1 preadipocytes without affecting cell growth. Mechanistically, LNX treatment at 6 $\mu$g/ml led to a reduction in phosphorylation levels of signal transducer and activator of transcription 3 (STAT3), whereas it did not influence the peroxisome proliferator-activated receptor gamma (PPAR-$\gamma$) and CCAAT enhancer binding protein alpha (C/EBP-$\alpha$) expression levels during 3T3-L1 preadipocyte differentiation. In addition, LNX treatment at 6 $\mu$g/ml led to a decrease in fatty acid synthase (FAS) expression levels on day (D) 2, but not D5 and D8, during preadipocyte differentiation. Treatment with LNX at 6 $\mu$g/ml did not affect the expression levels of perilipin A during preadipocyte differentiation. In differentiated 3T3-L1 adipocytes, LNX treatment at 6 $\mu$g/ml did not stimulate glycerol release and hormone-sensitive lipase phosphorylation, which are known lipolysis hallmarks. Furthermore, LNX treatment at the doses tested had no effect on tumor necrosis factor alpha-induced COX-2 expression in 3T3-L1 preadipocytes. Collectively, these results demonstrated that LNX has an anti-adipogenic effect on differentiating 3T3-L1 preadipocytes, which is mediated by the downregulation of STAT3 phosphorylation and FAS expression.

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## Introduction

Obesity is a significant public health concern that causes a high disease burden in developed countries (1). According to statistical data from 2015, obesity rates continue to rise worldwide, with 600 million adults and 100 million children reported to be obese in 195 countries (1). Moreover, obesity is a well-known risk factor for many chronic diseases such as type 2 diabetes, cardiovascular disease and degenerative brain disease (2). Obesity is caused by hereditary characteristics, endocrine disorders, dietary and environmental factors and an imbalance in energy intake and output (3). It is documented that abnormal fat accumulation in adipocytes, also called hypertrophic adipocytes, leads to the development of obesity. Hence, any material that suppresses excessive fat accumulation in adipocytes can be considered a potential anti-obesity agent.

Hypertrophic adipocytes are formed by the differentiation of pre-adipocytes into adipocytes or fat-laden cells, also known as adipogenesis. Preadipocyte differentiation is regulated by expression and activation (phosphorylation) of numerous transcription factors, enzymes and proteins. For example, the family members of peroxisome proliferator-activated receptors (PPARs) and CCAAT enhancer binding proteins (C/EBPs) are essential regulators of preadipocyte differentiation (4). Members of the signal transducer and activator of transcription (STAT) family are other important transcriptional factors that regulate preadipocyte differentiation (5,6). Notably, the expression and phosphorylation levels of these transcription factors increase significantly during adipogenesis (7). In addition, the expression of lipogenic enzymes such as fatty acid

synthase (FAS), acetyl-CoA carboxylase (ACC) and perilipin A, a lipid droplet (LD)-binding and stabilizing protein, is required for preadipocyte differentiation (8,9).

Lipolysis is the metabolic pathway through which lipids, mainly triglycerides (TG), are hydrolyzed into glycerol and free fatty acids and is controlled by a group of lipases, including hormone-sensitive lipase (HSL) expressed in adipocytes (10). Therefore, the promotion of lipolysis in lipid-laden adipocytes is regarded as another key strategy for preventing or treating obesity. A wealth of information strongly supports the link between chronic inflammation in adipose tissue (AT) and preadipocytes and the development of obesity (11). Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine that is expressed and secreted in preadipocytes (12). It has been revealed that TNF-α induces and aggravates inflammation in the AT (13). Cyclooxygenase-2 (COX-2) is an inducible and inflammatory enzyme responsible for the biosynthesis of prostaglandins (14). It is further documented that TNF-α is a strong inducer of COX-2 in various cells, including preadipocytes (15,16). Indeed, the authors of the present study as well as other groups have demonstrated that the short-term exposure of TNF-α leads to induction of COX-2 expression in 3T3-L1 preadipocytes (16-18). Thus, inhibition of the TNF-α-induced COX-2 expression in preadipocytes is considered as a possible target to manage obesity inflammation.

Nutmeg (*Myristica fragrans*) is a seed derived from an evergreen tree belonging to the *Nutmegaceae* family. It is a dioecious plant that grows to a height of 10-20 m and is cultivated in the Maluku province of Indonesia (19). Nutmeg is often used as a spice to enhance food flavor. Notably, nutmeg byproducts, such as seeds and mace, have been reported to have many potential bioactivities, including anti-obesity, anti-diabetic, anti-fungal, anti-microbial, antioxidant and anti-inflammatory (20-25). Evidence indicates that nutmeg seeds contain several alkylbenzene derivatives, such as myristicin, elemicin and safrole, which are toxic to the human organism (26,27). By contrast, lignans in nutmeg seeds are known to contain bioactive compounds, including nectandrin B, a nutmeg lignan with anti-aging and anti-diabetic effects (25,28). Interestingly, a previous study demonstrated that nutmeg lignans induce the IGF-1-AKT-mTOR pathway in an aging rat model, indicating their potential anti-sarcopenic effects (29). These findings illustrated that the nutmeg extraction method for concentrating bioactive lignan compounds, while removing toxic substances, has great potential for the treatment of various metabolic diseases. In previous studies, the authors prepared lignan-enriched nutmeg extract (LNX), which would comprise minimal levels of toxic myristicin (<0.5%) and maximum nectandrin B (28) and reported that LNX restores muscle proteins in aged mice (30), suggesting the potential of LNX as a therapeutic agent to overcome sarcopenia. However, the anti-obesity (antiadipogenic, prolipolytic and anti-inflammatory) effects of LNX on preadipocytes remain unclear. In the present study, the aim was to primarily demonstrate the LNX-contained whole active component's potential effects on inhibition of lipid accumulation to evaluate it as an anti-obesity drug candidate; therefore the authors focused on LNX, but not on nectandrin B. Therefore, the regulatory effects of LNX on lipid accumulation in differentiating preadipocytes, glycerol release and HSL phosphorylation in differentiated adipocytes and the TNF-α-induced COX-2 expression in preadipocytes by using 3T3-L1 cells, a mouse white preadipocyte cell line, were investigated. In the present study, it was reported that LNX exerts an anti-adipogenic effect on differentiating 3T3-L1 preadipocytes, which is mediated by the downregulating of STAT3 phosphorylation and FAS expression.

