

Polyamine metabolism is involved in adipogenesis of 3T3-L1 cells

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Abstract Polyamines spermidine and spermine are known to be required for mammalian cell proliferation and for embryonic development. Alpha-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC) a limiting enzyme of polyamine biosynthesis, depleted the cellular polyamines and prevented triglyceride accumulation and differentiation in 3T3-L1 cells. In this study, to explore the function of polyamines in adipogenesis, we examined the effect of polyamine biosynthesis inhibitors on adipocyte differentiation and lipid accumulation of 3T3-L1 cells. The spermidine synthase inhibitor *trans*-4-methylcyclohexylamine (MCHA) increased spermine/spermidine ratios, whereas the spermine synthase inhibitor *N*-(3-aminopropyl)-cyclohexylamine (APCHA) decreased the ratios in the cells. MCHA was found to decrease lipid accumulation and GPDH activity during differentiation, while APCHA increased lipid accumulation and GPDH activity indicating the enhancement of differentiation.

The polyamine-acetylating enzyme, spermidine/spermine *N*¹-acetyltransferase (SSAT) activity was increased within a few hours after stimulus for differentiation, and was found to be elevated by APCHA. In mature adipocytes APCHA decreased lipid accumulation while MCHA had the opposite effect. An acetylpolyamine oxidase and spermine oxidase inhibitor MDL72527 or an antioxidant *N*-acetylcysteine prevented the promoting effect of APCHA on adipogenesis. These results suggest that not only spermine/spermidine ratios but also polyamine catabolic enzyme activity may contribute to adipogenesis.

Keywords Adipogenesis · Polyamine metabolism · Spermine · Spermidine · Polyamine oxidation · 3T3-L1

Abbreviations

AcPAO	Acetylpolyamine oxidase
APCHA	<i>N</i> -(3-aminopropyl)-cyclohexylamine
CS	Calf serum
DEX	Dexamethasone
DFMO	α -Difluoromethylornithine
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
GPDH	Glycerol-3-phosphate dehydrogenase
Gy	Gyro
IBMX	Isobutylmethylxanthine
INS	Insulin
MAO	Monoamine oxidase
MCHA	<i>trans</i> -4-Methylcyclohexylamine
MDL72527	<i>N,N</i> ¹ -Bis(2,3-butadienyl)-1,4-butanediamine
NAC	<i>N</i> -acetylcysteine
ODC	Ornithine decarboxylase
SSAO	Semicarbazide-sensitive amine oxidase
SSAT	Spermidine/spermine <i>N</i> ¹ -acetyltransferase
SMO	Spermine oxidase

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Introduction

The ubiquitous polycationic polyamines spermidine and spermine are known to be required for embryonic development and for the maintenance of mammalian cell proliferation (Heby 1981; Pegg et al. 1982; Agostinelli et al. 2009). Male Gyro (Gy) mice with profound spermine deficiency due to the extensive disruption of both phosphate regulating endopeptidase and spermine synthase gene (Meyer et al. 1998), exhibit the features of smaller size and lower weight than normal mice, whereas spermine synthase overexpressing mice are slightly larger than normal mice (Wang et al. 2004). Mutations in the spermine synthase gene are reported to cause Snyder-Robinson syndrome, an X linked mental retardation disorder condition accompanied by thin habitus and skeletal defects such as osteoporosis (Cason et al. 2003). The polyamine-acetylating enzyme, spermidine/spermine N^1 -acetyltransferase (SSAT), participates in polyamine homeostasis by regulating polyamine export and catabolism. SSAT transgenic mice characteristically lack hair, and have a lean body mass. In contrast, SSAT knock-out mice tend to accumulate fat in their bodies (Jell et al. 2007), suggesting that altered SSAT expression can affect fat metabolism. Furthermore, both spermine and spermidine at physiological concentrations, have been shown to inhibit lipolysis by suppressing cyclic AMP levels and to facilitate glucose transport, which is accompanied by up-regulated conversion of glucose into triacylglycerols in adipocytes of Zucker obese rats (Lockwood et al. 1974; Jamdar et al. 1996).

