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RESEARCH ARTICLE

Anti-adipogenesis by 6-thioinosine is mediated by downregulation of PPAR γ through JNK-dependent upregulation of iNOS

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Abstract Adipocyte dysfunction is associated with the development of obesity. This study shows that 6-thioinosine inhibits adipocyte differentiation. The mRNA levels of PPAR γ and C/EBPα, but not C/EBPβ and δ, were reduced by 6-thioinosine. Moreover, the mRNA levels of PPAR γ target genes (LPL, CD36, aP2, and LXRα) were downregulated by 6-thioinosine. We also demonstrated that 6-thioinosine inhibits the transactivation activity and the mRNA level of PPAR γ. Additionally, attempts to elucidate a possible mechanism underlying the 6-thioinosine-mediated effects revealed that 6-thioinosine induced iNOS gene expression without impacting eNOS expression, and that this was mediated through activation of AP-1, especially, JNK. In addition, 6-thioinosine was found to operate upstream of MEKK-1 in JNK activation signaling. Taken together, these findings suggest that the inhibition of adipocyte differentiation by 6-thioinosine occurs primarily through the reduced expression of PPAR γ, which is mediated by upregulation of iNOS via the activation of JNK.

Keywords 3T3-L1 adipocytes · Adipocyte differentiation · iNOS · JNK · PPAR gamma

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Introduction

Adipose tissue biology studies have led to improved understanding of the mechanisms that link obesity to metabolic syndrome and other complications. Adipocytes play a central role in the maintenance of lipid homeostasis and energy balance in vertebrates by storing triglycerides (TGs) or by releasing free fatty acids in response to changes in energy demands [1, 2]. However, obesity is associated with a number of pathological disorders including non-insulin (INS)-dependent diabetes, hypertension, hyperlipidemia, and cardiovascular diseases [3]. In addition, several lines of evidence have suggested that TG accumulation in skeletal muscles and pancreatic islets is casually related to skeletal muscle INS resistance and pancreatic β-cell dysfunction in obese patients [4–6]. Obesity is not only caused by adipose tissue hypertrophy, but also by adipose tissue hyperplasia, which triggers the transformation of preadipocytes into adipocytes [7]. However, the molecular basis for these associations remains to be elucidated, which has made the search for compounds with anti-obesity activity difficult.

Adipogenesis is a complex process that is accompanied by coordinated changes in morphology, hormone sensitivity, and gene expression. Members of the CCAAT/enhancer binding protein (C/EBP) transcription factor family and peroxidase proliferator-activated receptor γ (PPAR γ) act in concert to regulate the adipocyte differentiation program [8]. Retinoic acid and its derivatives, which are possible anti-obesity agents, are able to inhibit adipogenesis [9].

Purine nucleotides and their metabolites are essential to cell function. There is also increasing evidence that purine nucleosides or nucleobases (e.g., inosine and adenine) play physiologically important roles in tissues as a result of cell

surface receptor signaling [10–13]. For example, adenosine may exert a neuroprotective effect through glial cells via the production of trophic factors [14]. Furthermore, it has been reported that adenosine protects astrocytes from the cytotoxicity of peroxynitrite by maintaining the intracellular ATP level, not by interacting with their receptors [15]. Inosine was recently implicated in cellular responses to ischemia in the brain and skeletal muscle, and it has been reported to activate A_3 receptors [10–13]. Additionally, 6-benzylaminopurine (6-BAP), which is a purine derivative, has been found to induce melanogenesis in B16 melanoma cells [16]. These various functions of purine and its derivatives led us to evaluate their inhibitory effect on adipogenesis. To accomplish this, a cell-based, purine compound library screen that was intentionally biased to select relative compounds with low toxicity and high activity was conducted. Triglyceride and glycerol-3-phosphate dehydrogenase (GPDH) activity assays were used as a screening tool in mouse preadipocytes (3T3-L1) to evaluate the anti-adipogenesis effects of purines and their derivatives. During this screening, 6-thioinosine was selected as a candidate for use as an anti-adipogenesis agent.

In the present study, we assessed the effects of 6-thioinosine on the adipogenesis on 3T3-L1 cells. The results indicated that 6-thioinosine inhibited adipogenesis in 3T3-L1 cells. Specifically, 6-thioinosine downregulated PPAR γ and C/EBPα, as well as PPAR γ target genes such as LPL, CD36, aP2, and LXRα. Prior to this study, no possible mechanisms of 6-thioinosine-induced inhibition of adipogenesis had been identified; however, the results of this study demonstrated that 6-thioinosine-induced inhibition of adipogenesis was mediated by the upregulation of iNOS via activation of JNK. Taken together, these results suggest that 6-thioinosine has the potential for use in the treatment of obesity.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and anti-peroxisome proliferator-activated receptor-γ 2 (PPAR γ 2 (N-19)) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin monoclonal Ab, 6-thioinosine, isobutylmethylxanthine, dexamethasone, and insulin were purchased from Sigma-Aldrich (St. Louis, MO). TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). Anti-iNOS was purchased from Millipore (Temecula, CA). Anti-phospho-p38 MAPK antibody (Thr180/Tyr182) (28B10), anti-phospho-JNK antibody, anti-phospho-ERK1/2 (p42/44 MAPK) antibody (Thr202/Tyr204) (E10), anti-p38 MAPK antibody, anti-JNK antibody, and anti-ERK1/2 (p42/44 MAPK) antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). L-N^G-monomethyl arginine citrate [L-NMMA (citrate)] and aminoguanidine were obtained from Cayman chemical (Ann Arbor, MI).

3T3-L1 cell culture and stimulation

3T3-L1 preadipocytes (ATCC, Manassas, VA) were seeded in 6-cm diameter dishes at a density of 15×10^4 cells/well. Cells were grown in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS at 37°C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were incubated for 3 days with differentiation medium (0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 1 μg/ml insulin in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS). The preadipocytes were then maintained in and re-fed every 3 days with maintenance medium (phenol red-free DMEM supplemented with 10% charcoal-stripped FBS and 1 μg/ml insulin). To examine the effects of 6-thioinosine on adipocyte differentiation, 2-day post-confluent 3T3-L1 preadipocytes were stimulated with differentiation medium in the presence or absence of the indicated concentrations of 6-thioinosine for 3 days. The medium was then replaced with maintenance media in the presence or absence of the indicated concentrations of 6-thioinosine every 3 days until the end of the experiment at day 9. 6-Thioinosine was reconstituted in DMSO and stored at −20°C until use.

Human adipose tissue-derived stem cell culture and stimulation

Human adipose tissue-derived stem cells (Invitrogen, Carlsbad, CA) were seeded in 6-cm diameter dishes at a density of 15×10^4 cells/well. Cells were grown in MesenPro RS™ media (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2-day post-confluent human adipose tissue-derived stem cells (day 0) were incubated for 21 days with STEM PRO® adipocyte differentiation media (Invitrogen, Carlsbad, CA). To examine the effect of 6-thioinosine on adipocyte differentiation of human adipose tissue-derived stem cells, 2-day post-confluent human adipose tissue-derived stem cells were stimulated with STEM PRO® adipocyte differentiation media (Invitrogen, Carlsbad, CA) in the presence or absence of the indicated concentrations of 6-thioinosine for 21 days. The medium was then replaced with STEM PRO® adipocyte differentiation

media (Invitrogen, Carlsbad, CA) in the presence or absence of the indicated concentrations of 6-thioinosine every 3 days until the end of the experiment at day 21.

Oil Red O staining

3T3-L1 adipocytes or human adipose tissue-derived stem cells that were treated as described above were washed with PBS and fixed with 10% formalin for 30 min. Next, the cells were then washed twice with distilled water, after which they were stained for at least 1 h at room temperature in freshly diluted Oil Red O solution (six parts Oil Red O stock solution and four parts H_2O; Oil Red O stock solution is 0.5% Oil Red O in isopropanol). The results were confirmed by three independent experiments.

Triglyceride assay

3T3-L1 adipocytes or human adipose tissue-derived stem cells that were treated as described above were washed with PBS and then harvested into 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA. The samples were then sonicated three times for 15 s using UP50H with MS7 (Hielscher Ultrasonics GmbH, Germany) to homogenize the cell suspension after the total triglyceride content was evaluated using a triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). The protein content of an aliquot of the homogenate was also determined using a protein assay kit (Pierce, Rockford, IL). The results were confirmed by three independent experiments.

