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

Katsuhiko Takahashi, Natsumi Uchida, Chisato Kitanaka, Chiaki Sagara, Masahiko Imai, Noriko Takahashi *

Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, Shinagawa, Tokyo 142-8501, Japan

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ABSTRACT

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 adipogenerating 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.

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 adipogenetic 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

Abbreviations: ATRA, all-*trans* retinoic acid; DIM, dexamethasone, insulin and 3-isobutylmethylxanthine; FXR, farnesoid X Receptor; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; GPNA, L-γ-glutamyl-*p*-nitroanilide; PPARγ, peroxisome proliferator-activated receptor γ; RAR, retinoic acid receptor; RXR, retinoid X receptor

* Corresponding author at: Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan. Tel./fax: +81 3 5498 5950.

E-mail address: t-noriko@hoshi.ac.jp (N. Takahashi).

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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 *glut4*, 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 glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin in 5% CO_2 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 DIM (1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 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, followed by treatment with oil Red-O (2.1 mg/ml 60% isopropyl alcohol) at room temperature. Cells were then washed in turn with 60% isopropyl alcohol and PBS. To perform quantitative measurement of the accumulated lipids, the oil Red-O stained wells were treated with 100% isopropyl alcohol to extract oil Red-O, and the solutions were then measured for absorbance at 510 nm.

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 (Piece, 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′-GTC ACA GCC ACA GCA TCC A-3′, reverse 5′-CCA GCC CCA AAA GCA TCA C-3′), *adipoq* (forward 5′-TGG CAG AGA TGG CAC TCC TG-3′, reverse 5′-GGT CGT AGG TGA AGA GAA CG-3′), *pparg1* (forward 5′-TGA AAG AAG CGG TGA AC-3′, reverse 5′-TAG TGT GGA GCA GAA AT-3′), *pparg2* (forward 5′-GCT GTT ATG GGT GAA AC-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 GAG 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 treated with DIM and ATRA as previously mentioned, and the cells were fixed with methanol. The fixed cells were 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 DIM-stimulated 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 adipo-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 DIM-stimulated 3T3-L1 cells, 1 μM ATRA treatment suppressed *asct2* gene expression by