SPANDIDOS PUBLICATIONS

Diallyl disulfide accelerates adipogenesis in 3T3-L1 cells

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Received April 2, 2007; Accepted May 7, 2007

Abstract. Adipocyte differentiation is regulated by the sequential activation of transcription factors such as the CAAT/enhancer binding protein and peroxisome proliferator-activated receptor-γ (PPAR-γ). Several recent studies have shown that regulators of chromatin structure are also involved in adipocyte differentiation. Here we investigated the effects of diallyl disulfide (DADS), an oil-soluble sulfur compound found in processed garlic and an inhibitor of histone deacetylase (HDAC), on adipogenesis. Treatment with DADS accelerated terminal differentiation of 3T3-L1 cells into adipocytes as evidenced by Oil red O staining and cellular triglyceride assay results. Notably, the inhibition of HDAC during the first 2 days was sufficient to stimulate adipogenesis. Western blot analysis revealed that DADS increased the level of acetylated histones H3 and H4. In addition, DADS increased the expression of adipogenesis-related genes; LPL, FAS, SREBP1c, aP2 and PPAR-γ, and decreased the expression of pref-1, a preadipocyte marker gene. Taken together, our results suggest that DADS affects adipocyte differentiation through histone acetylation at an early phase of adipocyte differentiation.

Introduction

Adipocytes are generally considered to be derived from undetermined mesenchymal stem cells (1), and cell culture systems employing preadipocyte cell lines (such as 3T3-L1) have been extensively used to study adipocyte differentiation (2). Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine are inducers well known for their ability to undergo complete differentiation into mature adipocytes (3,4). The early responses to insulin and cAMP include transient induction of CAAT/enhancer binding proteins (C/EBP)ß and C/EBPδ, and sufficient overexpression of C/EBPß to force the early phase of adipocyte differentiation (5). Completion of the differentiation program is accomplished by C/EBPα and peroxisome proliferator-activated receptor-γ (PPAR-γ) (6-8). C/EBPα is required for the activation and maintenance of PPAR-γ expression and the conferment of insulin sensitivity to the mature adipocyte (9). The differentiated cells exhibit many of the morphological and biochemical characteristics of adipocytes found in white adipose tissue. These characteristics include accumulation of triglycerides, insulin-regulated metabolism, and expression of characteristic adipocyte genes such as PPAR-γ, fatty acid synthase (FAS), lipoprotein lipase (LPL), sterol response element binding protein (SREBP), and adipocyte-specific lipid binding protein (aP2) (2,10).

Protein acetylation has been implicated in the regulation of gene expression and protein-protein interactions. Two groups of enzymes, histone acetyltransferase and histone deacetylase (HDAC) modulate the acetylation status of histones and nonhistone proteins (11). Generally, acetylation is associated with active transcription, whereas HDAC is found in repressive complexes of transcription (11,12). HDAC inhibitors such as butyrate and trichostatin A have been shown to have anticancer properties and to stimulate adipogenesis and osteogenesis (12-16).

A compound native to garlic, diallyl disulfide (DADS) is metabolized to S-allylmercaptocysteine, which resembles butyrate, a well-known HDAC inhibitor (Fig. 1) (17). In human colon and HL-60 cells, DADS inhibited HDAC activity and increased the acetylation status of histones (18,19). The present study was designed to determine if DADS had the ability to accelerate differentiation events in 3T3-L1 cells.

Materials and methods

Cell culture. 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml of penicillin in a humidified atmosphere of 5% CO_2/95% air

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Key words: diallyl disulfide, adipocyte, differentiation, histone deacetylase

Table I. Sequences of primers and the probes used in this study.