Glycerol-3-phosphate dehydrogenase (GPDH) activity

3T3-L1 adipocytes that were treated as described above were washed twice with PBS and then harvested into 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT. The cells were then disrupted by sonication, after which they were centrifuged at 12,000 *g* for 20 min at 4°C. Next, the supernatants were assayed for GPDH activity using GPDH activity assay kits (Takara Bio Inc., Japan) following the method described by Kozak et al. [17]. The results were confirmed by four independent experiments.

RNA preparation and real-time quantitative RT-PCR

Total cellular RNA was extracted from the 3T3-L1 cells 1, 4, or 9 days after inducing differentiation with TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction mix using random primers and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses for the genes described in Table 1 were then conducted using the Mx3005P Real-time PCR System (Stratagene, La Jolla, CA). Reactions were performed in a 25-μl mixture composed of 12.5 μl of 2 × SYBR Green reaction buffer, 5 μl of cDNA (corresponding to 50 ng of reverse transcribed total RNA) and 300 nM of each primer. After an initial incubation for 2 min at 50°C, the cDNA was denatured at 95°C for 10 min, followed by 40 cycles of PCR (95°C, 15 s; 60°C, 60 s). Each expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (C_T) method [18]. Results were confirmed by four independent experiments.

Plasmids

A luciferase reporter construct driven by 908 bp of human adiponectin promoter [p(-908)/LUC wt], a mutated construct [p(-908)/LUC PPRE mut] containing mutations in the PPAR response element (PPRE), and PPREx3 luciferase reporter were provided by Dr. A. Fukuhara (Osaka, Japan) [19]. A luciferase reporter construct driven by the

Table 1 Sequences of primers used for real-time PCR

Gene name	Accession no.	Forward primer	Reverse primer
18S	X00686	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG
PPARγ	NM_011146	CAAGAATACCAAAGTGCGATCAA	GAGCTGGGTCTTTTCAGAATAATAAG
C/EBPα	NM_007678	AGCAACGAGTACCGGGTACG	TGTTTGGCTTTATCTCGGCTC
C/EBPβ	NM_009883	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
C/EBPδ	NM_007679	TTCCAACCCCTTCCCTGAT	CTGGAGGGTTTGTGTTTTCTGT
LPL	NM_008509	GGCCAGATTCATCAACTGGAT	GCTCCAAGGCTGTACCCTAAG
aP2	NM_024406	AGTGAAAACTTCGATGATTACATGAA	GCCTGCCACTTTCCTTGTG
CD36	NM_007643	TTGTACCTATACTGTGGCTAAATGAGA	CTTGTGTTTTGAACATTTCTGCTT
LXRα	NM_013839	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT
iNOS	NM_010927	GAGGATGCCTTCCGCAGCTG	CATTGGAAGTGAAGCGTTTCG
eNOS	NM_008713	CCTTCCGCTACCAGCCAGA	CAGAGATCTTCACTGCATTGGCTA

iNOS promoter was provided by Dr. I. Singh (Charleston, SC) [20]. Renilla luciferase expression vector driven by a thymidine kinase promoter was purchased from promega (Madison, WI). AP-1 Luc and NF-κB-Luc reporter plasmids were purchased from Stratagene. Flag-tagged dominant negative of MEKK-1 (Dn-MEKK-1) and flag-tagged dominant negative MKK4 (Dn-MKK4) are gifts from Dr. Won (Mogam Biotech Research Institute, Korea).

Luciferase reporter assay

To assay for human adiponectin, PPRE, AP-1, NF-κB, and iNOS promoter activities, 3T3-L1 cells or COS-7 cells were transfected with human adiponectin, PPRE, AP-1, NF-κB, or iNOS-Luc reporter along with 1 μg of Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega, Madison, WI) (internal standard) using Superfect transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Four hours later, the cells were incubated with DMEM containing the indicated concentrations of 6-thioinosine for 20 h, after which the luciferase activities were assayed using a Luciferase Assay System (Promega, Madison, WI). The cells were then harvested, lysed, and centrifuged. Next, the supernatants were assayed for luciferase activity using a Dual Luciferase Assay system (Promega, WI) and a LB953 luminometer (Berthold, Germany), after which the activity was expressed as a ratio of the adiponectin, PPRE, AP-1, NF-κB, or iNOS-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity (% control). Results were confirmed by eight independent transfections.

Assay for NO production

NO synthesis was estimated by the accumulation of nitrite, a stable reaction product of NO and molecular oxygen [21, 22]. Briefly, 600 μl of culture supernatant was incubated with 100 ml of Griess-Romijin reagent (Wako Pure Chemicals, Japan). After 15 min of incubation at 25°C, the optical density of the assay samples was measured spectrophotometrically at 520 nm. The nitrite concentrations were calculated from a standard curve derived from nitrite ion standard solution (Kanto Chemical Co., Tokyo, Japan). DMEM was used as the blank. The protein concentration of the cells was then determined by the method described by Bradford [23].

Immunoblotting

Two-day post-confluent 3T3-L1 preadipocytes were stimulated with differentiation medium in the presence or absence of the indicated concentrations of 6-thioinosine for 3 days. The medium was then replaced with maintenance media in the presence or absence of the indicated concentrations of 6-thioinosine every 3 days until the end of the experiment at day 9. On day 9, the cells were washed twice with cold PBS, after which they were lysed in 150 μl of sample buffer [100 mM Tris–HCl, pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate (SDS), 1% bromophenol blue, 10% β-mercaptoethanol]. Next, the samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA). The membranes were then incubated overnight at 4°C with anti-PPAR γ 2 (N-19) antibody, anti-phospho-p38 MAPK antibody (Thr180/Tyr182) (28B10), anti-phospho-JNK antibody, and anti-phospho-ERK1/2 (p42/44 MAPK) antibody (Thr202/Tyr204) (E10) or anti-p38 MAPK antibody, anti-JNK antibody, anti-ERK1/2 (p42/44 MAPK) antibody and anti-β-actin antibody. The membranes were then washed three times with Tris-buffered saline containing Tween-20 (Sigma) (TBST), probed with horseradish peroxidase-conjugated secondary antibody, and developed using an ECL (enhanced chemiluminescence) Western blotting detection system (Amersham Biosciences).

ELISA assay for TNF α

Concentrations of TNF α in the culture supernatant were measured using Enzyme-linked Immunosorbent Assay (ELISA) kits (Genzyme, Minneapolis, MN). Culture supernatants were added to 96-well plates, and then diluted biotinylated TNF α was added to the sample wells. The samples were then incubated at room temperature for 3 h, after which the sample wells were washed. Next, streptavidin-HRP was distributed to the sample wells, and the plate was then incubated for 30 min at room temperature. The wells were then washed and 3, 3', 5, 5'-tetramethylbenzidine (TMBZ) substrate solution was added. Finally, the samples were then incubated in the dark, after which the absorbance was read at 450 nm for 12–15 min according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the means ± SD. Differences between the control and the treated group were evaluated by a Student's *t*-test using the Statview software (Abacus Concepts, Cary, NC). For all analyses, $P < 0.05$ was considered to be statistically significant.

Results

6-Thioinosine inhibits 3T3-L1 adipocyte differentiation

Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. No significant effects were observed in response to treatment with 6-thioinosine at concentrations less than 5 μM. After the preadipocytes differentiated into adipocytes, morphological alterations were observed due to the accumulation of lipid droplets in the cytoplasm. Specifically, Oil Red O staining revealed that the lipid accumulation in 6-thioinosine-treated cells was significantly lower than the lipid accumulation in control cells (Fig. 1c). To further characterize the effects of 6-thioinosine on differentiation, the cellular triglyceride content and GPDH enzyme activity were measured. Treatment with 6-thioinosine inhibited triglyceride accumulation on day 9 after full differentiation had occurred ($P < 0.05$) (Fig. 1a). Consistent with the observed reduction in triglyceride accumulation, GPDH activity was also reduced by treatment with 6-thioinosine ($P < 0.05$) (Fig. 1b).