It has also been suggested that in some cases the polyamines may play an essential role in cellular differentiation (Pegg and McCann 1982; Erwin et al. 1983). The process of adipogenesis or the formation of adipose tissue has become better understood by the study of several cell types such as 3T3-L1 that can be induced to undergo differentiation into adipocytes. Alpha-difluoromethylornithine (DFMO), which is an irreversible inhibitor of ornithine decarboxylase (ODC) (Metcalfe et al. 1977), the enzyme producing putrescine and a limiting step in polyamine biosynthesis, prevented the differentiation of 3T3-L1 fibroblasts into adipocytes (Bethell et al. 1981). In 3T3-L1 cells stimulation with insulin (INS) and isobutylmethylxanthine (IBMX) increased twofold putrescine, threefold spermidine within 48 h, and spermine slightly (Erwin et al. 1984). The cells stimulated with INS and IBMX had significantly higher activity of ODC than not stimulated, but the activities of both spermidine synthase and spermine synthase were not altered. Therefore, it appears that the elevations of polyamine contents in the differentiating cells might be not only due to changes in the biosynthetic enzymes but also due to the degradation and/or excretion of these polyamines affected by the stimulation medium

(Erwin et al. 1984). Spermidine is required in 3T3-L1 differentiation, shown by using methylspermidine as spermidine analogues (Vuohelainen et al. 2010). However, the specific roles of individual polyamines during adipogenesis have remained unclear.

trans-4-Methylcyclohexylamine (MCHA) and *N*-(3-aminopropyl)-cyclohexylamine (APCHA) selectively and potently inhibited the activities of spermidine synthase and spermine synthase, respectively. Previous studies showed that each inhibitor markedly decreases cellular spermidine and spermine, respectively, both in rat tissues when they were orally administered to rats and after treatment of HTC cells (Shirahata et al. 1993; Beppu et al. 1995).

In this study, to explore the possible function of spermidine and spermine in adipogenesis, we describe the effect of MCHA and APCHA on lipid accumulation and their opposite effects on 3T3-L1 lipid accumulation during differentiation and maturation. Moreover, we also demonstrate that polyamine catabolism may be involved in lipid metabolism.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), calf serum (CS), penicillin and streptomycin were purchased from Invitrogen (NY, USA). Fetal bovine serum (FBS) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). Dexamethasone (DEX), IBMX, INS and all other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). MCHA, APCHA, *N,N*¹-bis(2,3-butadienyl)-1,4-butanediamine (MDL72527) and *N*¹-dansylspermine were prepared in this laboratory (Shirahata et al. 1988, 1991; Bey et al. 1985; Takao et al. 2008). *N*-acetylcysteine (NAC) was purchased from Sigma (MO, USA).

Cell culture and differentiation

Mouse fibroblast line 3T3-L1 preadipocytes were maintained in DMEM, 50 U/ml penicillin and 50 µg/ml streptomycin, 3.7 mg/ml NaHCO₃, and 10% CS, with a replacement of media every other day. To induce differentiation, 3T3-L1 preadipocytes were cultured in differentiation medium containing DMEM and 10% FBS. After confluence, cells were induced to differentiate (designated day 0) by addition of 1 µM DEX, 0.5 mM IBMX for the first 48 h. From day 2 to day 4, the medium was supplemented with 10 µg/ml INS after which the cells were fed every other day with DMEM containing 10% FBS from day 4 to day 11. In order to examine the effect of

polyamines on adipocyte differentiation process, the cells were pretreated 3 days prior to treatment with differentiation medium with the spermidine synthase inhibitor MCHA and spermine synthase inhibitor APCHA. This pretreatment was needed in order to change the cellular polyamine contents efficiently. In order to investigate the effect of polyamines on lipid accumulation in mature 3T3-L1 cells, the cells were treated with MCHA and APCHA from day 6. The inhibitor concentration was set up to 100 μM , which did not have any influence on cell growth.

Oil Red O staining

The cells were fixed in 10% formaldehyde in PBS at room temperature for 30 min, and washed with PBS and stained with filtered Oil Red O solution at room temperature for 5 min. Oil Red O solution was prepared to be dissolved at concentration of 60% in isopropyl alcohol and to be diluted with an equal volume of water freshly at every experiment. After washing with PBS, the stained triglyceride droplets were photographed.

