Inhibition of ASCT2 is essential in all-trans retinoic acid-induced reduction of adipogenesis in 3T3-L1 cells

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A R T I C L E   I N F O

Article history:
Received 26 March 2015
Revised 21 May 2015
Accepted 28 June 2015

Keywords:
Adipocyte
All-trans retinoic acid
Asct2
L-glutamine
Obesity

A B S T R A C T

Vitamin A has preventive effects on obesity. All-trans retinoic acid (ATRA), the active form of vitamin A, inhibits lipid accumulation in 3T3-L1 cells in an experimental adipogenesis model. We found that ATRA suppressed up-regulation of the amino acid transporter, Asct2, in adipogenating 3T3-L1 cells. We observed that Asct2 was up-regulated at 1 day after adipogenesis stimuli. The Asct2 inhibitor L-γ-glutamyl-p-nitroanilide (GPNA) decreased lipid accumulation. Glutamine-free conditions also suppressed adipogenesis. Suppression of adipogenesis by ATRA may be through Asct2 reduction. These results indicate that Asct2 could be a target for obesity prevention and treatment.

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1. Introduction

Obesity is the leading risk for the development of type 2 diabetes mellitus, hypertension and cardiovascular disease. Experiments have shown that molecular mechanisms of adipogenesis form a link between obesity and insulin resistance [1]. Vitamin A is an important nutrient that has multiple biological functions that affect vision, embryonic development, reproduction, and immune function [2–4]. The bioactive form of vitamin A is all-trans retinoic acid (ATRA); a nutrient derivative with many remarkable effects on adipocyte biology affecting preadipocyte survival, and adipogenesis in preadipocyte clonal cell lines [5,6]. ATRA treatment reduces body weight and adiposity [7,8]. ATRA is a specific ligand for retinoic acid receptors (RARs), and in part, it suppresses adipogenesis through activation of RARs. Several reports have indicated that various adipogenic signaling pathways are involved in the ATRA-dependent regulation of adipogenesis [6–12].

The mouse preadipocyte cell line, 3T3-L1 provides a model for differentiation process associated with adipogenesis, including activation of the adipogenic signaling pathway. The temporal expression pattern of important adipogenic factors is regulated by specific adipogenous transcription factors [13]. In particular, peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor that up-regulates adipocyte-specific genes involved in lipid synthesis, and insulin signaling. It also regulates adipokine production for terminal differentiation, and PPARγ agonists, including pioglitazone, promote 3T3-L1 cell differentiation to mature adipocytes [14,15].

Asct2 (SLC1A5) is a neural amino acid transporter belonging to the solute carrier 1 family. Asct2 is expressed in many tissues, including placenta, lung, intestine and adipose-tissue [16,17]. Lane et al. have shown that Asct2 expression is activated by insulin-treatment [17]. Since Asct2 shows high affinity toward glutamine, Asct2 is essential for glutamine uptake in growing epithelial cells [18]. Bungard and McGivan have shown that Asct2 expression is regulated by farnesoid X Receptor (FXR)/retinoid X receptor (RXR) heterodimers in hepatoma cell line HepG2 cells [19,20]. Recently Palmada et al. have reported that Asct2 activity is regulated by serine-threonine kinases stimulated by insulin and insulin-like growth factor (IGF) [21]. We hypothesized that Asct2 in adipocytes may represent a new anti-obesity target.

When using differentiating 3T3-L1 cells as an experimental adipogenesis model, bio-synthesis of triacylglycerol, which is made from glucose and amino acids, is accelerated, resulting in enlarged...
intracellular lipid droplets. We observed that ATRA down-regulates Asct2 expression in these cells. In addition, we found in 3T3-L1 cells that the specific Asct2 inhibitor, L-γ-glutamyl-p-nitroanilide (GPNA) [22], inhibits adipogenesis in an additive fashion with ATRA. While GPNA has little effect on the expression levels of asct2 and glu4, GPNA decreases the protein levels of PPARγ. We hypothesized that inhibition of Asct2 may prevent obesity by suppression of glutamine-uptake. Inhibition of Asct2 by daily vitamin A intake may be sufficient to reduce obesity.