6-Thioinosine inhibits the expression and transactivation activity of PPAR γ

As previously mentioned, we found that 6-thioinosine has anti-adipogenesis activity. Therefore, we examined its effect on the expression of adipokines. To accomplish this, the effects of 6-thioinosine on the expression and transactivation of PPAR γ, one of major adipogenic transcription factors, was investigated. As shown in Fig. 2a, the mRNA level of PPAR γ was significantly lower in cells that were treated with 6-thioinosine during adipocyte differentiation than in the control cells. These results were confirmed by

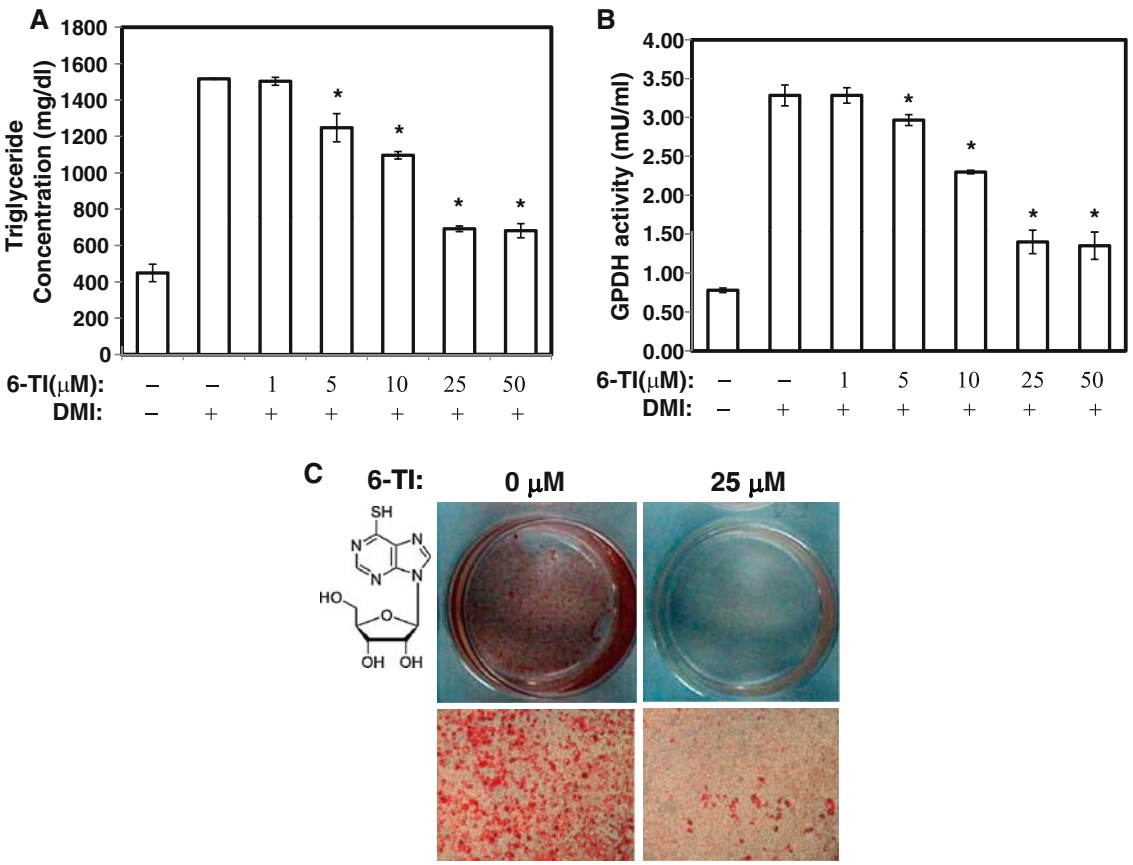

Fig. 1 6-Thioinosine inhibits adipocyte differentiation of 3T3-L1 cells. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. The assays were performed on fully differentiated adipocytes (day 9). **a** The triglyceride content was measured using a triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). DMI: differentiation media. Data are expressed as the means ± SD. $*P < 0.05$ versus controls. The results were verified by three repetitions of the experiments, which were each conducted in triplicate. **b** GPDH activity was measured using GPDH activity assay kits (Takara Bio Inc., Japan). Data are expressed as the means ± SD. $*P < 0.05$ versus controls. The results were verified by four repetitions of the experiments, each in triplicate. 6-TI: 6-thioinosine. DMI: differentiation media. **c** Intracellular lipids were stained with Oil Red O. The results were confirmed by three independent experiments, which were each conducted in duplicate

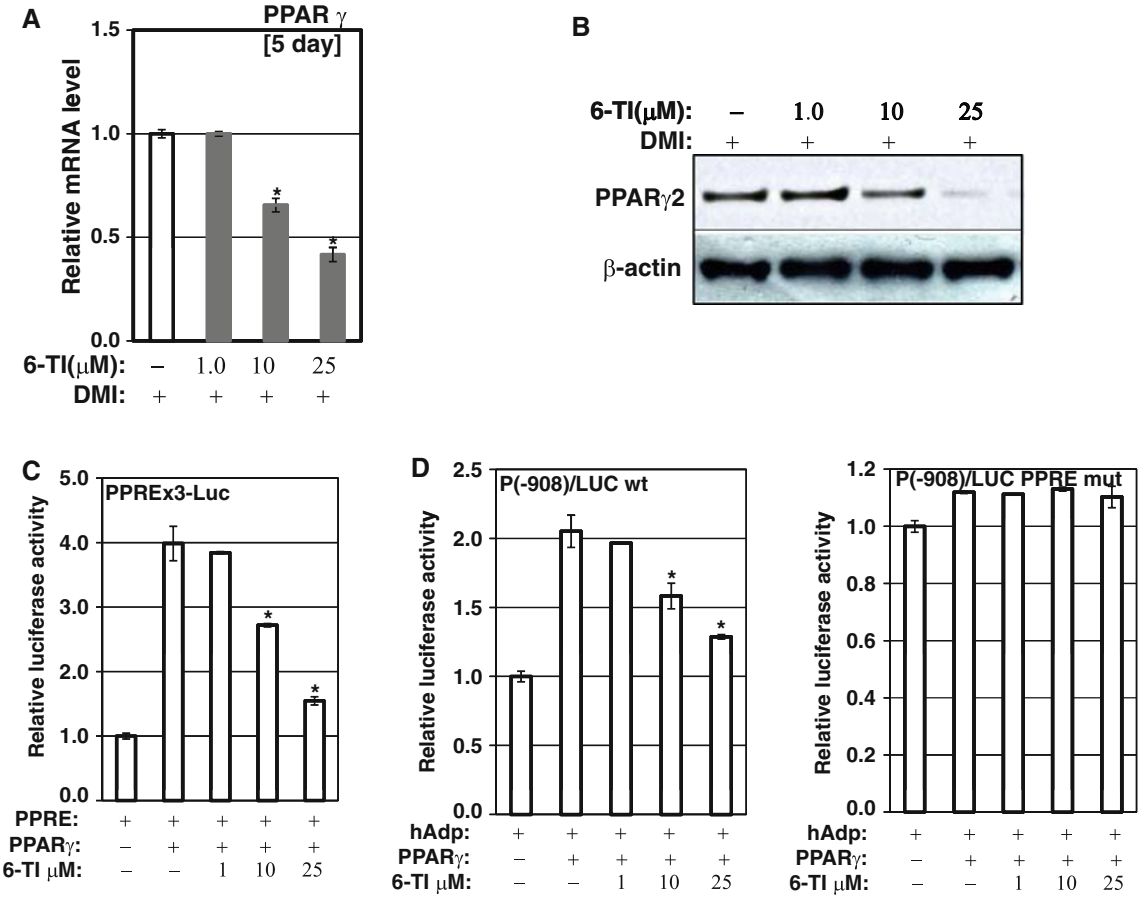

Fig. 2 Effects of 6-thioinosine on the expression and transactivation activity of PPAR γ. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. **a** At 5 days after the induction of differentiation, the total RNA was isolated, and the mRNA level of the PPAR γ gene was measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. **b** At day 5 after the induction of differentiation, the cell lysates were analyzed by Western blot analysis using the indicated antibodies. The results were verified by three repetitions of the experiments, each of which was conducted in duplicate. **c** 3T3-L1 cells were cotransfected with luciferase constructs that contained PPREx3-Luc reporter and PPAR γ cDNA plasmid. After 16 h, the transfected cells were incubated for 20 h with the indicated concentrations of 6-thioinosine. The cells were then harvested and lysed. Luciferase activity is expressed as the ratio of PPREx3 promoter-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase units). Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The experiments were repeated eight times, with each experiment being conducted in triplicate. **d** COS-7 cells were cotransfected with luciferase constructs that contained adiponectin promoter [p(-908)/LUC wt] and PPAR γ cDNA plasmid or mutated promoter [p(-908)/LUC PPRE mut] and PPAR γ cDNA plasmid. After 16 h, the transfected cells were incubated for 20 h with the indicated concentrations of 6-thioinosine. The cells were then harvested and lysed. Luciferase activity is expressed as the ratio of adiponectin promoter-dependent firefly luciferase activity divided by control thymidine kinase *Renilla* luciferase activity (relative luciferase units). Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The experiments were repeated eight times, with each experiment being conducted in triplicate. 6-TI: 6-thioinosine. DMI: differentiation media

Western blot analysis for PPAR γ, which revealed that 6-thioinosine inhibited PPAR γ2 protein expression in a concentration-dependent manner at day 9 of differentiation (Fig. 2b). Furthermore, we evaluated 6-thioinosine to determine if it had an effect on the transactivation activity of PPAR γ in mature adipocytes. As shown in Fig. 2c, cotransfection assays in 3T3-L1 cells that used PPAR γ cDNA and PPREx3-Luc reporter revealed that 6-thioinosine exerted an inhibitory effect on PPAR γ-mediated transactivation. In addition, as shown in Fig. 2d, cotransfection assays in COS-7 that used PPAR γ cDNA and human adiponectin reporters with PPRE site revealed that human adiponectin promoter activation was inhibited by 6-thioinosine. Taken together, these results indicate that PPAR γ is involved in the anti-adipogenesis effect of 6-thioinosine.