Measurement of triglyceride contents and glycerol-3-phosphate dehydrogenase activity

Cells at the designated stage of induction of differentiation were washed twice with PBS and lysed by brief sonication. The triglyceride levels in the lysates were quantified using the Triglyceride E Test WAKO kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's protocol. Glycerol-3-phosphate dehydrogenase (GPDH) activity was assayed by recording the initial rate of oxidation of NADH at 340 nm at 30°C (Wise et al. 1979). The cells were washed twice with PBS, and removed by scraping into enzyme extraction buffer (50 mM Tris-HCl buffer pH 7.5 at 30°C, 1 mM EDTA and 1 mM β -mercaptoethanol). After centrifugation at 9,200 $\times g$ for 5 min at 4°C, GPDH specific activity in the supernatant was measured using a GPDH Assay Kit (Takara Bio. Inc., Shiga, Japan) as an estimation for adipogenesis.

Determination for SSAT activity

SSAT enzyme activity was assayed as described previously (Takao et al. 2008). Cells were washed twice with PBS and removed by scraping into SSAT extraction buffer (1 mM EDTA, 10 mM Tris-HCl pH 7.0 at 4°C and 2.5 mM DTT) and lysed by brief sonication. The homogenate was centrifuged 15,600 $\times g$ for 20 min at 4°C. Aliquots of the supernatant containing the same amounts (50 μg) of protein were mixed with 0.1 M Tris-HCl pH 7.8, 50 μM acetyl-CoA and 40 μM N^1 -dansylnormospermine for the assay. After incubation at 37°C for 10 min, TCA was

added immediately to stop the reaction, and the mixture centrifuged 15,600 $\times g$ for 20 min at 4°C. An aliquot of the supernatants was injected onto fluorescence-detective HPLC (Takao et al. 2008).

Polyamine analysis

Cells were washed twice with PBS and polyamines were extracted in 10% trichloroacetic acid. The extracts were directly injected onto the OPA-postcolumn ion-exchange HPLC system (Shirahata et al. 1993).

Statistical analysis

Results were expressed as mean \pm SE, and compared using Student's *t* test. $p < 0.05$ was considered statistically significant.

Results and discussion

Changes in polyamine contents and SSAT activity during 3T3-L1 adipose conversion

3T3-L1 cells were stimulated to differentiate into adipocytes by the addition of IBMX, DEX, and INS. This

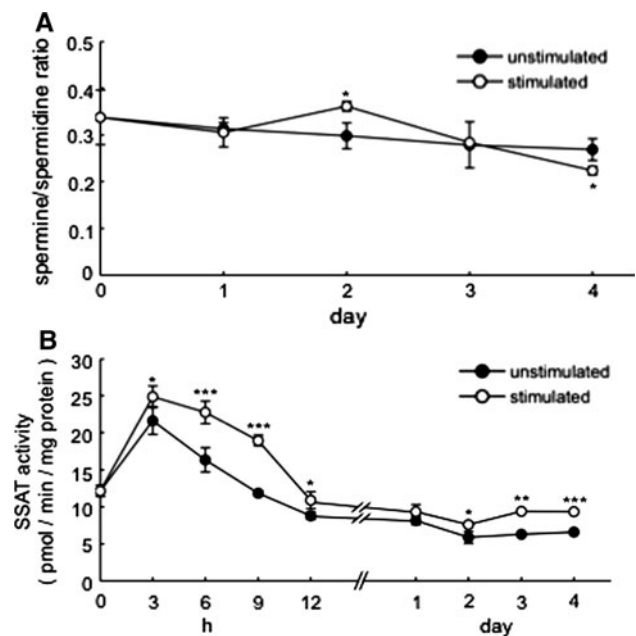


Fig. 1 Changes in spermine/spermidine ratios and SSAT activity of 3T3-L1 during differentiation with IBMX, DEX and INS. Spermine/spermidine ratios (a) and SSAT activity (b) in 3T3-L1 cells at indicated times from day 0 to day 4 were measured. Results shown for control cells stimulated with IBMX, DEX and INS (open circles) and blank cells without the stimulus (filled circles) at times shown after start of stimulation. Results represent the mean \pm SE. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with control