2. Materials and method

2.1. Cell culture

Murine preadipocyte, 3T3-L1 cells (obtained from Dr. Fumio Fukai, Department of Pharmaceutical Sciences, Tokyo University of Science), were routinely cultured in growth medium consisting of DMEM (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 2 mM glucose, 100 units/ml penicillin, and 100 units/ml streptomycin in 5% CO2 incubator at 37 °C. For glutamine free conditions, DMEM high glucose without glutamine (Wako) was supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 units/ml streptomycin.

2.2. Adipocyte differentiation

Routinely cultured 3T3-L1 cells were differentiated according to an established protocol [23]. Briefly, cells were cultured to full confluence. At 2 days post confluence (referred to as day 0), cells were stimulated with induction medium consisting of growth medium with DM (1 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, and 1 μg/ml insulin). After 20 h, cells were switched to “insulin medium”, consisting of growth medium with 1 μg/ml insulin, and were maintained for 7 days. The cells normally differentiated into mature adipocytes on day 7. For examining the effects on adipogenesis of ATRA (Sigma, St. Louis, USA) and L-γ-glutamyl-p-nitroanilide (GPNA, Wako), compounds were added to the insulin medium.

2.3. Oil Red-O staining

To evaluate the extent of lipid accumulation in DIM-stimulated 3T3-L1 cells, the oil Red-O staining method was utilized as previously reported [23]. In brief, cells were fixed with 10% formalin at room temperature, and were then washed with PBS and 60% isopropyl alcohol. After 20 h, cells were switched to “insulin medium”, consisting of growth medium with 1 μg/ml insulin, and were maintained for 7 days. The cells normally differentiated into mature adipocytes on day 7. For examining the effects on adipogenesis of ATRA (Sigma, St. Louis, USA) and L-γ-glutamyl-p-nitroanilide (GPNA, Wako), compounds were added to the insulin medium.

2.4. Immunoblotting analysis

Whole cell lysates were prepared with NP40 lysis buffer [23,24]. The protein concentration of the whole cell lysates was determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL, USA). Whole lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were probed with either polyclonal antibodies against ASCT2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PPARγ (Cell Signaling, Danvers, MA, USA), or glyceraldehyde 3-phosphate dehydrogenase (Gapdh, Abcam, Cambridge, MA, USA). Proteins were visualized using ECL-plus (Invitrogen).

2.5. Quantitative RT-PCR

Total RNA of cultured 3T3-L1 cells was prepared with ISOGEN reagent (Nippon gene, Toyama, Japan). Complementary DNA (cDNA) of each tissue was prepared by reverse transcriptase (RT) reactions from 1 μg total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The sequences of the PCR primers were as follows: asct2 (forward 5′-TGG ACC ACA GCC GTA TCC A-3′, reverse 5′-CCA GCC CCA AAA GGA TCA C-3′), adipon (forward 5′-TGG CAG AAG TGG CAC TCC TG-3′, reverse 5′-GCT AGG TGA AGA CG-3′), pparg1 (forward 5′-TGA AAG AAG CCG TGA AC-3′, reverse 5′-TAG TGT GGA GCA GAA AT-3′), pparg2 (forward 5′-GCT GTT ATG GGT GAA AAG CCG C-3′, reverse 5′-TAG TGT GGA GCA GAA AT-3′), b-actin (forward 5′-AGC CAT GTA CGT AGC CAT CC-3′, reverse 5′-ATT ACC GAG GAC GAC CCC AGA C-3′). Quantitative mRNA expression analysis was performed by a real-time PCR system (StepOne™, Applied Biosystems Inc., Foster City, CA, USA). To estimate the level of transcripts quantitatively, b-actin transcript was used as an internal control for the each prepared sample.

2.6. Confocal microscopic observation

Intracellular Asct2 localization was visualized by immunofluorescent staining with anti-ASCT2 antibodies (Cell Signaling). In brief, harvested cells on 8-well chamber slides were fixed with DIFM and ATRA as previously mentioned, and the cells were fixed with methanol. The fixed cells were then treated with anti-ASCT2 antibodies, and subsequently immunostained with Alexa546-conjugated anti-rabbit IgG antibody (Invitrogen). Confocal microscopy was performed using an Olympus FV1200 instrument (Olympus, Tokyo, Japan).