## Materials and methods

*Preparation of LNX*. LNX used in the present study was obtained from Daehan Cell Pharm, Inc. (Guri, Republic of Korea). Briefly, the original seeds of 100 g nutmeg (*Myristica fragrans*) were pulverized and dissolved in 500 ml of 80% ethanol (EtOH). The optimal extraction conditions were determined by monitoring the minimal toxicity of myristicin and maximal bioactivity of nectandrin B. The extract was subjected to a mobile phase of the methanol (MeOH)-$H_2O$ (0-32 min, 63% MeOH; 32-37 min, 63-100% MeOH) mixture. The final extract was then adsorbed through Diaion HP-20 (Merck KGaA), one of the ion exchange resin, and eluted in a serial concentration of 30-90% EtOH to monitor the contents of myristicin and nectandrin B. The nutmeg alcohol extract was detected at 234 nm at a flow rate of 1.5 ml/min using a Waters HPLC Optima Pak $C_{18}$ reverse-phase column (cat. no. OP C18-51002546; 4.6x250 mm, 5 $\mu$m pore size, RS Tech. Corp.). The mobile phase in the analytical column was composed of the mixture (the ratio of 4:6; absolute acetonitrile and 0.1% formic acid). In addition, nectandrin B concentration used in the present study is 4.2 from 91% of dry weight of LNX, which is lower than the concentration used in the previous study (28) with <0.5% of myristicin in a 91% of dry weight of LNX.

*Chemicals and reagents*. Primary antibodies against PPAR-γ (cat. no. sc-7272), C/EBP-α (cat. no. sc-61), phosphorylated (p)-STAT3 (cat. no. sc-8059) and total (T)-STAT3 (cat. no. sc-8019) were purchased from Santa Cruz Biotechnology, Inc. The primary antibody against FAS (cat. no. 9452) was obtained from BD Biosciences. Primary antibodies against p-HSL (S563) (cat. no. 4139) and p-HSL (S565) (cat. no. 4137) were acquired from Cell Signaling Technology, Inc. Primary antibodies against COX-2 (cat. no. 160106) and T-HSL (cat. no. 10006371) were purchased from Cayman Chemical Company. Primary antibody against perilipin A (cat. no. 3948-200) was purchased from BioVision, Inc. The primary antibody of β-actin (A5441), iso-butylmethylxanthine (IBMX), dexamethasone, insulin, Oil Red O solution, Isoproterenol, and Free Glycerol Reagent were obtained from MilliporeSigma. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Welgene, Inc. Fetal calf serum (FCS) and trypan blue dye were purchased from Gibco; Thermo Fisher Scientific, Inc. Enhanced chemiluminescence (ECL) reagents were purchased from Advansta Inc.

*Cell culture and differentiation*. 3T3-L1 mouse white preadipocytes (American Type Culture Collection) were cultured in DMEM containing 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% $CO_2$. 3T3-L1 preadipocytes were seeded with

DMEM containing 10% FCS and 1% penicillin/streptomycin mixture and maintained up to the contact inhibition stage for 2 days. The differentiation of 3T3-L1 preadipocytes was initiated by replacing the media with new DMEM containing 10% heat-inactivated FBS plus a cocktail of hormones (MDI): 0.5 mM IBMX (M), 0.5 $\mu$M dexamethasone (D) and 5 $\mu$g/ml insulin (I) either with or without LNX at the designated concentrations for 2 days (Fig. 1A). After 2 days, the first differentiation medium was removed from the cells and the cells were subsequently cultured in new DMEM supplemented with 10% FBS and 5 $\mu$g/ml insulin either with or without LNX at the designated concentrations for an additional 3 days. After 3 days, the second differentiation medium was removed from the cells and the cells were further supplemented with new DMEM containing 10% FBS with or without LNX at the designated concentrations for another 3 days.

*Oil red O staining*. On day 8 post the differentiation induction, the control or LNX-treated 3T3-L1 cells underwent phosphate-buffered saline (PBS) washing and 10% formaldehyde fixation for 2 h at room temperature (RT). Following a 60% isopropanol washing, the cells were thoroughly dried. The fixed cells were treated with Oil Red O working solution for 1 h at RT, followed by washing with distilled water. Light microscopy was used to identify the lipids or fats that accumulated in the conditioned cells (Nikon Corporation).

*Cell count analysis*. On day 8 post differentiation induction, control and LNX-treated 3T3-L1 cells were stained with 0.4% trypan blue dye at RT. Only cells with intact membranes could effectively block the dye. Once dead cells with damaged membranes were exposed to the dye, they were stained and counted under a light microscope.

*Western blot analysis*. At the designated time, the control or LNX-treated 3T3-L1 cells were washed with PBS and lysed in a modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. The whole-cell lysates were collected and centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was collected and protein concentrations were determined using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.).

An aliquot of protein (40 $\mu$g per lane) was separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (MilliporeSigma). The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween 20 (TBST) and blocked with blocking buffer (TBST containing 5% (w/v) non-fat dried milk) at 4˚C overnight. The membranes were incubated overnight with corresponding primary antibodies for C/EBP-α (1:2,000), PPAR-γ (1:2,000), p-STAT3 (1:2,000), STAT3 (1:2,000), phosphorylated (p)-STAT-5 (1:2,000), STAT-5 (1:2,000), FAS (1:1,000), perilipin A (1:2,000), p-HSL (S563) (1:2,000), p-HSL (S565) (1:2,000), T-HSL (1:2,000), COX-2 (1:2,000) or β-actin (1:10,000) at 4°C. The membranes were then washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody with either anti-mouse IgG (115-035-062; 1:5,000) or anti-rabbit IgG (111-035-045; 1:5,000) (Jackson ImmunoResearch Laboratories, Inc.) for 2 h at RT. The membranes were washed with TBST. ECL reagents were used to develop the images. Equal protein loading per lane was quantified based on the relative intensity of β-actin, an internal control protein. ImageJ software (v.1.6.0.24; National Institutes of Health) was used to intensify the bands for proteins that were standardized to the internal control.

*Reverse transcription polymerase chain reaction (RT-PCR)*. Total RNA was isolated from the control and LNX-treated 3T3-L1 cells after 4 h using RNAiso Plus (Takara Bio, Inc.). A total of three micrograms of total RNA were used to prepare complementary DNA using random hexadeoxynucleotide primers, reverse transcriptase (M-MLV RT), reverse transcriptase buffer (M-MLV RT 5X Buffer) and dNTPs (Promega Corporation). The cDNA was amplified by PCR with the following primers (Bioneer Corporation): COX-2 forward, 5'-TTGAAGACCAGGAGTACAGC-3' and reverse, 5'-GGTACAGTTCCATGACATCG-3'; and β-actin forward, 5'-TCATGAAGTGTGACGTTGACATCCGT-3' and reverse, 5'-CCTAGAAGCACTTGCGGTGCACGATG-3'. The PCR conditions applied for COX-2 were as follows; 30 cycles of denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 30 sec and for β-actin; 30 cycles of denaturation at 95˚C for 30 sec, annealing at 63˚C for 30 sec and extension at 72˚C for 1 min. Levels of β-actin mRNA expression were used as an internal control.