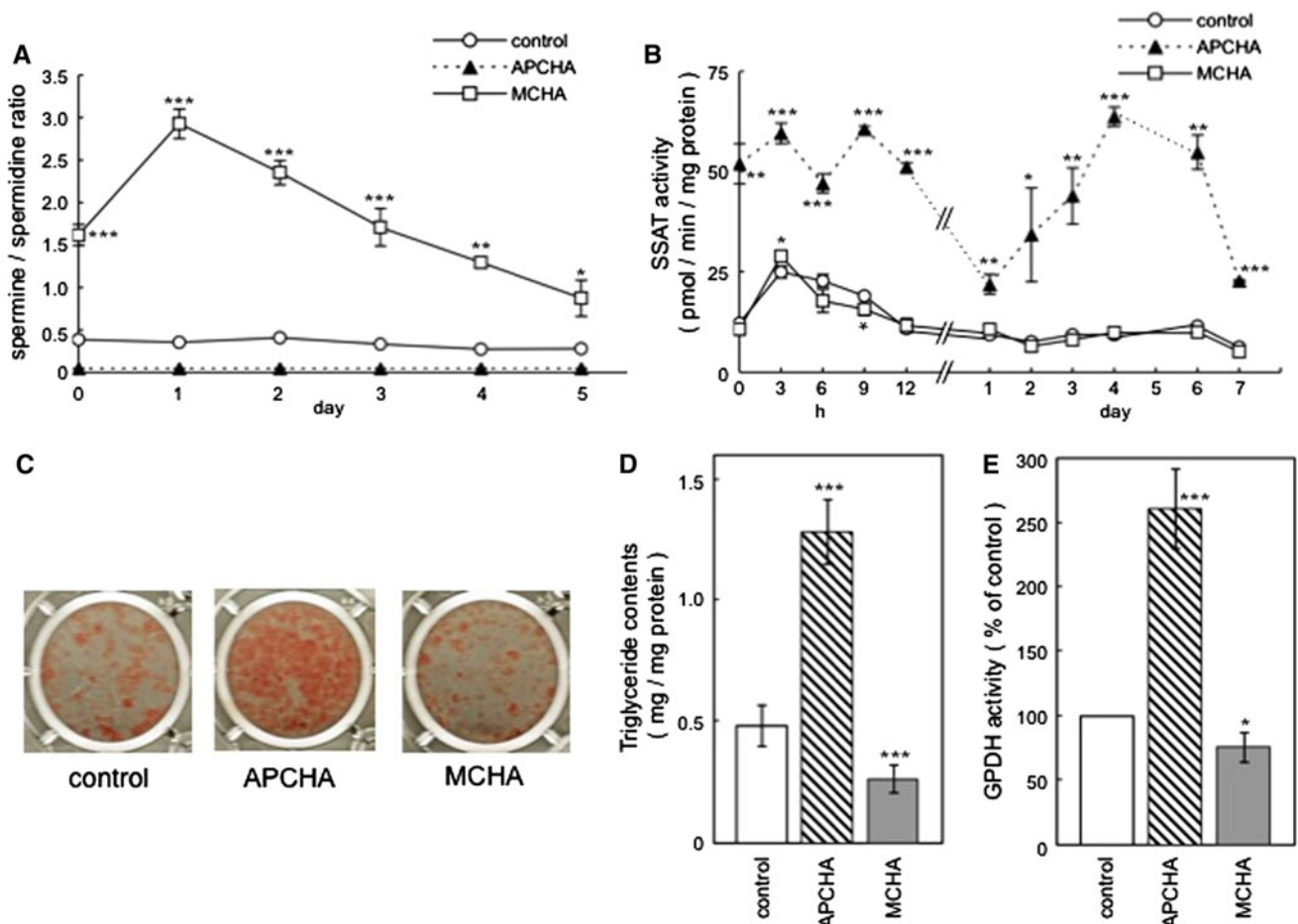


Fig. 2 The effect of APCHA or MCHA on adipocyte differentiation. Spermine/spermidine ratios (**a**) and SSAT activity (**b**) in 3T3-L1 cells treated with MCHA or APCHA for day-3 to day-11 according to “Materials and methods”, were measured during differentiation. Results shown for control cells (open circles with solid line), MCHA-treated cells (open squares with solid line) and APCHA-treated cells