Effects of 6-thioinosine on the expression of the adipogenic transcription factors C/EBPα, β, and δ and PPAR γ target genes

The mRNA levels of C/EBPα were significantly lowered in cells that were treated with 6-thioinosine during adipocyte differentiation when compared with the control cells (Fig. 3a). However, the mRNA levels of the upstream regulators of PPAR γ and C/EBPα, C/EBPβ and δ, were not reduced by treatment with 6-thioinosine (Fig. 3a). In addition, because the level of PPAR γ mRNA was decreased by 6-thioinosine, we evaluated the expression of PPAR γ target genes to determine if they were also downregulated. We found that the mRNA levels of LPL, CD36, aP2, and LXRα were significantly lower during adipocyte differentiation following treatment with 6-thioinosine (Fig. 3b). Collectively, these results indicate that PPAR γ expression plays an important role in the regulation of adipocyte differentiation by 6-thioinosine.

6-Thioinosine upregulates iNOS expression during adipocyte differentiation

Several molecular targets for anti-adipogenesis were reported, namely β-catenin, protein kinase A (PKA), and mitogen-activated protein kinases (ERK, p38MAPK) [24–26]. Recently, Kawachi et al. reported that nitric oxide inhibits adipogenesis in 3T3-L1 cells [27]. In addition, adenosine has been shown to increase NO production [28]. Therefore, we evaluated 6-thioinosine to determine if it exerted a stimulatory effect on nitric oxide production. To accomplish this, we performed an iNOS luciferase reporter assay in 3T3-L1 cells. As shown in Fig. 4a, we found that 6-thioinosine activated iNOS promoter activity in a

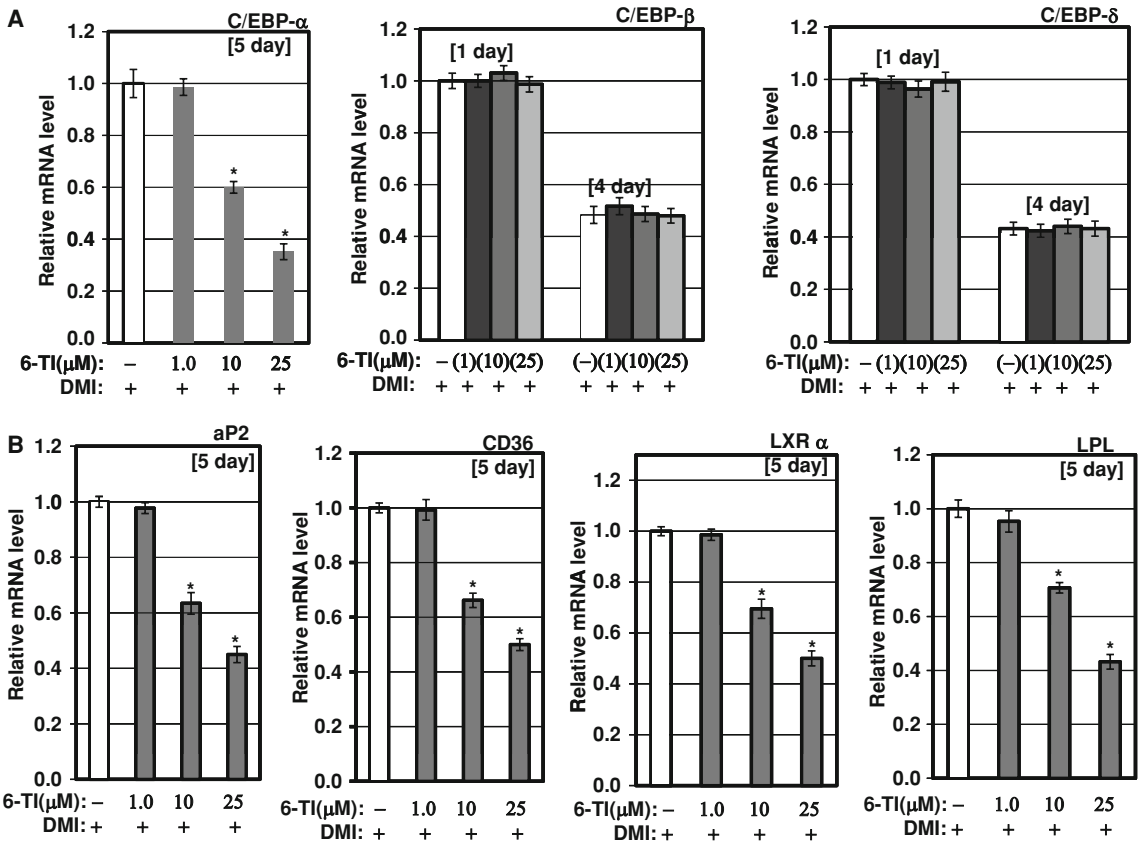

Fig. 3 Effects of 6-thioinosine on gene expression patterns during adipogenesis. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. At 1 day, 4 days, or 5 days after differentiation was induced, the total RNA was isolated, and the mRNA levels of the indicated genes were measured by real-time quantitative RT-PCR. **a** mRNA expression of the adipogenic transcription factors C/EBPα, C/EBPβ, and C/EBPδ. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. **b** mRNA expression of the PPAR γ target genes LPL, CD36, aP2, and LXRα. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. 6-TI: 6-thioinosine. DMI: differentiation media