*Quantification of glycerol content*. On day 8 of the post-differentiation induction, 3T3-L1 cells were serum-starved for 2 h and further treated without or with LNX (6 $\mu$g/ml) or isoproterenol (ISO; 20 $\mu$M), a known lipolysis inducer for another 3 h. The culture medium from the control, LNX- or ISO-treated cells was stored and the glycerol content in the respective culture medium was measured using a Free Glycerol Reagent according to the manufacturer's instructions. The absorbance was measured at 540 nm using a microplate reader.

*Statistical analysis*. Cell counting, western blotting and RT-PCR analysis were conducted in triplicates and repeated two times. The mean and SD values were used to express the data. One-way ANOVA was employed to compare the significance of the differences, followed by a Bonferroni test for the post hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference. The statistical software used in the present study was IBM SPSS Statistics 25 software (IBM Corp.).

## Results

*Treatment with LNX leads to a concentration-dependent suppression of lipid accumulation in differentiating 3T3-L1 preadipocytes*. Initially, in the preliminary studies, LNX was tested at higher concentrations such as 50, 100 and 200 $\mu$g/ml for 3T3-L1 preadipocyte differentiation (data not shown). However, LNX treatment at 100 and 200 $\mu$g/ml had been demonstrating cytotoxicity in differentiating 3T3-L1 cells. Hence, LNX was tested in lower concentrations as revealed in the present study (6.25, 12.5 and 25 $\mu$g/ml) to

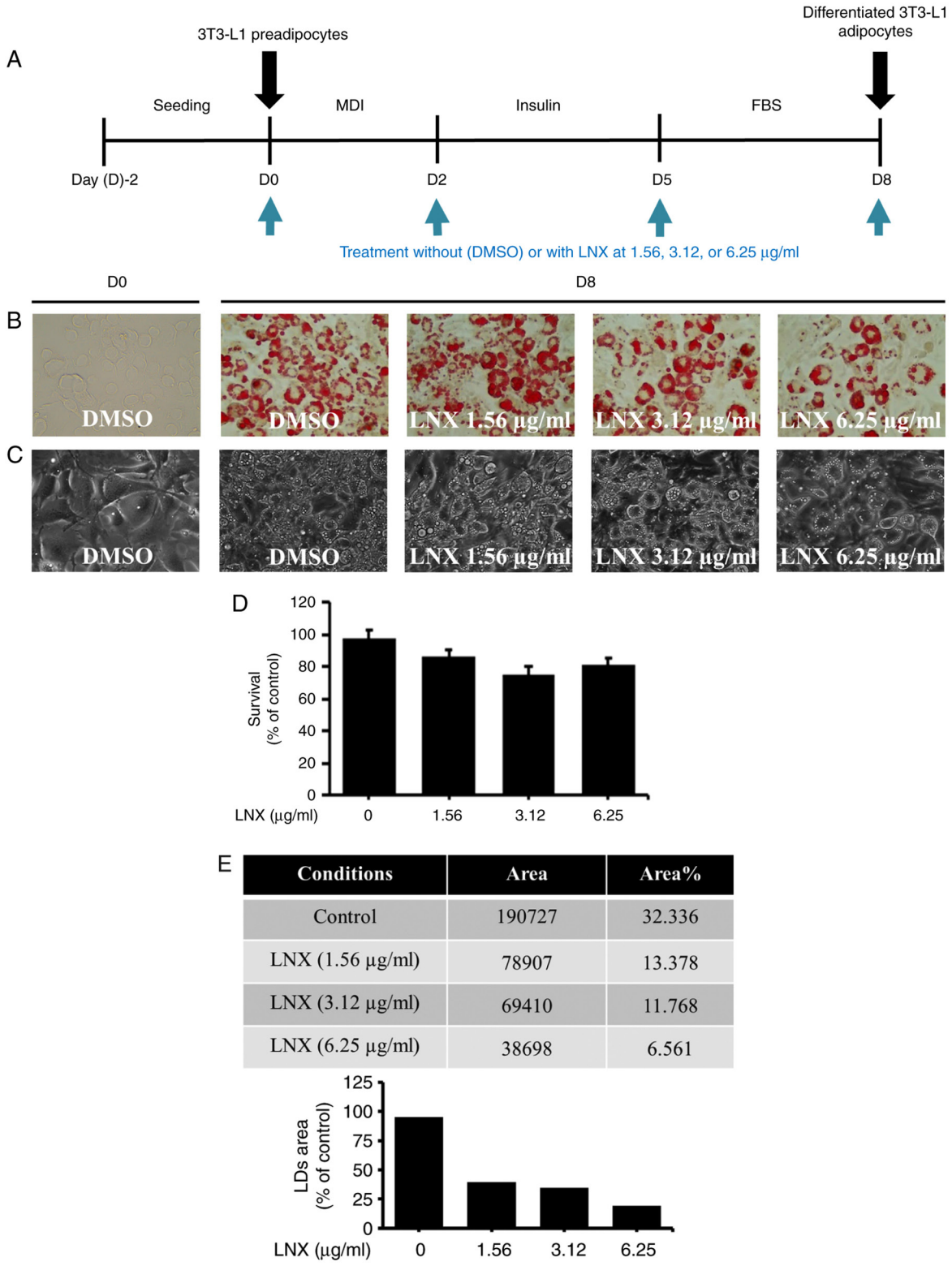


Figure 1. Effects of LNX on lipid accumulation during 3T3-L1 preadipocyte differentiation. (A) Experimental scheme for 3T3-L1 preadipocyte differentiation. (B) Measurement of intracellular LDs' accumulation in undifferentiated 3T3-L1 preadipocytes on D0 or differentiated adipocytes on D8 in the absence (control; 0.1% DMSO) or presence of LNX at the designated concentrations using Oil Red O staining. (C) Phase-contrast microscopic images are illustrated for the aforementioned conditions in panel B. (D) Cell survival assay was performed. (E) Quantification of Oil Red O staining images (B) to determine the percentage of LDs using ImageJ software. Data are presented as the mean ± SD (n=3). LNX, lignan-enriched nutmeg extract; LDs, lipid droplets; D, day; MDI; IBMX, dexamethasone and insulin.

further check the deposition rate of LDs during 3T3-L1 differentiation. Of interest, it was found that the accumulation of LDs appeared to be increased in the higher concentration of LNX in a dose-dependent manner (data not shown). Therefore, since the LNX effects on the inhibition of lipid accumulation during 3T3-L1 preadipocyte differentiation using Oil Red O staining