(filled triangles with dotted line). **c** Cells were stained with Oil Red O at day 10 after the stimulus. **d** Triglyceride contents were measured at day 11. **e** GPDH activity was measured at day 7. Results represent the mean \pm SE. *** p < 0.001, ** p < 0.01, * p < 0.05 compared with control

mixture, which is frequently used to induce differentiation, had never been tested for study on polyamine to our knowledge. The adipocyte differentiation status was estimated by staining with Oil Red O and quantification for triglyceride. The spermine/spermidine ratios were found to increase slightly at day 2, and then decreased at day 4 after the treatment with the mixture of the inducers (Fig. 1a). To test whether the polyamine metabolism could be involved in the changes in cellular polyamine ratios after stimulation, spermidine/spermine- N^1 -acetyltransferase (SSAT) activity, which is one of major polyamine metabolic enzymes, inducible in many normal and disease processes (Min et al. 2002), was measured during 3T3-L1 adipogenesis. The activity of SSAT in stimulated cells taken as a control was found to be significantly higher than that in unstimulated cells taken as a blank. The SSAT activity of the control transiently peaked at 3 h and was 1.9-fold that of the blank cells at 9 h after stimulus for differentiation

(Fig. 1b). The results indicate that both spermine/spermidine ratios and activity of its metabolic enzymes could be involved in adipogenesis.

Effects of MCHA or APCHA on polyamine contents, SSAT activity and differentiation of 3T3-L1 cells

There is no report at present on studies of the effect of cellular spermine/spermidine ratios on differentiation of 3T3-L1 cells using spermidine synthase inhibitor MCHA and spermine synthase inhibitor APCHA. Addition of 100 μ M MCHA or APCHA, which had no effect on cell growth (data not shown), was used to study the functions of polyamines by treatment starting from 3 days prior to induction for differentiation. MCHA maintained a high spermine/spermidine ratio during differentiation in 3T3-L1 cells (Fig. 2a). Little difference was seen in the SSAT activity between in MCHA-treated cells and control