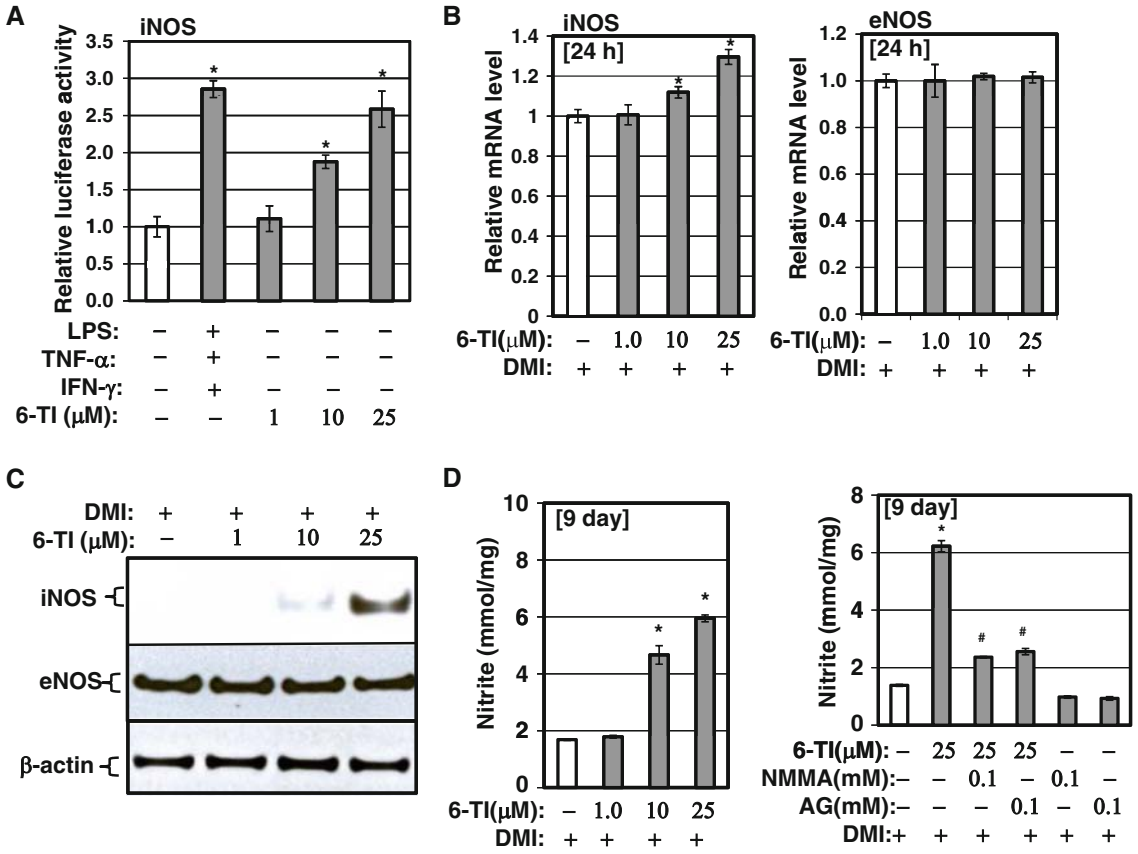

Fig. 4 6-Thioinosine upregulates expression of the iNOS gene. **a** Confluent 3T3-L1 cells were transfected with iNOS-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells were stimulated with the indicated concentrations of 6-thioinosine for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the untreated control. LPS: 1 μg/ml. TNF-α: 10 ng/ml. IFN-γ: 10 ng/ml. **b** Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. At day 1 after the induction of differentiation, the total RNA was isolated and subjected to quantitative analysis of iNOS and eNOS by real-time PCR. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were confirmed by four independent experiments, each of which was conducted in triplicate. **c** At day 1 after the induction of differentiation, the cell lysates were analyzed by Western blot using the indicated antibodies. The results were verified by three repetitions of the experiments, each of which was conducted in duplicate. **d** At day 9 after differentiation was induced, a NO production assay was performed. Data are expressed as the means ± SD. $^*P < 0.05$ versus controls. The results were verified by three repetitions of the experiments, each of which was conducted in duplicate. *6-TI* 6-thioinosine, *DMI* differentiation media, *NMMA* L-NMMA, *AG* aminoguanidine

concentration-dependent manner. Consistent with these findings, 3T3-L1 cells that were treated with 6-thioinosine had significantly higher levels of iNOS mRNA during adipocyte differentiation than control cells (Fig. 4b). These findings were confirmed by Western blot analysis for iNOS, which revealed that 6-thioinosine increased iNOS protein expression in a concentration-dependent manner (Fig. 4c). However, we found that eNOS gene expression is not induced by thioinosine (Fig. 4b, c). Finally, the production of NO by 6-thioinosine was examined. In this study, treatment of 3T3-L1 cells with 6-thioinosine was found to lead to a significant increase in NO production during adipocyte differentiation (Fig. 4d). In addition, 6-thioinosine-induced production of NO was attenuated by L-NMMA (non-specific inhibitor of NOSs) and aminoguanidine (a specific inhibitor of iNOS). Collectively, these results indicate that NO production was induced by 6-thioinosine through upregulation of the iNOS gene.

iNOS expression by 6-thioinosine is mediated through JNK activation

To determine if the stimulatory effect of 6-thioinosine on iNOS gene expression is mediated by the activation of NF-κB or AP-1, we employed the NF-κB and AP-1 luciferase reporter in confluent 3T3-L1 cells. As shown in Fig. 5a, 6-thioinosine activated AP-1 promoter, but NF-κB activation was not induced. These results suggest that

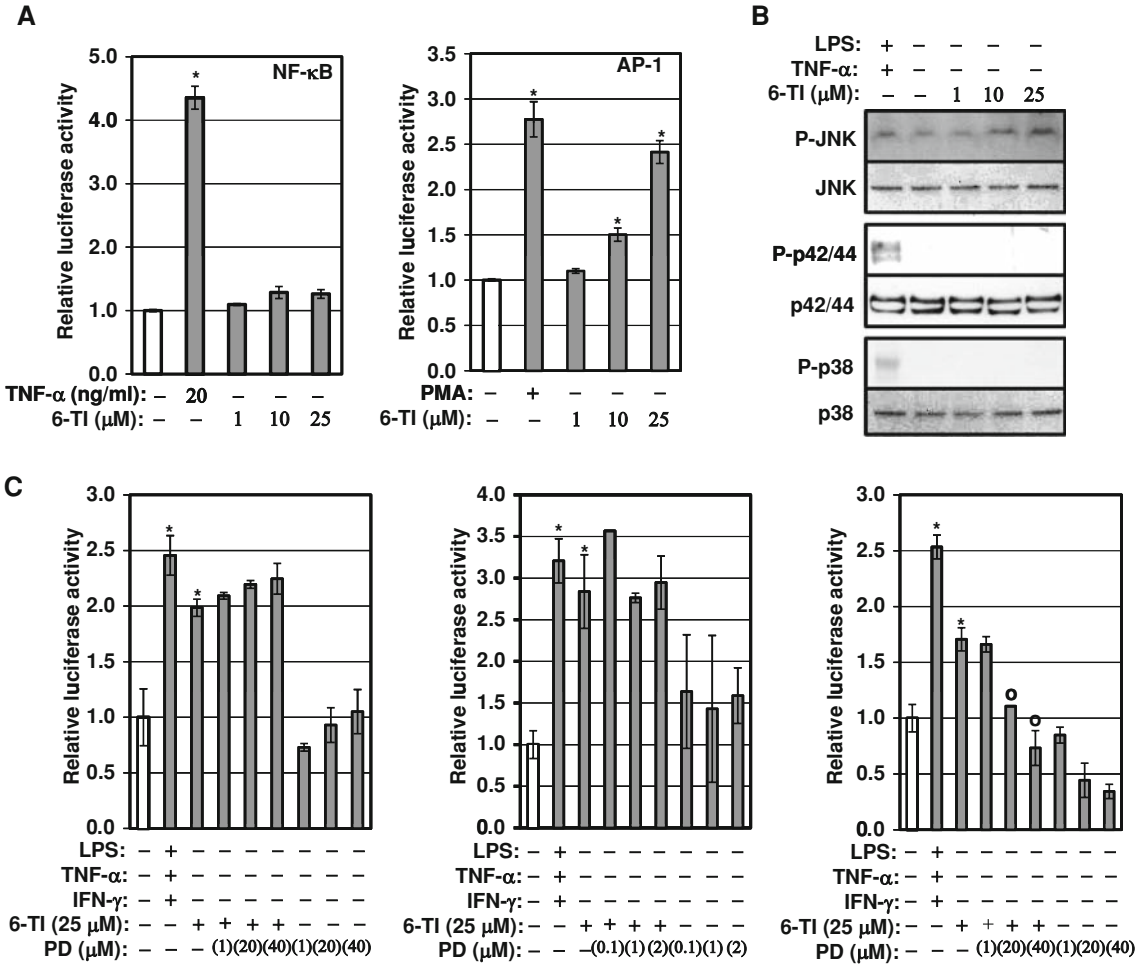

Fig. 5 iNOS expression by 6-thioinosine is mediated through JNK activation. **a** Confluent 3T3-L1 cells were transfected with AP-Luc or NF-κB-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells were stimulated with the indicated concentrations of 6-thioinosine for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the untreated control. *PMA* Phorbol myristate acetate (100 nM). **b** Confluent 3T3-L1 cells were treated with the indicated concentrations of 6- thioinosine for 2 h. The cell lysates were then analyzed by Western blot using the indicated antibodies. The experiments were repeated three times. LPS: 1 μg/ml. TNF-α: 10 ng/ml. **c** Confluent 3T3-L1 cells were transfected with iNOS-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells pretreated with or without the indicated MAPK inhibitors for 1 h were incubated with 25 μM 6-thioinosine for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the 6-thioinosine-treated control. LPS: 1 μg/ml. TNF-α: 10 ng/ml. IFN-γ: 10 ng/ml

6-thioinosine acts on the AP-1 pathway to activate expression of the iNOS gene. Therefore, we designed an experiment to determine if 6-thioinosine induced the phosphorylation of p42/44 MAPK, p38 MAPK, and JNK because these events are known to be involved in the activation of AP-1 [29]. As shown in Fig. 5b, 6-thioinosine induced JNK phosphorylation, but it did not induce phosphorylation of p38 MAPK or p42/44 MAPK. These results suggest that iNOS expression in response to treatment with 6-thioinosine is mediated through activation of JNK. To confirm this, luciferase activity driven by the iNOS promoter was measured in confluent 3T3-L1 adipocytes that were treated with or without AP-1 inhibitors in the presence of 6-thioinosine. The results of this assay revealed that iNOS promoter activation by 6-thioinosine was attenuated by treatment with the JNK inhibitor, SP600125, but that there were no changes observed in response to treatment with PD98059 (a p42/44 MAPK inhibitor) or SB203580 (a p38 MAPK inhibitor). These findings suggest that the increase in iNOS expression in response to 6-thioinosine may be dependent on JNK activation (Fig. 5c). Additionally, to elucidate a possible mechanism underlying the 6-thioinosine-mediated JNK activation signaling, the AP-1 and iNOS luciferase reporter assays were employed in confluent 3T3-