Gene	Sequence for primers	Accession no.
ß-actin	Forward: 5'-AAGGCCAACCGTGAAAAGAT-3' Reverse: 5'-GTGGTACGACCAGAGGCATAC-3'	NM007393
PPAR-γ	Forward: 5'-GAAAGACAACGGACAAATCACC-3' Reverse: 5'-GGGGGTGATATGTTTGAACTTG-3'	NM011146
aP2	Forward: 5'-AGCCTTTCTCACCTGGAAGA-3' Reverse: 5'-TTGTGGCAAAGCCCACTC-3'	BC054426
SREBP1c	Forward: 5'-GGTTTTGAACGACATCGAAGA-3' Reverse: 5'-CGGGAAGTCACTGTCTTGGT-3'	BC056922
LPL	Forward: 5'-GCTGGTGGGAAATGATGTG-3' Reverse: 5'-TGGACGTTGTCTAGGGGGTA-3'	NM008509
Pref-1	Forward: 5'-CGGGAAATTCTGCGAAATAG-3' Reverse: 5'-TGTGCAGGAGCATTCGTACT-3'	NM010052
FAS	Forward: 5'-TGATGTGGAACACAGCAAGG-3' Reverse: 5'-GGCTGTGGTGACTCTTAGTGATAA-3'	BC046513

at 37˚C. For the 3T3-L1 differentiation experiments, confluent cells were treated with differentiation medium (MDI: DMEM, 10% FBS, 1 µM dexamethasone, 10 µg/ml insulin, and 0.5 mM isobutylmethylxanthine) for 2 days as described previously (20). The medium was then replaced with standard medium containing 5 µg/ml insulin and changed every other day for the following 4 days. Cellular triglyceride (TG) content was assayed using a TG assay kit (Sigma, St. Louis, MO) as described previously (21).

Oil red O staining. Cells were washed twice with PBS and fixed with 10% formaldehyde for 45 min at room temperature. After washing with distilled water twice and 50% isopropanol once, the cells were stained for 1 h at room temperature with filtered Oil red O/60% isopropanol solution. The cells were washed twice with distilled water and twice with PBS. Adipocytes which stained red were observed by light microscopy.

Quantitative real-time PCR. Total RNA was extracted from 3T3-L1 cells using Trizol reagent (Life Technologies, Grand Island, NY). RNA was precipitated with isopropanol, dissolved in DEPC-treated distilled water and kept at -80˚C until used. One µg of RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies). The cDNA was diluted 10-fold prior to PCR amplification. After cDNA synthesis, all PCR reactions were performed using the LightCycler System (Roche Diagnostics Ltd., UK) in a total volume of 20 µl containing 1X LightCycler TaqMan Master mix, 200 nM of each primer, 100 nM probe and 5 µl cDNA. Reaction mixtures were incubated for an initial denaturation at 94˚C for 10 min, followed by 45 PCR cycles. Each cycle consisted of 10 sec at 95˚C and 30 sec at 60˚C. Sequences of each primer and probe set were confirmed by visualization of a single PCR product by agarose gel electrophoresis. Results were expressed relative to the number of ß-actin transcripts used as an internal control. All experiments were performed in triplicate. Each primer was designed by using LightCycler Probe Design Software 2.0 (Roche Diagnostics Ltd.) and each probe was selected from a Mouse probe library (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Primer sequences are shown in Table I.

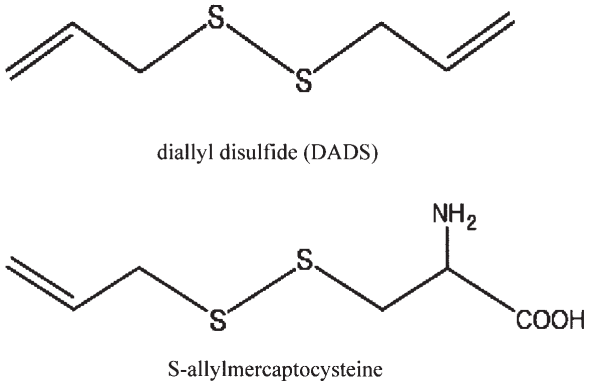

Figure 1. Chemical structures of DADS and S-allylmercaptocysteine.