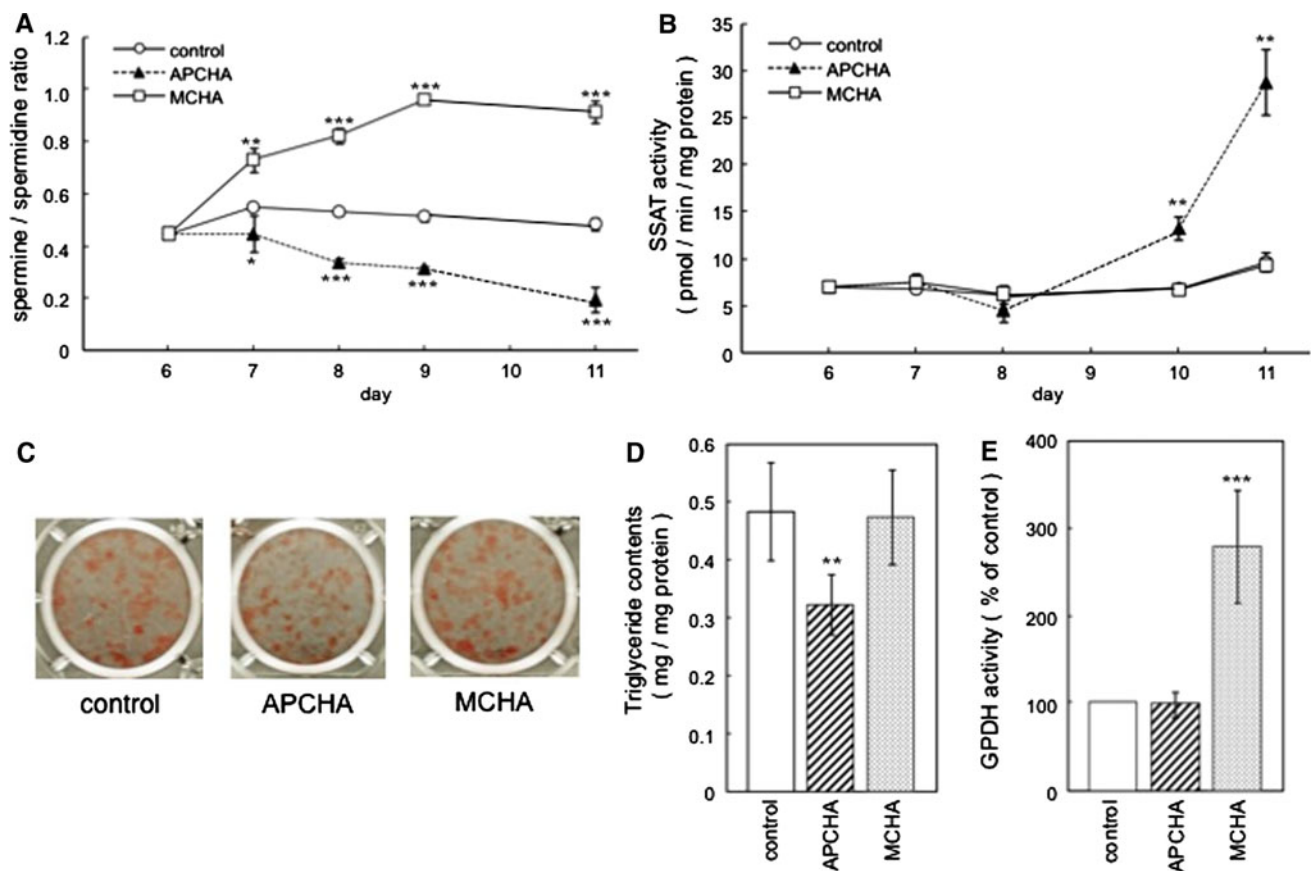


Fig. 3 The effect of MCHA or APCHA on lipid accumulation in mature adipocytes. The differentiated cells were treated with MCHA or APCHA from day 6. Results shown for control cells (open circles with solid line), MCHA-treated cells (open squares with solid line) and APCHA-treated cells (filled triangles with dotted line). Spermine/spermidine ratios (a) and SSAT activity (b) were measured at

indicated times from day 6 to day 11. c The effect of inhibitors on lipid accumulation. Cells were fixed and stained with Oil Red O at day 10. d Triglyceride contents at day 11 and e the activity of GPDH at day 14 were measured. Results represent the mean \pm SE. *** p < 0.001, ** p < 0.01, * p < 0.05 compared with control

(Fig. 2b). In contrast to MCHA, APCHA decreased spermine/spermidine ratios in 3T3-L1 cells significantly (Fig. 2a). SSAT activity in APCHA-treated cells was significantly enhanced during the adipocyte differentiation by 2.4-, 3.2- and 6.8-fold than that in control at 3 h, 9 h and day 4, respectively (Fig. 2b).

The effect of the inhibitors on adipocyte differentiation was investigated by Oil Red O staining for lipid drops and by the measurement of intracellular triglyceride contents. MCHA was found to decrease Oil Red O staining (Fig. 2c). Both triglyceride contents and GPDH activity in MCHA-treated cells were lower (0.5-fold and 0.7-fold, respectively) than those in control, respectively (Fig. 2d, e). On the other hand, APCHA significantly increased Oil Red O staining (Fig. 2c), and also increased both triglyceride contents and GPDH activity by 2.7-fold and by 1.7-fold, respectively (Fig. 2d, e). In mouse preadipocyte Ob1771 cells, the decrease in cellular spermine/spermidine ratios by treatment with putrescine or spermidine and exposure to growth hormone enhanced GPDH activity (Amri et al.

1986a, b). The elevated GPDH activity could therefore be explainable by decrease in cellular spermine/spermidine ratios by APCHA. The decline in GPDH activity by MCHA-treatment is consistent with an increase in the ratios.

In our previous study, to research the effect of MCHA and APCHA on enzyme activity on rat hepatoma cells (HTC cells), no significant changes in the SSAT activities occurred during the treatment (Beppu et al. 1995). The results shown in this paper illustrate that the alternation of spermine/spermidine ratios and the SSAT enzyme activity could be involved in adipocyte differentiation.