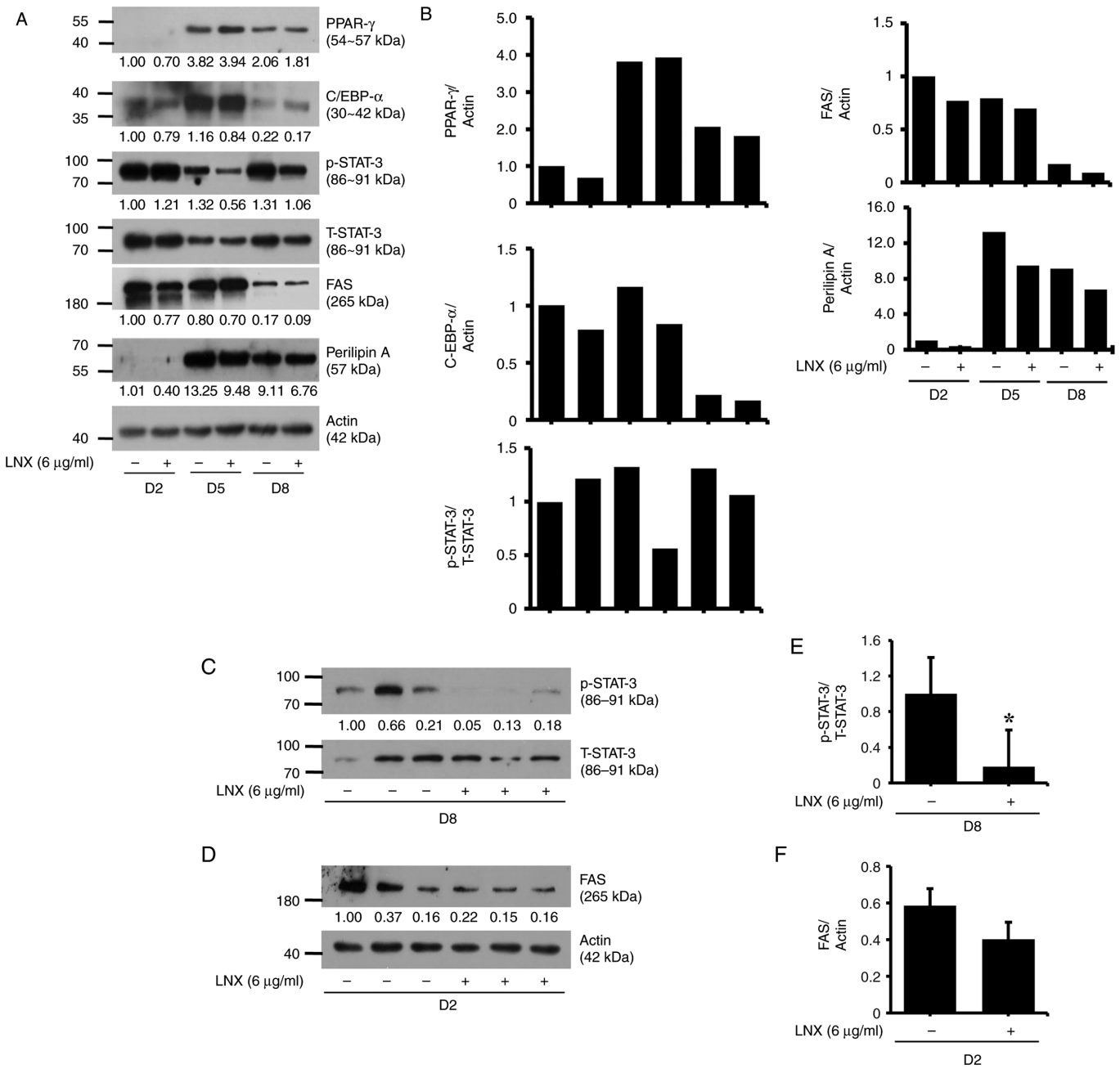


Figure 2. Effects of LNX on the expression and/or phosphorylation levels of C/EBP-α, PPAR-γ, FAS, Perilipin A and STAT3 during 3T3-L1 preadipocyte differentiation. (A, C and D) 3T3-L1 preadipocytes were differentiated using induction medium containing IBMX, dexamethasone and insulin and FBS in the presence or absence of LNX and harvested at day 2, 5 and 8, respectively. Cellular proteins at the indicated time points were extracted and analyzed using western blot analysis. (B) Densitometric analysis of panel A. (C and D) Western blot analysis in triplicate experiments on D8. (E and F) The densitometric analysis of (C) and (D), respectively. *P<0.05 compared with vehicle control. LNX, lignan-enriched nutmeg extract; PPAR-γ, peroxisome proliferator-activated receptor gamma; C/EBP-α, CCAAT enhancer binding protein alpha; p-STAT3, phosphorylated signal transducer and activator of transcription 3; T-STAT3, total-STAT3; FAS, fatty acid synthase; D, day.

had to be assessed, various concentrations of LNX (1.56, 3.12 and 6.25 $\mu$g/ml) have been selected for the present study. The timetable for 3T3-L1 pre-adipocyte differentiation used in the present study is depicted in Fig. 1A. As demonstrated in Fig. 1B, Oil Red O staining revealed markedly higher lipid accumulation in differentiated 3T3-L1 cells (D8) than in undifferentiated cells (D0). Notably, compared with mock-treated cells, treatment with LNX led to a dose-dependent inhibition of lipid accumulation in differentiated 3T3-L1 cells. The ability of LNX to downregulate lipid accumulation in differentiated 3T3-L1 cells was confirmed using phase-contrast microscopy (Fig. 1C). Additionally, the effects of 1.56, 3.12 or 6.25 $\mu$g/ml LNX on cell growth during the differentiation of 3T3-L1 preadipocytes into adipocytes, were examined by using cell count analysis. As illustrated in Fig. 1D, treatment with LNX at the tested doses did not significantly affect cell growth during 3T3-L1 preadipocyte differentiation. Quantification of the Oil Red O staining images to determine the LDs percentage using ImageJ software also revealed that LNX reduced lipid accumulation in a dose-dependent manner (Fig. 1E). Hence,