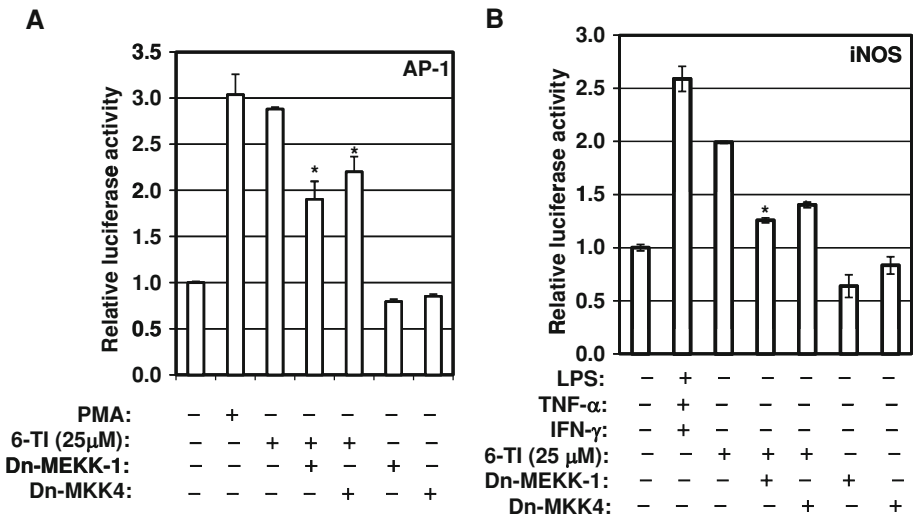

Fig. 6 6-Thioinosine operates upstream of MEKK-1 in JNK activation signaling. **a** Confluent 3T3-L1 cells were cotransfected with AP-Luc, and dominant negatives of MEKK-1 and MKK4, along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells were stimulated with the indicated concentrations of 6-thioinosine for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the 6-thioinosine-treated controls. PMA: Phorbol myristate acetate (100 nM). **b** Confluent 3T3-L1 cells were cotransfected with AP-Luc, and dominant negatives of MEKK-1 and MKK4, along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells were stimulated with the indicated concentrations of 6-thioinosine for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as the means ± SD. $^*P < 0.05$ compared to the 6-thioinosine-treated controls. LPS: 1 μg/ml. TNF-α: 10 ng/ml. IFN-γ: 10 ng/ml. 6-TI: 6-thioinosine. Dn-MEKK-1: dominant negative of MEKK-1. Dn-MKK4: dominant negative of MKK4

L1 cells. As shown in Fig. 6a, b, 6-thioinosine-induced reporter activation of AP-1 and iNOS was attenuated by dominant negatives of MEKK-1 and MKK4. These results indicate that 6-thioinosine operates upstream of MEKK-1 and MKK4.

SP600125, a JNK inhibitor, attenuates inhibition of adipocyte differentiation by 6-thioinosine

The results described above demonstrated that JNK activation plays an important role in the anti-adipogenesis effect of 6-thioinosine. To characterize the effects of SP600125, a JNK inhibitor, on reduced differentiation by 6-thioinosine, the cellular triglyceride content, GPDH enzyme activity, and Oil Red O staining assays were performed. Treatment with SP600125 attenuated the reduction in both triglyceride accumulation and GPDH activity that occurred in response to treatment with 6-thioinosine ($P < 0.05$) (Fig. 7a). Consistent with these findings, the reduction in lipid accumulation that was induced by 6-thioinosine was also significantly recovered by SP600125 ($P < 0.05$) (Fig. 7c). In addition, the mRNA level of PPAR γ was significantly higher in cells that were treated with SP600125, a JNK inhibitor, in the presence of 6-thioinosine during adipocyte differentiation than in cells that were treated with 6-thioinosine (Fig. 7d). The increased mRNA level of iNOS by 6-thioinosine was also reduced by SP600125 (Fig. 7e). These results were confirmed by Western blot analysis for PPAR γ2 and iNOS (Fig. 7f). Taken together, these findings suggest that JNK activation by 6-thioinosine inhibits adipocyte differentiation.

Effects of NO, a product of iNOS enyme, on adipocyte differentiation

The results described above demonstrated that 6-thioinosine inhibits adipogenesis through the reduced expression of PPAR γ, which is mediated by upregulation of iNOS via the activation of JNK. To characterize the effects of NO, a product of iNOS enzyme, on adipocyte differentiation, the Oil Red O staining and cellular triglyceride content assays were performed. Oil Red O staining revealed that the lipid accumulation in NOC18 (NO donor) treated cells was significantly lower than the lipid accumulation in control cells (8A). In addition, treatment with NOC18 inhibited triglyceride accumulation on day 9 after full differentiation had occurred ($P < 0.05$) (Fig. 8b). Consistent with these results, the mRNA level of PPAR γ was significantly lower in cells that were treated with NOC18 during adipocyte differentiation than in the control cells ($P < 0.05$) (Fig. 8c). These results support that anti-adipogenesis