Western blot analysis. Histone proteins were isolated from 3T3-L1 cells by acid extraction according to the protocol provided by Upstate Biotechnology (Upstate Biotechnology, Lake Placid, NY). The proteins were separated by SDS-PAGE with 15% resolving and 3% acrylamide stacking gels, and transferred to nitrocellulose sheets (Millipore, Bedford, MA) in a Western blot apparatus (Bio-Rad, Hercules, CA). The nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 h with 1 µg/ml of primary antibodies for acetylated histone H3 (Lys^9), histone H3, acetylated histone H4 (Lys^8), or histone H4 (Cell Signaling, Danvers, MA). Horseradish peroxidase-conjugated IgG

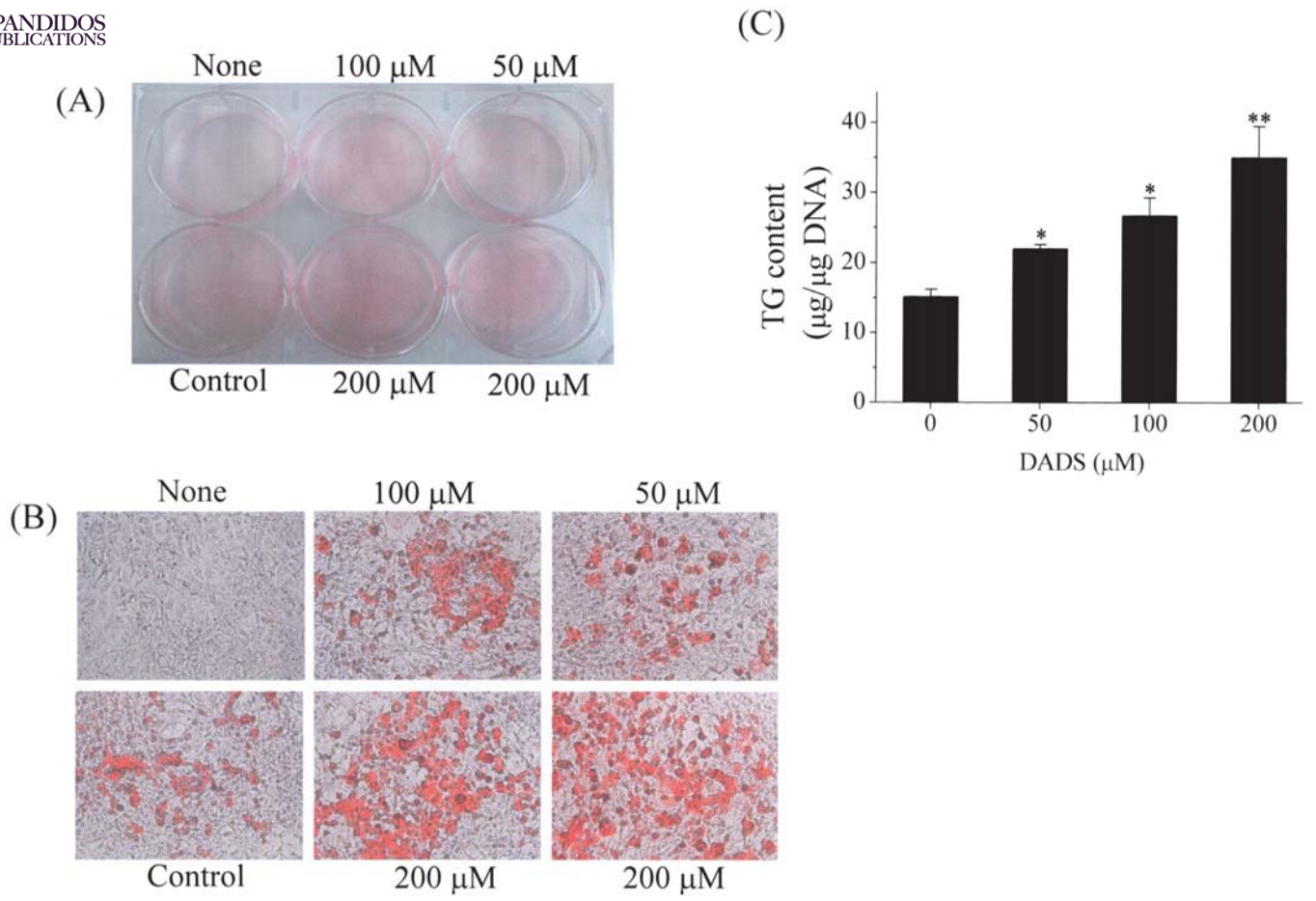

Figure 2. Acceleration of adipocyte differentiation by DADS. 3T3-L1 preadipocytes were grown in the MDI medium for the first 2 days and treated with the indicated concentrations of DADS. Four days after initiation of differentiation, visualization of triacylglycerol levels by Oil red O staining (A) and high magnification (x200) of cells (B) was conducted. (C) Cells were harvested and the TG contents were measured. Each value was expressed as the mean ± SEM of 4 independent experiments. *P<0.05 and **P<0.01 vs. the control.

(Zymed, San Francisco, CA) was used as secondary antibody. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad).

Statistical analysis. Statistical analysis of the data was performed by ANOVA and Duncan's test. Differences of P<0.05 were considered statistically significant.

Results