2.7. Statistical analysis

All data were analyzed using Student’s t-test. Differences were considered to be statistically significant if the p-value was <0.001.

3. Results

3.1. ATRA suppresses up-regulation of ASCT2 during adipogenesis

Several reports have shown that Asct2 transcripts are detected in adipose tissues and that insulin up-regulates Asct2 expression in adipogenesis–stimulated 3T3-L1 cells [16,17]. Since we hypothesized that ATRA could potentially suppress 3T3-L1 adipogenesis through Asct2 down-regulation, we added ATRA into the culture medium of 3T3-L1 cells to examine the effects of ATRA on the adipogenesis. The lipid droplets of the cultured cells were stained with oil Red-O, and a quantitative estimation of the accumulated lipid bodies was determined by measuring the oil Red-O under each culture condition.

Inhibition of adipogenesis by ATRA was observed in a dose-dependent manner. The quantity of lipid droplets decreased by 63% with 1 nM, 30% with 10 nM, 15% with 100 nM, and by 2% with 1 μM ATRA during adipogenesis, respectively (Fig. 1A). In adipogenic-differentiating cells in vitro, the source of lipids is mainly from glucose and amino acids in the culture medium. The neutral amino acid transporter, Asct2, is an essential glutamine transporter in proliferating cells. We found that, in DM-stimulated 3T3-L1 cells, 1 μM ATRA treatment suppressed asct2 gene expression by
59% (vs 0.1% ethanol treatment) at day 5 (Fig. 1B). The levels of asct2 genes increased until day 3, and then the levels decreased in the late stage of adipogenesis (Supplementary Fig. 1). ASCT2 protein levels in the adipogenerating 3T3-L1 were suppressed by 54% following ATRA treatment (Fig. 1C). From immunofluorescent stain analysis, DIM-stimulated 3T3-L1 cells displayed Asct2 on the surface of cells at 60 min following DIM-stimulation (Supplementary Fig. 2A). DIM-stimulated cells treated with ATRA had little Asct2 detection on cell surface (Fig. 1D). These results indicate that ATRA may suppress expression and intracellular localization of Asct2 during adipogenesis.

3.2. An Asct2 inhibitor suppresses adipogenesis of 3T3-L1 cells

Next, we examined the role of Asct2 in adipogenesis. To measure the contribution of Asct2 to adipogenesis, we used the Asct2 inhibitor, L-γ-glutamyl-p-nitroanilide (GPNA) [22]. We performed analysis by oil Red-O staining (Fig. 2), on DIM-stimulated 3T3-L1 cells following treatment with 500 μM GPNA, which is sufficient to achieve ~100% inhibition of glutamine uptake [22]. We found that under these conditions, lipid accumulation was reduced by approximately 12% during adipogenesis (Fig. 2A and B). Treatment with 10 nM ATRA suppressed lipid accumulation by 34%, and co-treatment of 500 μM GPNA with 10 nM ATRA resulted in 5% greater inhibitory effects on adipogenesis (Fig. 2A and B), which equaled the effects of 1 μM ATRA (Fig. 1A and B). Based on immunofluorescent stain analysis, DIM-stimulated 3T3-L1 cells treated with GPNA displayed the same level of Asct2 on the cell surface as with ATRA treatment (Supplementary Fig. 2B). GPNA suppressed adipogenesis in a dose-dependent manner (Supplementary Fig. 3).

We investigated the effects of 500 μM GPNA on asct2 gene expression. We found that GPNA addition down-regulated asct2 expression by 73% (Fig. 2C). GPNA also showed suppressive influence on the expression levels of adipogenic marker molecules at day 5, including adipocytokine (adipoq) and peroxisome proliferator-activated receptor γ (pparg2). This is consistent with the fact that expression of adipocytokine genes is regulated by PPARγ [25,26]. The suppressive effects of GPNA were similar to those seen after treatment with 1 μM ATRA (Fig. 2C). GPNA reduced Asct2 and PPARγ protein levels in differentiating 3T3-L1 cells (Fig. 2D). The inhibition of
amino acid intake by GPNA could potentially decrease lipid accumulation by down-regulating Asct2 and PPARγ2, which would be reflected in reduced adipogenesis.