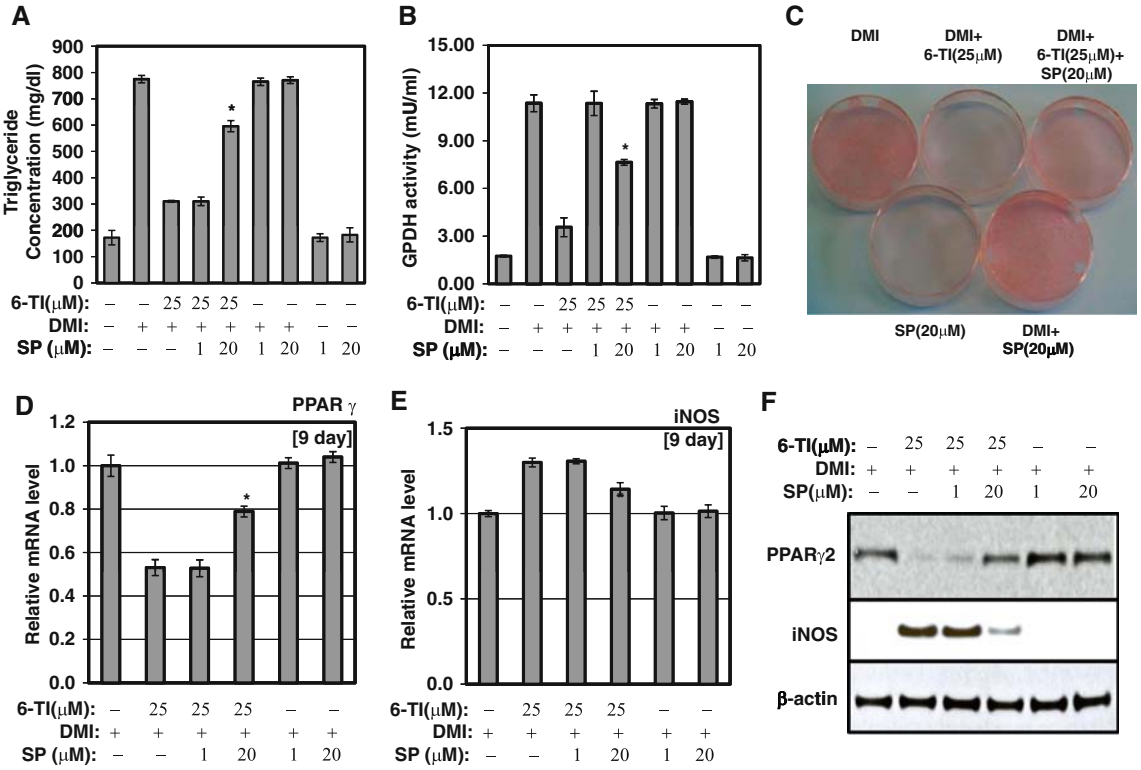

Fig. 7 SP60019, a JNK inhibitor, attenuates inhibition of adipocyte differentiation by 6-thioinosine. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were pre-treated with the indicated concentrations of SP60019 and then incubated with 25 μM 6-thioinosine for 3 days. After 3-day incubation, cells (day 3) were further treated with 25 μM 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. The assays were performed on fully differentiated adipocytes (day 9). **a** The triglyceride content was measured using a Triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). DMI: differentiation media. Data are expressed as the means ± SD. $*P < 0.05$ versus 6-thioinosine-treated controls. The results were verified by three repetitions of the experiments, which were each conducted in triplicate. **b** GPDH activity was measured using GPDH activity assay kits (Takara Bio Inc., Japan). Data are expressed as the means ± SD. $*P < 0.05$ versus 6-thioinosine-treated controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. 6-TI: 6-thioinosine. DMI: differentiation media. **c** Intracellular lipids were stained with Oil Red O using SP600125, a JNK inhibitor. The results were confirmed by three independent experiments, which were each conducted in duplicate. **d** At day 9 after the induction of differentiation, the total RNA was isolated, and the mRNA level of the iNOS gene was measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $*P < 0.05$ versus 6-thioinosine-treated controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. **e** At day 9 after the induction of differentiation, the total RNA was isolated, and the mRNA level of the PPAR γ gene was measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. $*P < 0.05$ versus 6-thioinosine-treated controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. **f** At day 9 after the induction of differentiation, the cell lysates were analyzed by Western blot analysis using the indicated antibodies. The results were verified by three repetitions of the experiments, each of which was conducted in duplicate. *6-TI* 6-thioinosine, *DMI* differentiation media, *SP* SP60019

mechanisms of 6-thioinosine are mediated through upregulation of iNOS via the activation of JNK.

Effect of 6-thioinosine on differentiation of adipose tissue-derived stem cells into adipocytes

The aforementioned results demonstrated that 6-thioinosine inhibits adipogenesis through upregulation of the iNOS gene via activation of JNK in 3T3-L1 cells. To further investigate the role of 6-thioinosine, we studied its effects on the differentiation of adipose tissue-derived stem cells into adipocytes. Two-day post-confluent adipose tissue-derived stem cells (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 21 days. Similar to the results observed in the 3T3-L1 cells, Oil Red O staining revealed that the level of lipid accumulation in 6-thioinosine-treated cells was significantly lower than the level of lipid accumulation in control cells (Fig. 9a). To further characterize the effects of 6-thioinosine on differentiation, the cellular triglyceride content was measured. Treatment with 6-thioinosine inhibited triglyceride accumulation on day 21 after full differentiation had occurred

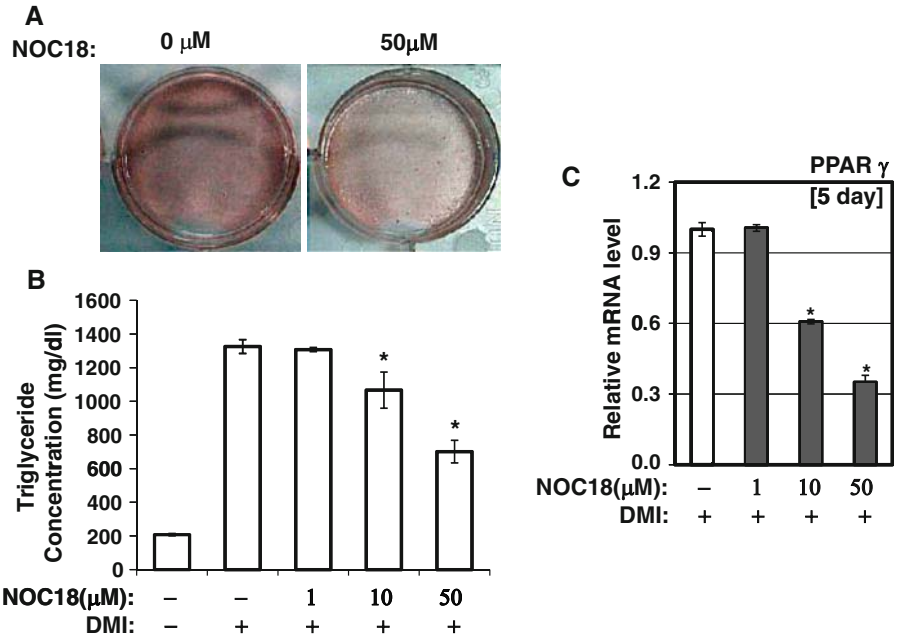

Fig. 8 NOC18 inhibits adipocyte differentiation of 3T3-L1 cells. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the 50 μM NOC18 every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. The assays were performed on fully differentiated adipocytes (day 9). **a** Intracellular lipids were stained with Oil Red O. The results were confirmed by three independent experiments, which were each conducted in duplicate. **b** The triglyceride content was measured using a triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). DMI: differentiation media. Data are expressed as the means ± SD. *$P < 0.05$ versus controls. The results were verified by three repetitions of the experiments, which were each conducted in triplicate. **c** Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of NOC18 every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. At 5 days after the induction of differentiation, the total RNA was isolated, and the mRNA level of the PPAR γ gene was measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± SD. *$P < 0.05$ versus controls. The results were verified by four repetitions of the experiments, each of which was conducted in triplicate. *DMI* differentiation media

($P < 0.05$) (Fig. 9b). Taken together, these results suggest that 6-thioinosine exerts an anti-adipogenic effect on adipose tissue-derived stem cells and presdipocytes.

Effect of 6-thioinosine on TNF α production

It is well known that TNF-α activates JNK and inhibits adipogenesis, and also utilizes the iNOS signaling pathway for its action. These actions are similar to the effects of 6-thioinosine; therefore, we investigated the effects of 6-thioinosine on the production of TNF-α in 3T3-L1 cells. In this study, treatment of 3T3-L1 cells with 6-thioinosine was found to lead to a significant increase in TNF α production during adipocyte differentiation (Fig. 10). In addition, 6-thioinosine-induced production of TNF α was not attenuated by L-NMMA (non-specific inhibitor of NOSs) or aminoguanidine (a specific inhibitor of iNOS). Collectively, these results suggest that 6-thioinosine-induced production of TNF α is an upstream step of iNOS activation and that 6-thioinosine also exerts an anti-adipogenic effect via the JNK-iNOS signaling pathway through TNF α.

Discussion

The results of this study provide direct evidence of the effects of 6-thioinosine on adipocyte differentiation, as well as its inhibitory mechanisms on adipogenesis in mouse 3T3-L1 cells. Specifically, the results of this study demonstrated that 6-thioinosine upregulates iNOS gene expression through activation of JNK, after which it reduces adipogenic gene expression, consequently inhibiting adipocyte differentiation. The cytosolic enzyme, GPDH, plays a central role in the pathway of triglyceride synthesis and is linked to the characteristic changes that occur during adipose conversion [17]. Here, we demonstrated that 6-thioinosine led to significant reductions in GPDH activity and the triglyceride content of adipocytes.

At the molecular level, adipogenesis is driven by a complex transcriptional cascade that involves the sequential activation of C/EBPs and PPAR γ [3], which are rapidly and transiently expressed after the hormonal induction of differentiation. These factors act synergistically to induce the expression of C/EBPα and PPAR γ, the master adipogenic transcription regulators. C/EBPα and PPAR γ, in turn,