Effects of DADS on adipocyte differentiation of 3T3-L1 cells. We first examined the effects of DADS on adipocyte differentiation. Two days after incubation with the MDI differentiation mix, 3T3-L1 cells treated with DADS accumulated significant amounts of lipids compared to the vehicle-treated group. An accelerating effect on adipocyte differentiation was evident with as little as 50 μM DADS and differentiation increased in a manner directly related to DADS concentration as demonstrated by Oil red O staining and measurement of TG (Fig. 2). Next, we treated the 3T3-L1 cells with different time-based protocols to determine the critical periods at which DADS causes increased accumulation of lipids. As shown in Fig. 3, treatment with 100 μM DADS for the first 2 days of differentiation induction was sufficient to accelerate MDI-induced adipocyte differentiation and lipid accumulation. However, treatment of 3T3-L1 cells with DADS subsequent to the initial 2 days of exposure to MDI showed little effect, suggesting that DADS enhances the adipocyte differentiation itself, and not during the later lipid accumulation stage.

Effects of DADS on the expression of adipocyte differentiation markers. We further investigated the effects of DADS on the expression of adipocyte markers by quantitative real-time PCR. We compared the expression profiles of 3T3-L1 cells treated with DADS with those of the control cells. In the DADS-treated cells, PPAR-γ, aP2, LPL, and SREBP1c mRNA were several folds higher than the controls, whereas FAS mRNA was only slightly increased (Fig. 4). Preadipocyte factor-1 (pref-1), a preadipocyte marker that normally disappears during adipocyte maturation (22), declined significantly compared to the control (Fig. 4).

Increases in histone acetylation in DADS-treated 3T3-L1 cells. Chromatin modifications by histone acetylation and deacetylation are crucial for specific gene expression and thereby crucial in regulating adipocyte differentiation (16).