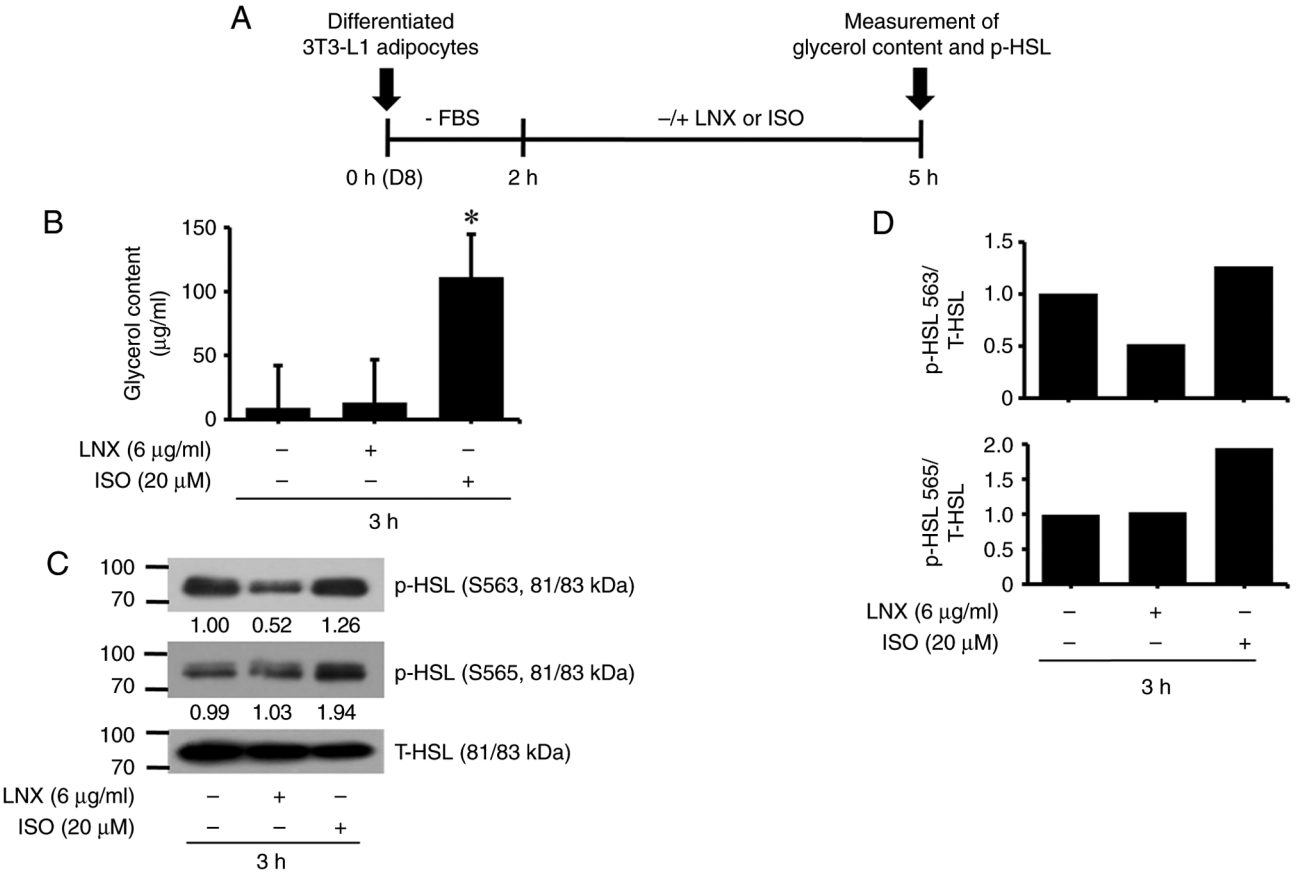


Figure 3. Effects of LNX on glycerol release and the phosphorylation levels of HSL in differentiated adipocytes. (A) The experimental scheme for measurement of glycerol content and HSL phosphorylation in differentiated 3T3-L1 cells. (B) Differentiated 3T3-L1 cells on D8 (0 h) were serum-starved for 2 h and then cultured in the absence (control; 0.1% DMSO) or presence of LNX (6 $\mu$g/ml) or ISO (20 $\mu$M) for an additional 3 h. Glycerol content in culture medium from control or drug (LNX or ISO)-treated cells was analyzed in triplicates. Data are presented as the mean ± SD of three independent experiments. $^{*}$P<0.05 vs. control. (C) After the treatment aforementioned, whole-cell lysates were extracted and analyzed using western blot analysis with corresponding antibodies. (D) The densitometric analysis of panel C. LNX, lignan-enriched nutmeg extract; HSL, hormone-sensitive lipase; ISO, isoproterenol; p, phosphorylated; T, total.

based on the maximal lipid-lowering effect on differentiating 3T3-L1 preadipocytes with no cytotoxicity, the concentration of 6 $\mu$g/ml of LNX was selected for further experiments.

*LNX treatment at 6 μg/ml selectively downregulates STAT3 phosphorylation and FAS expression in differentiating 3T3-L1 preadipocytes*. Subsequently, to explore the fundamental molecular and cellular mechanisms underlying this LNX-induced anti-adipogenic effect, LNX (6 $\mu$g/ml) was investigated on the expression and phosphorylation levels of major adipogenic transcription factors, including C/EBP-α, PPAR-γ and STAT3/5, using immunoblotting analysis. As revealed in Fig. 2A, LNX treatment did not modulate significantly the expression levels of C/EBP-α and PPAR-γ proteins in 3T3-L1 preadipocyte differentiation at the assessed timepoints; however, it led to the slight elevation of the expression levels of C/EBP-α and PPAR-γ on D5 and D8, respectively. In addition, C/EBP-β was also assessed during 3T3-L1 differentiation. However, a band for this protein could not be obtained, which was possibly due to an antibody error.

In the present study, the day courses such as D2, D5 and D8 in 3T3-L1 preadipocyte differentiation were observed to determine the p/T-STAT3 and FAS protein expression (Fig. 2A and B). It was revealed that STAT3 was highly phosphorylated during 3T3-L1 preadipocyte differentiation. Of note, p-STAT3 expression level was decreased on D5. In addition, on D8, STAT3 protein levels were strongly phosphorylated again during differentiation period (as compared with the control group of D5). Notably, the mid-phase of STAT3 decline affected the adipocyte differentiation. Meanwhile, FAS protein was greatly attenuated on D2 compared with D5 or D8. Hence, based on these day course experiment results, different day courses were investigated on each target (D8 for STAT3 and D2 for FAS protein expression levels) (Fig. 2C-F).

Interestingly, while treatment with LNX had no effect on the phosphorylation and total expression levels of STAT3 on D2 of 3T3-L1 cell differentiation, it markedly reduced the phosphorylation of STAT3 without altering its total expression levels on D5 of the cell differentiation. However, LNX treatment further reduced the phosphorylation and total expression levels of STAT3 on D8 of cell differentiation. Moreover, LNX treatment slightly inhibited the expression of FAS on D2, D5 and D8, during 3T3-L1 preadipocyte differentiation. LNX treatment did not influence perilipin A expression during cell differentiation. Expression levels of the control actin protein remained constant under these experimental conditions. The densitometric data from Fig. 2A are demonstrated in Fig. 2B. The results of the triplicate experiments, as demonstrated in Fig. 2C and D,