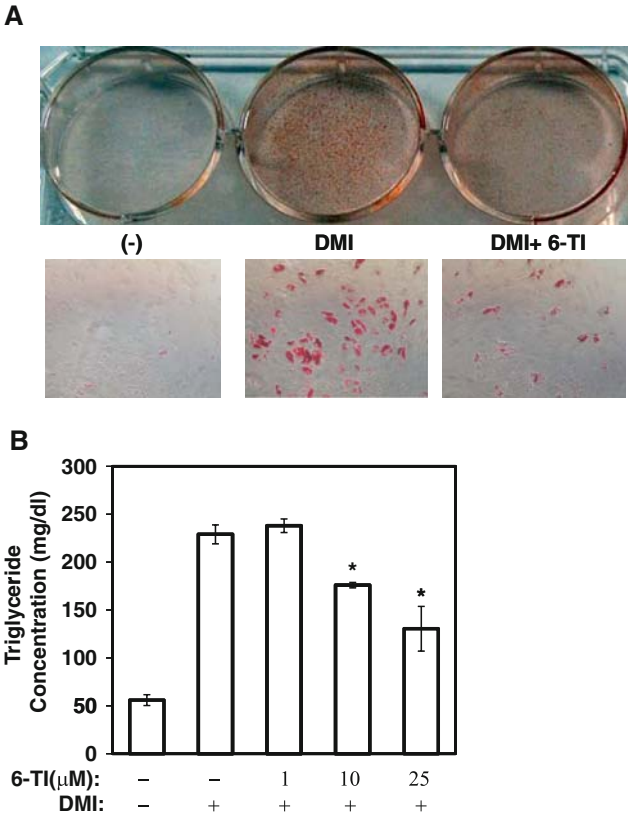

Fig. 9 6-Thioinosine inhibits adipocyte differentiation of human adipose tissue-derived stem cells. Two-day post-confluent human adipose tissue-derived stem cells (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 21 days. Cells treated with 0.1% DMSO were used as controls. The assays were performed on fully differentiated adipocytes (day 21). **a** Intracellular lipids were stained with Oil Red O. The results were confirmed by three independent experiments, which were each conducted in duplicate. **b** The triglyceride content was measured using a Triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). Data are expressed as the means ± SD. $*P < 0.05$ versus controls. The results were verified by three repetitions of the experiments, each of which was conducted in triplicate. *6-TI* 6-thioinosine, *DMI* differentiation media

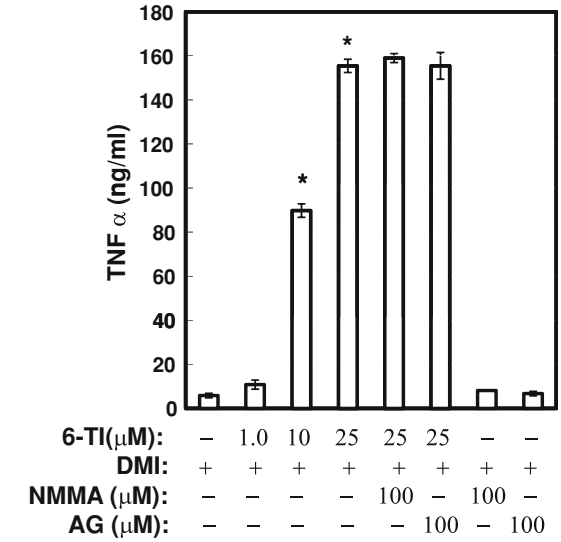

Fig. 10 6-Thioinosine induces production of TNF α in 3T3-L1 cells. Two-day post-confluent 3T3-L1 preadipocytes (day 0) were treated with the indicated concentrations of 6-thioinosine every 3 days for 9 days. Cells treated with 0.1% DMSO were used as controls. At day 1 after the induction of differentiation, the supernatants were harvested for TNF-α measurement. Data are expressed as the means ± SD. $*P < 0.05$ versus controls. The results were verified by three repetitions of the experiments, each of which was conducted in duplicate. *6-TI* 6-thioinosine, *DMI* differentiation media, *NMMA* L-NMMA, *AG* aminoguanidine

promote terminal differentiation by activating the transcription of genes involved in the creation and maintenance of the adipocyte phenotype. Loss-of-function studies have shown that PPAR γ is necessary as well as sufficient to promote adipogenesis and that C/EBPα is influential in maintenance of the expression of PPAR γ. The results of the present study indicate that exposing 3T3-L1 preadipocytes to 6-thioinosine during adipogenesis reduces the level of C/EBPα and PPAR γ mRNA, but that it does not affect the expression of C/EBPβ and C/EBPδ. In addition, it is well known that C/EBPβ upregulation is a very early event that is required for mitotic clonal expansion to occur [30] and that this event mediates the downstream upregulation of C/EBPα and PPAR γ expression [31]. Collectively, these results suggest that 6-thioinosine downregulates the expression of C/EBPα and PPAR γ through inhibition of the downstream signaling of C/EBPβ.

A recent study conducted by Yanagiya et al. [32] demonstrated that 18-α-glycyrrhetinic acid (AGA) reduced the level of C/EBPα and PPAR γ mRNA, but did not affect the expression of C/EBPβ, which is similar to the effects of 6-thioinosine. In addition, they found that AGA inhibited mitotic clonal expansion by blocking gap-junctional communication, which led to the suppression of adipogenesis. Another report also showed that tumor necrosis factor-α inhibits adipogenesis via a β-catenin/TCF4(TCFL2)-dependent pathway. In this case, even though TNF-α inhibited the expression of C/EBPα and PPAR γ, the expression of C/EBPβ was not altered [33]. Overall, the results of their study suggest a possible inhibitory mechanism of 6-thioinosine that warrants further investigation.

Activation of PPAR γ induces the expression of genes that control adipocyte fatty acid metabolism, including lipoprotein lipase (LPL), fatty acid translocase (CD36), and liver X receptor α (LXRα). In the present study, we demonstrated that 6-thioinosine reduces the expression of PPAR γ target genes during adipogenesis. Therefore, we evaluated this down-regulation to determine if it was related to the observed decrease in the level of PPAR γ mRNA. We found that 6-thioinosine reduced the expression of PPAR γ target genes through inhibition of the

expression and transactivation activity of PPAR γ, which indicates that PPAR γ plays a primary role in anti-adipogenesis induced by 6-thioinosine.

NO is a signaling molecule synthesized by NO synthase (NOS)-mediated oxidation of the terminal guanidine nitrogen atom of free-L-arginine [34]. This short-lived molecule plays a role in a variety of physiological processes, including neurotransmission, vasodilation, and immune response [35]. NO can also prevent or stimulate cell death by apoptosis depending on cell type and dose [36–39]. NO exerts many of these functions through posttranslational modifications of target proteins on cysteine (S-nitrosylation) and tyrosine (nitration) residues [40], and affects signaling pathways via modification of protein–protein interactions [41]. Controversial observations concerning the role of NO in adipocyte differentiation have been reported in several studies, and the production of NO has been shown to promote adipocyte differentiation [42–44]. Similarly, the results of other studies have suggested that NO production may have opposing effects in the process of adipogenesis [21, 27]. In the present study, 6-thioinosine was found to upregulate expression of the iNOS gene through JNK activation, which resulted in the inhibition of adipocyte differentiation. The 6-thioinosine-induced blockade of adipocyte differentiation was also reversed by the inhibition of JNK activation. However, we found that 6-thioinosine has no effect on the expression of eNOS. These results suggest that anti-adipogenesis by 6-thioinosine is mediated by eNOS-independent, iNOS-dependent NO production. Our data also suggest that the controversial role of NO in adipocyte differentiation may be attributed to the source of NO. Namely, NO can be either a pro-adipogenic or anti-adipogenic factor depending on whether it is produced via eNOS or iNOS. This hypothesis is supported by a study conducted by Yan H et al., in which NO produced by eNOS was found to promote adipogenesis [42] and the results of our present study in which iNOS was found to inhibit adipogenesis. Taken together, these findings suggest that iNOS-dependent NO inhibits adipogenesis, while eNOS-dependent NO promotes adipogenesis.

Many attempts have been made to correct the metabolic disparity that occurs in obesity using reagents such as Sibutramine (appetite suppressor), Orlistat (gastrointestinal lipid inhibitor), and Fibrates (PPAR α agonists) [45, 46]. However, the administration of these drugs frequently causes undesirable side effects such as a dry mouth, anorexia, constipation, insomnia, dizziness, and nausea [47]. Therefore, there is a high demand for therapeutically potent, yet safe anti-obesity reagents. Our in vitro experimental data indicate that 6-thioinosine inhibits adipogenesis and the expression of adipokines through the PPAR γ pathway via upregulation of iNOS gene expression, implying that 6-thioinosine may be beneficial for reducing diet-induced obesity via its ability to regulate adipocyte differentiation. However, the data describing the production of TNF α induced by 6-thioinosine suggest that 6-thioinosine exerts an anti-adipogenic effect via the JNK-iNOS signaling pathway through TNF α. This indicates that, similar to TNF α, which is a negative regulator of adipogenesis, 6-thioinosine may contribute to the pathogenesis of cachexia, sepsis, and obesity-associated conditions such as insulin resistance, hyperlipidemia, and type II diabetes [48, 49]. To establish the physiological relevance of 6-thioinosine-induced anti-adipogenesis, further investigation will be required.

Taken together, the results of this study demonstrate that 6-thioinosine inhibits 3T3-L1 adipocyte differentiation by reducing C/EBPα and PPAR γ mRNA levels. Additionally, these results show that the anti-adipogenesis induced by 6-thioinosine is mediated by upregulation of iNOS gene expression through JNK activation.

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