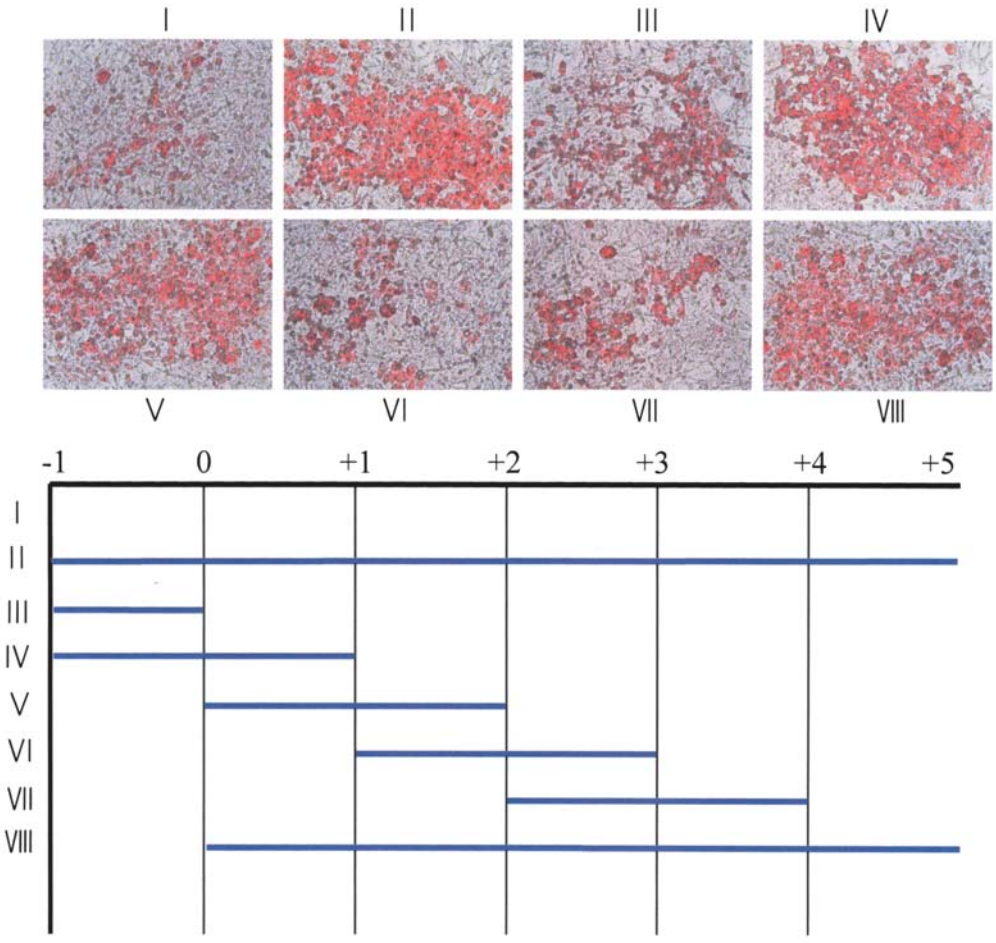

Figure 3. Time-dependent induction of adipocyte differentiation by DADS. Differentiating 3T3-L1 preadipocytes were treated with 100 μM DADS for various time periods and stained with Oil red O. Bars indicate the duration of DADS treatment. Representative data from three separate experiments are shown.