Effects of MCHA or APCHA on lipid accumulation in mature adipocytes

In order to examine the effect of the inhibitors on lipid accumulation in the mature stage, the differentiated cells were treated with 100 μ M MCHA or APCHA from day 6. Spermine/spermidine ratios were gradually increased by

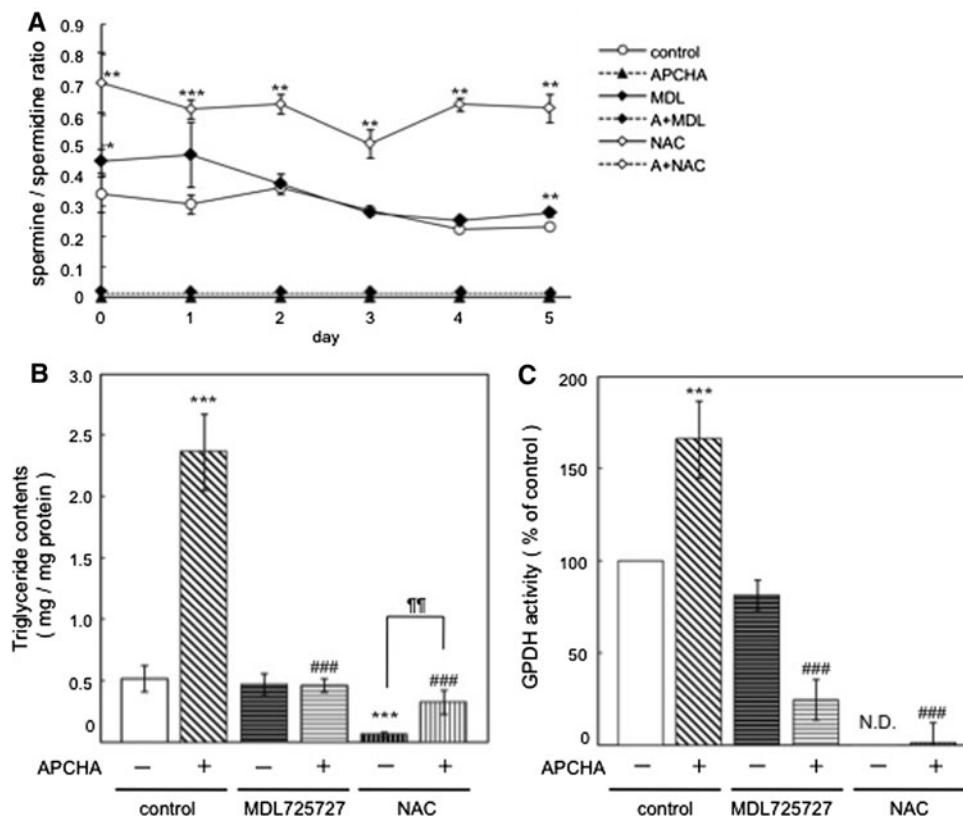


Fig. 4 The effect of MDL72527 and NAC on APCHA-enhanced differentiation of 3T3-L1 cells. Cells were treated with MDL72527 (5 μ M) in the presence or absence of APCHA. Cells were pretreated with NAC (5 mM) 1 day prior to induction for differentiation. **a** spermine/spermidine ratios, **b** triglyceride contents at day 11 and **c** GPDH activity at day 8 were analyzed after stimulus for differentiation. Results shown for control cells (open circles with solid line), treated with APCHA (filled triangles with dotted line) and

MDL72527 (filled diamonds with solid line), MDL72527 with APCHA (filled diamonds with dotted line), NAC (open diamonds with solid line) and NAC with APCHA (open diamonds with dotted line). Results represent the mean \pm SE. *** p < 0.001, ** p < 0.01, * p < 0.05 compared with control. ### p < 0.001, ## p < 0.01, # p < 0.05 compared with APCHA-treated cells. ^{††} p < 0.01 compared with NAC-treated cells. ND not detectable

MCHA, and decreased by APCHA at 24 h after treatment (Fig. 3a) because polyamine synthesis was affected by treatment of MCHA or APCHA even in mature cells with depression of cellular proliferation. APCHA elevated SSAT activity significantly up to 3.0-fold higher than control at day 11. In contrast to APCHA's effect, SSAT activities in MCHA-treated cells were similar to those in control (Fig. 3b).