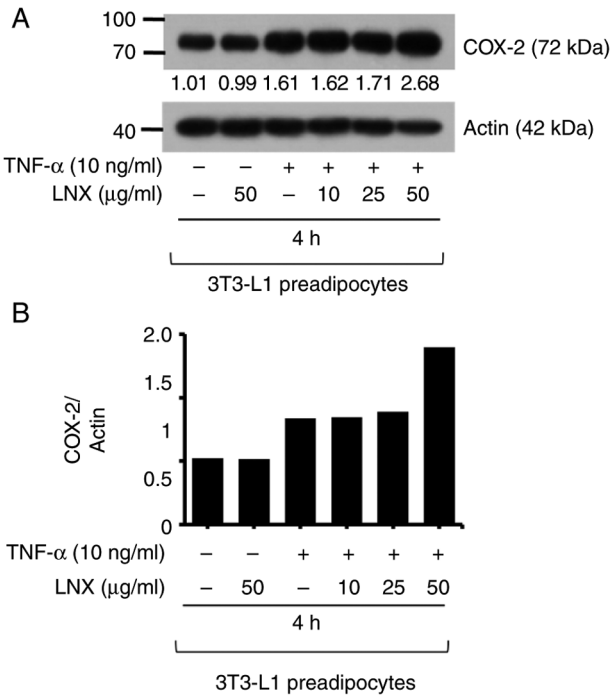


Figure 4. Effects of LNX treatment on TNF-α-induced COX-2 protein expression level in 3T3-L1 preadipocytes. (A) 3T3-L1 preadipocytes were treated with or without TNF-α (10 ng/ml) in the presence or absence of LNX at the designated concentrations for 4 h. After the aforementioned treatment, whole-cell lysate was prepared and analyzed using western blotting. (B) The densitometric analysis of panel A. LNX, lignan-enriched nutmeg extract; COX-2, cyclooxygenase-2.

revealed the capability of LNX (6 $\mu$g/ml) to vastly inhibit STAT3 phosphorylation and FAS expression on D8 and D2 of 3T3-L1 preadipocyte differentiation, respectively. The densitometric data from Fig. 2C and D are shown in Fig. 2E and F, respectively.

*LNX treatment at 6 μg/ml does not stimulate lipolysis in differentiated 3T3-L1 adipocytes*. Subsequently, it was explored whether treatment with LNX (6 $\mu$g/ml) modulated lipolysis in differentiated 3T3-L1 adipocytes by measuring the levels of glycerol content and HSL phosphorylation, two hallmarks of lipolysis. The experimental scheme and timescale for measuring glycerol content and HSL phosphorylation are exhibited in Fig. 3A. To this end, differentiated 3T3-L1 adipocytes on D8 were serum-starved (0% FBS) for 2 h and incubated in a fresh culture media containing 10% FBS in the absence or presence of LNX (6 $\mu$g/ml) or ISO (20 $\mu$M), a known inducer of lipolysis (31), for an additional 3 h. Culture media and whole-cell lysates from each condition were used to measure the two lipolysis hallmarks aforementioned. As illustrated in Fig. 3B, ISO treatment for 3 h significantly enhanced glycerol release from differentiated 3T3-L1 adipocytes, whereas LNX treatment did not. In addition, while ISO treatment for 3 h significantly elevated p-HSL levels at residues S563 and S565 in differentiated 3T3-L1 adipocytes, LNX treatment had no stimulatory effect. The total HSL protein expression levels remained unchanged under these experimental conditions. The densitometric data from Fig. 3C are demonstrated in Fig. 3D.

*LNX treatment has no effect on the TNF-α-induced expression of COX-2 in 3T3-L1 preadipocytes*. Next, it was further explored whether TNF-α at 10 ng/ml induces COX-2 expression in 3T3-L1 preadipocytes and whether treatment of LNX at different concentrations (1.5, 3 or 6 $\mu$g/ml) interferes with it by using RT-PCR analysis. As expected, the exposure of TNF-α for 4 h greatly induced COX-2 mRNA expression in 3T3-L1 preadipocytes. However, LNX treatment at the examined concentrations did not significantly influence cytokine-induced COX-2 mRNA expression in these cells (Fig. S1). Using immunoblotting analysis (Fig. 4A), it was investigated whether LNX treatment at higher doses (10, 25 and 50 $\mu$g/ml) could modulate cytokine-induced COX-2 expression in 3T3-L1 preadipocytes. As revealed in Fig. 4, while treatment with TNF-α for 4 h markedly enhanced COX-2 protein expression in 3T3-L1 preadipocytes, LNX treatment at such high doses tested did not interfere with the TNF-α-induced COX-2 protein expression in 3T3-L1 preadipocytes. Expression levels of the control actin protein remained constant under these experimental conditions. The densitometric data from Fig. 4A are illustrated in Fig. 4B.

## Discussion

Abnormal lipid accumulation, aggravated inflammation and the dysregulation of preadipocyte lipolysis are closely linked to the development of obesity. In the present study, LNX demonstrated anti-adipogenic, but not pro-lipolytic, and anti-inflammatory effects in differentiating 3T3-L1 preadipocytes through the control of STAT3 phosphorylation and FAS expression.

In the initial experiments, it was revealed that LNX treatment led to a concentration-dependent suppression of fat accumulation in differentiating 3T3-L1 preadipocytes with no cytotoxicity, supporting its anti-adipogenic effect. Mounting evidence illustrated a pivotal role of adipogenic transcription factors including C/EBP-α, PPAR-γ and STAT3/5 in adipogenesis of preadipocytes (32-34). To date, LNX regulation of the expression and phosphorylation levels of these transcription factors during the adipocyte differentiation of 3T3-L1 preadipocytes remains unknown. In the present study, LNX treatment greatly inhibited STAT3 phosphorylation without affecting total protein expression in the middle stage (D5) of cell differentiation. Previous studies have shown that STAT3 phosphorylation is involved in the early stages of 3T3-L1 preadipocyte differentiation (35,36). These findings suggested that LNX exerts its lipid-lowering effect on differentiating 3T3-L1 preadipocytes by inhibiting STAT3 and its downstream components or pathways. Previously, it has also been reported that STAT3 regulates the upregulation of C/EBP-α and PPAR-γ at middle or last stage of preadipocyte differentiation (37). However, in the present study, it was demonstrated that LNX treatment does not modulate the expression levels of both C/EBP-α and PPAR-γ throughout 3T3-L1 preadipocyte differentiation. It is therefore conceivable that the anti-adipogenic effect of LNX on differentiating 3T3-L1 preadipocytes is mediated not through the STAT3-dependent C/EBP-α and PPAR-γ pathway but via a different STAT3-dependent pathway.

Preadipocyte differentiation is also closely associated with lipogenesis and lipid or LD stabilization (38,39). FAS

is a key lipogenic enzyme responsible for catalyzing *de novo* synthesis of fatty acids and its expression is highly elevated during preadipocyte differentiation (40,41). Perilipin A is an LD-binding and stabilizing protein whose expression is greatly enhanced during preadipocyte differentiation (42,43). At present, little is known about the LNX regulation of FAS and perilipin A during preadipocyte differentiation. Notably, LNX treatment inhibited FAS expression in the early stages of 3T3-L1 preadipocyte differentiation. However, the present study demonstrated that LNX treatment does not interfere with perilipin A expression during 3T3-L1 cell differentiation. These results suggested that the anti-adipogenic effect of LNX on differentiating 3T3-L1 preadipocytes is, in part, mediated through interference with FAS-dependent lipogenesis.