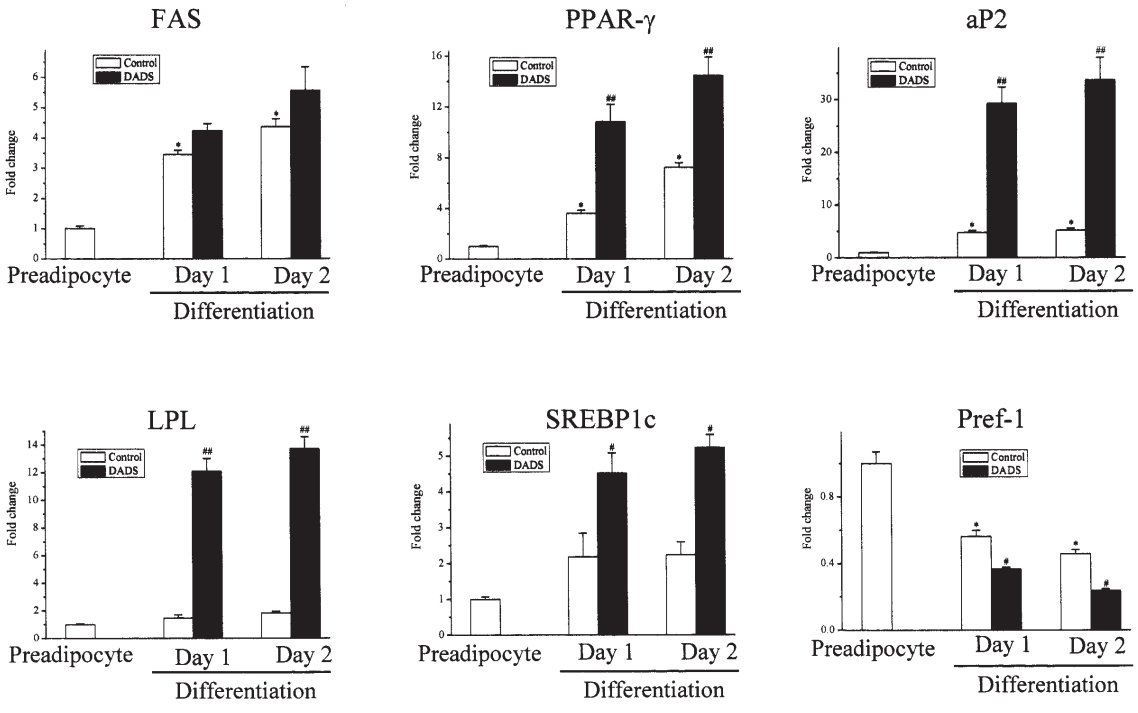

Figure 4. Real-time PCR analysis of adipocyte differentiation marker genes. Total RNA from 3T3-L1 cells incubated in the absence or presence of 100 μM DADS was isolated at the indicated time points after induction of differentiation, and real-time PCR analysis was performed. Each value was expressed as the mean ± SEM of three independent experiments. *P<0.05 vs. preadipocytes; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the control.

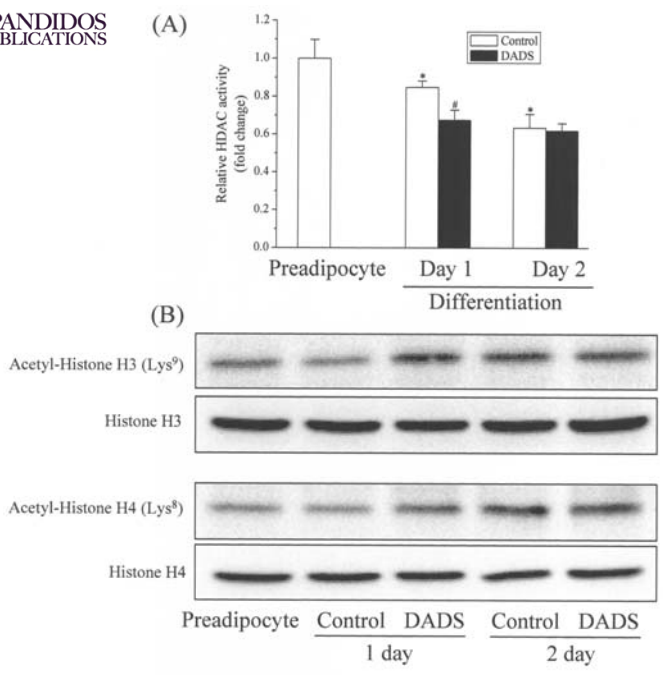

Figure 5. Changes in overall histone acetylation and HDAC activity during adipocyte differentiation. (A) Total nuclear HDAC enzyme activity of 3T3-L1 preadipocytes/adipocytes. (B) Changes of histone modification during adipocyte differentiation. Each value was expressed as the mean ± SEM of 4 independent experiments. *P<0.05 vs. preadipocytes; #P<0.05 and ##P<0.01 vs. the control.

We measured the HDAC enzyme activity in DADS-treated 3T3-L1 cells. HDAC activities were reduced to 85.7±4.6 and 67.7±9.3% by the 1st and 2nd days after MDI induction, respectively. Addition of DADS decreased the HDAC activity significantly compared with the control cells; 63.7±12.9 and 62.0±7.2% at the 1st and 2nd days, respectively (Fig. 5A); however, the difference was not significant at day 2. We next examined the effects of DADS on the acetylation status of histones in the same cell extracts (Fig. 5B). As expected, a significant increase of acetylated histone H3 and H4 was observed 2 days after adipogenesis induction. However, the presence of DADS increased the acetylation of H3 and H4 even at the 1st day of induction, which was correlated with lowered HDAC activities.