3.3. Early effects of ATRA and an Asct2 inhibitor on adipogenesis

We hypothesized that suppressing the action of ATRA might be through decreasing Asct2 in DIM-stimulated 3T3-L1 cells. To identify the time course for this activity, we examined the lipid accumulation in 3T3-L1 cells treated with 1 μM ATRA at various intervals (Fig. 3A). At 3 day ATRA treatment, ATRA-treated cells already showed significantly less lipid accumulation (53%) than control cells (0.1% ethanol) (Fig. 3A). This implies that ATRA affected adipogenesis of DIM-stimulated 3T3-L1 cells in early periods, when suppression of pparg up-regulation had not yet been detected (Supplementary Fig. 1). We also investigated the time course of lipid accumulation in GPNA-treated cells. We observed that treatment with 500 μM GPNA enhanced lipid accumulation until day 3, and showed further suppressive effects in later stages (day 5 and 7) in a dose dependent manner (Fig. 3A). It is possible that during the early stages of DIM-stimulation, Asct2 might be a target of ATRA. Asct2 inhibition by GPNA might also suppress lipid
accumulation in the later stages of adipogenesis, Kim et al. have shown that ATRA treatment was sufficient to significantly inhibit adipogenesis at early stages as well as later periods [27]. Accordingly, we altered GPNA-treatment time periods during adipogenesis (Fig. 3B and C). The initial 3 day treatment showed suppressive effects on adipogenesis equal to the full treatment (Fig. 3B and C). This data suggested that initial amino acid uptake through Asct2 could be the primary source of the lipid droplets in mature adipocytes. Since treatment with both ATRA and GPNA indicated that adipogenesis-stimuli occurred in early stages (~3 days), uptake of amino acids including glutamine during this time, might be indispensable for adipo-maturation and lipid droplet formation.

3.4. Effects of glutamine free and GPNA treatment on 3T3-L1 cells

Asct2 is a neural amino acid transporter that has high affinity for glutamine. We examined the effects of GPNA treatment on adipogenesis under glutamine free conditions. Under glutamine free conditions, lipid accumulation decreased in differentiating 3T3-L1 cells by 31% (Fig. 4A, +DIM). Contrary to our expectations, glutamine free conditions did not suppress adipogenesis as great
as treatment with 500 μM of GPNA (11%) (Fig. 4A, GPNA). Glutamine free conditions increased PPARγ levels in 3T3-L1 cells, and suppressed reduction of Asct2 protein levels in the DIM-treated cells (Fig. 4B). This data indicated that the degree of lipid accumulation was not concomitant with Asct2 and PPARγ levels under glutamine free condition.

4. Discussion

In our current work, we have investigated ATRA’s suppressive effects on adipogenesis of 3T3-L1 cells. We found that in the early stages of adipogenesis ATRA down-regulated transcripts of asct2, a neutral amino acid transporter. During adipogenesis, GPNA, an Asct2 inhibitor, enhanced the suppressive activities of ATRA. Treatment with GPNA at early time points during adipocyte differentiation, was sufficient to suppress adipogenesis at a level that was equal to full duration treatment with ATRA or GPNA. Our studies have revealed that Asct2 can effectively inhibit lipid accumulation in preadipocytes.