Lipolysis is a biochemical process in which lipids or TGs stored in adipocytes are broken down, resulting in the efflux of fatty acids and glycerol (44). Hence, any material that induces lipolysis in lipid-laden adipocytes can be utilized as a potential anti-obesity agent. Accordingly, the LNX regulation of lipolysis and its regulator in differentiated or mature adipocytes were further tested. However, considering the present findings that LNX treatment did not enhance glycerol release in differentiated 3T3-L1 adipocytes, it is likely that LNX has no lipolytic activity in mature 3T3-L1 adipocytes. Cellular lipases which trigger lipolysis HSL are key for TG degradation in mature adipocytes (45). It has also been revealed that HSL is phosphorylated at S563 and S565 and becomes active in adipocytes in response to certain endogenous or exogenous stimuli, such as norepinephrine or ISO (46,47). Given that, unlike ISO, LNX treatment does not induce the phosphorylation of HSL at residues S563 and S565 in differentiated 3T3-L1 adipocytes, it is further evident that LNX has no lipolytic effect on these cells.

Accumulating evidence strongly indicates that chronic inflammation contributes to development of obesity (48). A wealth of information illustrates that preadipocytes, the predominant cell types present in the AT, are able to express and secrete numerous pro-inflammatory cytokines [including TNF-α, interleukin (IL)-1β and IL-6] and enzymes (COX-2) (49-51), which can confer and aggravate obese inflammation, via the autocrine, paracrine and endocrine ways. As aforementioned, the authors and other groups have previously demonstrated that TNF-α is an inducer of COX-2 in not only 3T3-L1 preadipocytes but also in mature or differentiated adipocytes (15-19). To date, the regulation of pro-inflammatory cytokine-induced expression of COX-2 by LNX in 3T3-L1 preadipocytes remains unknown. In the present study, it was observed that LNX treatment at doses tested does not interfere with the TNF-α-induced COX-2 expression at the protein and mRNA levels in undifferentiated 3T3-L1 cells. These results indicated that LNX has no anti-inflammatory effect on 3T3-L1 preadipocytes. In addition, it would be interesting to investigate whether LNX could alter the cytokine-induced expression of COX-2 (and other inflammatory mediators) in mature adipocytes. Moreover, further studies using *in vivo* experiments will be able to address the limitations of this study.

In summary, it was firstly reported that LNX has a potent anti-adipogenic effect on differentiating 3T3-L1 preadipocytes, and this effect is achieved by downregulating STAT3 phosphorylation and FAS expression. The present study advocated that LNX may be a potential obesity-preventive agent.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

NP, SD, JL, HY, BJ and JC conceived and designed the experiments. NP, SD, GB, JL, GK and AM performed the experiments. HY, BJ, JC, SD, GB and AM analyzed and interpreted the data. HY, BJ, AM and JC wrote the manuscript; BJ, JL, GB and AM reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. HY, BJ and JC confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. GBD 2015 Obesity Collaborators; Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, Lee A, Marczak L, Mokdad AH, Moradi-Lakeh M, *et al*: Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med 377: 13-27, 2017.
2. Gregg EW and Shaw JE: Global health effects of overweight and obesity. N Engl J Med 377: 80-81, 2017.
3. Spiegelman BM and Flier JS: Obesity and the regulation of energy balance. Cell 104: 531-543, 2001.
4. Madsen MS, Siersbæk, Boergesen M, Nielsen R and Mandrup S: Peroxisome proliferator-activated receptor γ and C/EBPα synergistically activate key metabolic adipocyte genes by assisted loading. Mol Cell Biol 34: 939-954, 2014.
5. Zhao P and Stephens JM: Identification of STAT target genes in adipocytes. JAKSTAT 2: e23092, 2013.
6. Zhang K, Guo W, Yang Y and Wu J: JAK2/STAT3 pathway is involved in the early stage of adipogenesis through regulating C/EBPβ transcription. J Cell Biochem 112: 488-497, 2011.
7. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, McKeon C, Darlington GJ and Spiegelman BM: Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell 3: 151-158, 1999.
8. Janovská A, Hatzinikolas G, Staikopoulos V, Mcinerney J, Mano M and Wittert GA: AMPK and ACC phosphorylation: Effect of leptin, muscle fibre type and obesity. Mol Cell Endocrinol 284: 1-10, 2008.