MCHA slightly increased Oil Red O staining, and had no effect on triglyceride contents (Fig. 3c, d). The GPDH activity was increased up to 2.8-fold by MCHA in comparison with control (Fig. 3e). APCHA was found to decrease both Oil Red O staining and triglyceride contents by 33% in mature adipocytes (Fig. 3c, d), although APCHA did not change GPDH activity (Fig. 3e).

MCHA increased and APCHA decreased lipid accumulation in mature adipocytes whereas MCHA decreased and APCHA increased lipid accumulation in preadipocytes, which interestingly appeared to demonstrate an opposite effect of the two polyamine synthesis inhibitors

on adipogenesis in differentiating and mature adipocytes. The above results suggest that not only spermine/spermidine ratios but also polyamine-metabolizing enzymes leading to changes in spermine/spermidine ratios could be involved in adipogenesis at each stage, and might indicate an additional role of polyamine in a cellular model of adipogenesis.

MDL72527 and NAC inhibit the effect of APCHA-induced lipid accumulation

Mammalian polyamine catabolism is a recycling pathway that converts spermine to spermidine and spermidine to putrescine with the production of H_2O_2 and acetamido-propanal or aminopropanal, via the concerted action of SSAT and acetyl polyamine oxidase (AcPAO) or via spermine oxidase (SMO) without the need of the acetylation step, respectively. Considering the above results on the changes in SSAT activity during adipogenesis, especially in the presence of APCHA, we examined the influence of

the polyamine catabolic enzyme inhibitor MDL72527, which inhibits both AcPAO and SMO activity, to study adipogenesis promotion by APCA.

MDL72527 did not affect cell growth (data not shown) or spermine/spermidine ratios by itself (Fig. 4a). MDL72527 prevented an APCA-promoted effect on cellular triglyceride content (Fig. 4b), and GPDH activity (Fig. 4c). NAC, a scavenger for reactive oxygen species and a precursor for GSH reducing the adipogenic action by the amines (Faruqi et al. 1997), was found to increase spermine/spermidine ratios by 2.3-fold during adipogenesis (Fig. 4a). NAC completely inhibited triglyceride production (Fig. 4b) and APCA-upregulated GPDH activity (Fig. 4c). Several oxidative products such as an aldehyde and H₂O₂ are known to be involved in adipocytes differentiation in the insulin signaling system (Mahadev et al. 2001; Lin et al. 2006; Kim et al. 2006). The results suggest that the oxidative stress, produced from enhanced polyamine catabolism by APCA in addition to the change in spermine/spermidine ratios, could have a function similar to the insulin signal (Fontana et al. 2001). NAC itself fully suppressed adipocyte differentiation in control cells, but not completely in APCA-treated cells probably due to the insufficient inhibition of oxidants produced by APCA-treatment.

Amines such as tyramine or benzylamine can mimic or reinforce several of the insulin effects on adipocyte differentiation in 3T3-F442A and 3T3-L1 cells mainly by generating H₂O₂ produced by amine oxidases like semicarbazide-sensitive amine oxidase (SSAO) or monoamine oxidase (MAO) (Fontana et al. 2001; Mercier et al. 2001; Carpené et al. 2006). This fact was suggested by the suppression in differentiation obtained by using their inhibitors and antioxidants. Our results do not exclude the possibility that the aldehydes and H₂O₂ generated via polyamine oxidation by AcPAO or SMO could play a part of roles in adipogenesis.

In this study, we have shown that both spermine/spermidine ratios and activity of its metabolic enzymes could be involved in adipogenesis although there are issues to be resolved in process of adipogenesis. We found that MCHA and APCA have their own specific effects on both pre-adipocytes and mature adipocytes with the changes in SSAT activity, shown prominently in the APCA-treated cells. These findings also suggest that the control of polyamine metabolic enzyme activity could regulate adipogenesis and that the inhibitors are valuable tools for research on lipid metabolism.

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