Discussion

DADS, an oil-soluble sulfur compound in processed garlic is known to inhibit the proliferation of human colon, lung, and leukemia cells (23-25). Several mechanisms have been suggested for the anticancer effect of DADS including induction of apoptosis and increased acetylation of histones (18,19,23).

Computer modeling studies suggest that dietary HDAC inhibitors have common structural features appropriate for HDAC inhibition; either (i) a functional group that interacts with the buried zinc atom, or (ii) a spacer or linker arm that fits within the HDAC pocket (26). DADS is metabolized to S-allylmercaptocysteine (Fig. 1), which has a spacer ending with a carboxylic acid-functioning group and inhibits HDAC enzyme activity (17).

The association of histone acetylation with transactivation and deacetylation with repression was first suggested in 1964 by Allfrey *et al* (27). Many studies subsequently supported the correlation between histone acetylation and active gene transcription (12,14,28). The role of HDAC during adipocyte differentiation remains largely unknown compared with its role in the differentiation of other cell types such as osteoblasts (13,15). Recently, HDAC inhibitors have been proven to have an ability to regulate adipogenesis. Fajas *et al* showed that treatment with HDAC inhibitors resulted in dissociation of PPAR-γ, a master adipogenic transcription factor from the HDAC3 complex (29). PPAR-γ interacts with coactivators and corepressors such as HDAC and regulates the expression of its target genes (30). By modifying chromatin structure, an HDAC inhibitor can transactivate PPAR-γ target genes required for differentiation and adipogenesis. Two other research groups obtained similar results by demonstrating the importance of HDAC1 in an adipogenic program (16,31). In contrast, Lagace and Nachtigal demonstrated opposite results by showing an inhibitory effect of valproic acid, an HDAC inhibitor, on adipocyte differentiation (32). In the current study, we used DADS as a different kind of HDAC inhibitor present in a naturally occurring product. To our knowledge, the effects of DADS on adipocyte differentiation have not yet been reported. Treatment of 3T3-L1 cells with DADS accelerated differentiation of 3T3-L1 preadipocytes into adipocytes in a dose-dependent manner. The results of the TG measurement also suggested that DADS increased adipogenesis, therefore, our data support a positive relationship between HDAC1 inhibition and adipocyte differentiation.

3T3-L1 cells treated with DADS for the first 2 days accelerated MDI-induced adipocyte differentiation; but cells treated with DADS at a later stage were not different from the control cells. Total HDAC enzymatic activity was significantly decreased and accompanied by hyperacetylation of histones H3 and H4. In addition, we found that DADS enhanced adipogenic marker gene expression in 3T3-L1 cells, suggesting that modulation of endogenous HDAC enzymatic activity is important for the execution of the adipogenic program. Together, these results indicate that DADS affects adipocyte differentiation through histone acetylation at an early phase of adipocyte differentiation.

Maintaining the balance of acetylation and deacetylation of histones has tremendous importance for regulating transcription and cellular differentiation. Several short-chain fatty acid HDAC inhibitors, such as phenylbutyrate and valproic acid, are under phase I and II clinical trials to examine their therapeutic effects on hematological and solid tumors (33). In recent years, there has been growing interest in applying naturally occurring phytochemical-based compounds to medicine because they are relatively non-toxic, inexpensive and available as oral forms. Adipose tissue has emerged as a focus area for tissue engineering, encouraged by the increase in reconstructive, cosmetic and correctional surgery. Tissue engineering involves the wide spectrum of areas such as cell culture, differentiation, angiogenesis and polymer chemistry to regenerate adipose tissue *de novo* (34,35). The potential of DADS as an accelerator of adipogenesis might, therefore, confer a beneficial role in reconstructive surgery.

Acknowledgements

This study was supported by the Korean Research Foundation Grant (no. KRF-2005-042-E00005).

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