ATRA has long been known to be an inhibitor of adipocyte differentiation [6–12]. As shown in Fig. 3A, ATRA exhibits suppressive effects from day 3 of treatment. Since the detectable activity in early periods is concomitant with PPARγ alteration, we hypothesized a mechanism other than the nuclear receptor cascade. Based on our results, it appears that Asct2 may be an ATRA target for inhibiting adipogenesis. Asct2 is the primary amino acid transporter for glutamine uptake, which serves as major source of triacylglycerol. Accordingly, we anticipated that down-regulation of asct2 could be an important component of ATRA’s inhibitory effects on adipogenesis. In our current study, the inhibitory effects of ATRA on Asct2 expression might not be dependent on FXR/RXR (farnesoid X Receptor/retinoid X receptor). Rather these effects...
might arise from other mechanisms, including RA-modification [28–30], RAR activation or inhibition of glutamine influx. Kim et al. have reported that soon after treatment with ATRA, adipogenesis was significantly inhibited. In ATRA treated cells, Wnt/β-Catenin signaling components are up-regulated at 6 h after adipogenesis stimulation [27]. To our knowledge, the relationship between glutamine influx and Wnt/β-Catenin signaling on adipogenesis has not been reported. However, Olkkou and Mahonen have indicated that the Wnt cascade inhibits intracellular glutamine levels by regulating glutamine synthetase [31].

Rhoderick et al. have reported that among compounds synthesized as N-aryl glutamine analogues, the Asct2 inhibitor, GPNA, had the most potent inhibitory effect on glutamine uptake [22]. Administration of GPNA to differentiating 3T3-L1 cells inhibits glutamine uptake, resulting in decreased lipid synthesis. We examined the effects of varying the treatment duration with GPNA. At early time points, GPNA suppressed adipogenesis of DIM-stimulated 3T3-L1 cells (Fig. 3C). This result is similar to Kim et al.’s results examining different time points of ATRA addition [27]. At the early time points, ATRA might inhibit adipogenesis by suppressing uptake of glucose and amino acids. GPNA might increase lipid accumulation up to day 3 by suppressing the uptake of amino acids, but not glucose. We found that under glutamine-free conditions, the formation of lipid droplets was reduced (Fig. 4A). We hypothesized that glutamine free conditions might achieve effects similar to treating differentiating 3T3-L1 cells with GPNA. Although GPNA reduced PPARγ levels in differentiating 3T3-L1 cells (Fig. 2C and D), we also found that reduction of PPARγ proteins might have occurred by mechanisms other than those arising from glutamine free conditions (Fig. 4). This indicates that glutamine is the major source for triacylglycerol. Therefore, GPNA-upregulated 3T3-L1 cells are not able to intake insufficient glutamine to form lipid droplets under glutamine free conditions. Accordingly, PPARγ regulation and GPNA treatment are different from glutamine-free conditions in 3T3-L1 cells. Under glutamine free conditions, amino acids other than glutamine may be taken up in preadipocytes in sufficient quantities to maintain PPARγ levels, but not sufficient for forming lipid droplets.

We found that treatment with GPNA had a suppressive effect not only on the adipo-generating cells, but also on mature adipocytes (Supplementary Fig. 4). Inhibition of Asct2 activities in adipo-matured 3T3-L1 cells might be effective against lipid-accumulation. We had speculated that the GPNA might be toxic to mature adipocytes, however the number of GPNA-treated adipocytes (after 5 days) was not significantly different from the control-conditioned cultured cells. In contrast, ATRA did not decrease accumulated lipids.

Our results suggest that targeting Asct2 activity could be a means of preventing/treating obesity. Cooperation of vitamin A (or ATRA) and an Asct2 inhibitor could potentially result in further anti-obesity activity. Glutamine influx might be a novel target for the treatment/prevention of obesity.

Conflicts of interest

None declared.

Author contributions

K.T., M.I. and N.T. conceived and supervised the study; K.T., M.I. and N.T. designed experiments; K.T., N.U., C.K., and C.S. performed experiments; N.T. provided new tools and reagents; K.T., M.I. and N.T. analyzed data and wrote the manuscript.

Acknowledgments

We thank Dr. Terrence Burke, Jr. for helpful comments. We thank Takaya Matsumiya, Mieko Harada, and Yui Sato for their expert technical assistance. This investigation was supported in part by ‘the Ministry of Education, Culture, Sports, Science, and Technology, Japan’, ‘the Promotion and Mutual Aid Corporation for Private Schools of Japan’, and ‘JSPS Core-to-Core Program, A. Advanced Research Networks, Japan.’

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.06.012.

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