9. Garcia A, Sekowski A, Subramanian V and Brasaemle DL: The central domain is required to target and anchor perilipin A to lipid droplets. J Biol Chem 278: 625-635, 2003.
10. Schweiger M, Eichmann TO, Taschler U, Zimmermann R, Zechner R and Lass A: Measurement of lipolysis. Methods Enzymol 538: 171-193, 2014.
11. Hsieh PS, Jin JS, Chiang CF, Chan PC, Chen CH and Shih KC: COX-2-mediated inflammation in fat is crucial for obesity-linked insulin resistance and fatty liver. Obesity (Silver Spring) 17: 1150-1157, 2009.
12. Hotamisligil GS, Shargill NS and Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science 259: 87-91, 1993.
13. Fuster JJ, Ouchi N, Gokce N and Walsh K: Obesity-induced changes in adipose tissue microenvironment and their impact on cardiovascular disease. Circ Res 118: 1786-1807, 2016.
14. Kim HL, Ha AW and Kim WK: Effect of saccharin on inflammation in 3T3-L1 adipocytes and the related mechanism. Nutr Res Pract 14: 109-116, 2020.
15. Cheng, AW, Tan X, Sun JY, Gu CM, Liu C and Guo X: Catechin attenuates TNF-α induced inflammatory response via AMPK-SIRT1 pathway in 3T3-L1 adipocytes. PLoS One 14: e0217090, 2019.
16. Li Y, Yang P, Chang Q, Wang J, Liu J, Lv Y, Wang TTY, Gao B, Zhang Y and Yu LL: Inhibitory effect of piceatannol on TNF-α-mediated inflammation and insulin resistance in 3T3-L1 Adipocytes. J Agric Food Chem 65: 4634-4641, 2017.
17. Kwon HS, Jeong GS and Jang BC: Cudratricusxanthone A inhibits lipid accumulation and expression of inducible nitric oxide synthase in 3T3-L1 preadipocytes. Int J Mol Sci 22: 505, 2021.
18. Yadav AK and Jang BC: Inhibition of lipid accumulation and cyclooxygenase-2 expression in differentiating 3T3-L1 preadipocytes by pazopanib, a multikinase inhibitor. Int J Mol Sci 22: 4884, 2021.
19. Van Gils C and Cox PA: Ethnobotany of nutmeg in the spice islands. J Ethnopharmacol 42: 117-124, 1994.
20. Nguyen PH, Le TVT, Kang HW, Chae J, Kim SK, Kwon KI, Seo DB, Lee SJ and Oh WK: AMP-activated protein kinase (AMPK) activators from *Myristica fragrans* (nutmeg) and their anti-obesity effect. Bioorg Med Chem Lett 20: 4128-4131, 2010.
21. Cho JY, Choi GJ, Son SW, Jang KS, Lim HK, Lee SO, Sung ND, Cho KY and Kim JC: Isolation and antifungal activity of lignans from *Myristica fragrans* against various plant pathogenic fungi. Pest Manag Sci 63: 935-940, 2007.
22. Zhao W, Guo M, Feng J, Gu Z, Zhao J, Zhang H, Wang G and Chen W: *Myristica fragrans* extract regulates gut microbes and metabolites to attenuate hepatic inflammation and lipid metabolism disorders via the AhR-FAS and NF-κB signaling pathways in mice with non-alcoholic fatty liver disease. Nutrients 14: 1699, 2022.
23. Zhao W, Song F, Hu D, Chen H, Zhai Q, Lu W, Zhao J, Zhang H, Chen W, Gu Z and Wang G: The protective effect of *Myristica fragrans Houtt*. Extracts against obesity and inflammation by regulating free fatty acids metabolism in nonalcoholic fatty liver disease. Nutrients 12: 2507, 2020.
24. Lesmana R, Siannoto M, Nugraha GI, Goenawan H, Feinisa AK, Pratiwi YS, Veronica F, Tarawan VM, Susianti S and Supratman U: Nutmeg extract potentially alters characteristics of white adipose tissue in rats. Vet Med Sci 7: 512-520, 2021.
25. Hien TT, Oh WK, Nguyen PH, Oh SJ, Lee MY and Kang KW: Nectandrin B activates endothelial nitric-oxide synthase phosphorylation in endothelial cells: Role of the AMP-activated protein kinase/estrogen receptor α/phosphatidylinositol 3-kinase/Akt pathway. Mol Pharmacol 80: 1166-1178, 2011.
26. Smith M: Nutmeg. In: Encyclopedia of Toxicology. 3rd edition. Elsevier, Amsterdam, pp630-631, 2014.
27. Götz ME, Sachse B, Schäfer B and Eisenreich A: Myristicin and elemicin: Potentially toxicalkenylbenzenes in food. Foods 11: 1988, 2022.
28. Jang HJ, Yang KE, Oh WK, Lee SI, Hwang IH, Ban KT, Yoo HS, Choi JS, Yeo EJ and Jang IS: Nectandrin B-mediated activation of the AMPK pathway prevents cellular senescence in human diploid fibroblasts by reducing intracellular ROS levels. Aging (Albany NY) 11: 3731-3749, 2019.
29. Pratiwi YS, Lesmana R, Goenawan H, Sylviana N, Setiawan I, Tarawan VM, Lestari K, Abdulah R, Dwipa L, Purba A and Supratman U: Nutmeg extract increases skeletal muscle mass in aging rats partly via IGF1-Akt-mTOR pathway and inhibition of autophagy. Evid Based Complement Alternat Med 2018: 2810840, 2018.
30. Lee JH, Kang H, Ban KT, Kim BK, Lee JH, Hwang H, Yoo HS, Cho K and Choi JS: Proteome network analysis of skeletal muscle in lignan-enriched nutmeg extract fed mice. J Anal Sci Tech 14: 11, 2023.
31. Greenberg AS, Shen WJ, Muliro K, Patel S, Souza SC, Roth RA and Kraemer FB: Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. J Biol Chem 276: 45456-45461, 2001.
32. Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ and Spiegelman BM: C/EBPalpha induces adipogenesis through PPARgamma: A unified pathway. Genes Dev 16: 22-26, 2002.
33. Stephens JM, Morrison RF and Pilch PF: The expression and regulation of STATs during 3T3-L1 adipocyte differentiation. J Biol Chem 271: 10441-10444, 1996.
34. Gong Z, Huang C, Sheng X, Zhang Y, Li Q, Wang M-W, Peng L and Zang YQ: The role of tanshinone IIA in the treatment of obesity through peroxisome proliferator-activated receptor gamma antagonism. Endocrinology 150: 104-113, 2009.
35. Farmer SR: Transcriptional control of adipocyte formation. Cell Metab 4: 263-273, 2006.
36. Richard AJ and Stephens JM: The role of JAK-STAT signaling in adipose tissue function. Biochim Biophys Acta 1842: 431-439, 2014.
37. Wang D, Zhou Y, Lei W, Zhang K, Shi J, Hu Y, Shu G and Song J: Signal transducer and activator of transcription 3 (STAT3) regulates adipocyte differentiation via peroxisome-proliferator-activated receptor gamma (PPARgamma). Biol Cell 102: 1-12, 2009.
38. Collins JM, Neville MJ, Pinnick KE, Hodson L, Ruyter B, van Dijk TH, Reijngoud DJ, Fielding MD and Frayn KN: De novo lipogenesis in the differentiating human adipocyte can provide all fatty acids necessary for maturation. J Lipid Res 52: 1683-1692, 2011.
39. Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR and Londos C: The central role of perilipin a in lipid metabolism and adipocyte lipolysis. IUBMB Life 56: 379-385, 2004.
40. Swierczyński J and Sledziński T: Metabolic and regulatory function of fatty acid synthase. Postepy Biochem 58: 175-185, 2012 (In Polish).
41. Jensen-Urstad APL and Semenkovich CF: Fatty acid synthase and liver triglyceride metabolism: Housekeeper or messenger? Biochim Biophys Acta 1821: 747-753, 2012.
42. Itabe H, Yamaguchi T, Nimura S and Sasabe N: Perilipins: A diversity of intracellular lipid droplet proteins. Lipids Health Dis 16: 83, 2017.
43. Kern PA, Gregorio GD, Lu T, Rassouli N and Ranganathan G: Perilipin expression in human adipose tissue is elevated with obesity. J Clin Endocrinol Metab 89: 1352-1358, 2004.
44. Langin D: Control of fatty acid and glycerol release in adipose tissue lipolysis. C R Biol 329: 598-607, 2006.
45. Althaher AR: An overview of hormone-sensitive lipase (HSL). ScientificWorldJournal 2022: 1964684, 2022.
46. Kraemer FB and Shen WJ: Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. J Lipid Res 43: 1585-1594, 2002.
47. McDonough PM, Ingermanson RS, Loy PA, Koon ED, Whittaker R, Laris CA, Hilton JM, Nicoll JB, Buehrer BM and Price JH: Quantification of hormone sensitive lipase phosphorylation and colocalization with lipid droplets in murine 3T3L1 and human subcutaneous adipocytes via automated digital microscopy and high-content analysis. Assay Drug Dev Technol 9: 262-280, 2011.
48. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA and Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112: 1821-1830, 2003.
49. Roy PK, Islam J and Lalhlenmawia H: Prospects of potential adipokines as therapeutic agents in obesity-linked atherogenic dyslipidemia and insulin resistance. Egypt Heart J 75: 24, 2023.
50. Ouchi N, Parker JL, Lugus JJ and Walsh K: Adipokines in inflammation and metabolic disease. Nat Rev Immunol 11: 85-97, 2011.
51. Chan PC, Liao MT and Hsieh PS: The dualistic effect of COX-2-mediated signaling in obesity and insulin resistance. Int J Mol Sci 20: 3115, 2019.