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Regulated expression of acyl-CoA thioesterases in the differentiation of cultured rat brown adipocytes

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ABSTRACT

Acyl-CoA thioesterases (ACOTs) are enzymes that catalyze the hydrolysis of fatty acyl-CoAs to free fatty acids and CoA-SH. In this study, we show that the expression profile of the ACOT isoforms changes remarkably during the differentiation of cultured rat brown adipocytes. Immunocytochemistry suggested that cytosolic ACOT1 was present in the preadipocytes, while mitochondrial ACOT2 was additionally expressed as the cells differentiated, concurrent with the accumulation of lipid droplets in the cytoplasm. Western blotting confirmed that, in contrast to ACOT1, the ACOT2 expression level was very low in the preadipocytes. However, after differentiation, the ACOT1 level fell to one-half of the baseline level and ACOT2 increased 18-fold. ACOT2 expression in the differentiated adipocytes was further enhanced by treatment with lipids or troglitazone. These changes in the ACOT2 expression level correlated well with changes in the expression of carnitine palmitoyltransferase 2, a mitochondrial β-oxidation enzyme. These results indicate that, in differentiating brown adipocytes, cytosolic ACOT1 becomes downregulated as the cellular use of acyl-CoA increases, while mitochondrial ACOT2 is upregulated as the β-oxidation capacity increases. ACOT isoform expression may be regulated during brown adipocyte differentiation to support the fat storage and combustion characteristics of this cell type.

1. Introduction

Acyl-CoA thioesterases (ACOTs) are enzymes that catalyze the hydrolysis of fatty acyl-CoA thioesters to free fatty acids and CoA-SH, and are localized in multiple compartments in cells [1–3]. For example, ACOT1 (formerly known as CTE-I or ACH2) is localized in the cytosol while ACOT2 (MTE-I or ARTISt) is present in the mitochondrial matrix. The physiological functions of ACOTs remain incompletely defined. However, ACOT1 and ACOT2 have been implicated in fatty acid catabolism because their expression is upregulated in conditions of fatty acid overload, as seen in the liver and heart of diabetic, fasted or high fat diet-fed animals [1,4–6]. In the liver, these enzymes are markedly induced by treatment with ligands for peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor that regulates lipid metabolism-related genes [1,7,8]. Moreover, they are expressed in tissues highly active in fatty acid oxidation, and ACOT2 protein expression has been demonstrated in the brown adipose tissue (BAT) of rats [8,9].

In terms of fat disposal in the body, BAT is characterized as a fat combusting, thermogenic organ, where energy from fatty acid oxidation is dissipated as heat through the action of mitochondrial uncoupling protein 1 (UCP1) [10]. While BAT research has been principally focused on rodents and newborn mammals, recent analyses using noninvasive imaging technology have revealed that a significant number of adult humans possess metabolically active BAT [11–13]. This finding has attracted attention to the protective and therapeutic potential of BAT against obesity and its related metabolic disorders. Our interest is in elucidating the roles of ACOT in acyl-CoA metabolism to support the efficient storage and combustion of fat in the BAT, to elicit the beneficial aspects of ACOT function applicable to consuming excessive fat in the body. Using cultured brown adipocytes, we observed drastic changes in the expression levels of the ACOT isoforms, which correlated with the changes in expression of a β-oxidation enzyme as well as lipid accumulation. We report the switching of ACOT isoform expression from ACOT1 to ACOT2 during the differentiation of rat brown

Abbreviations: ACOT, acyl-CoA thioesterase; BAT, brown adipose tissue; COX IV, cytochrome c oxidase subunit IV; CPT, carnitine palmitoyltransferase; DAPI, 4′,6-diamidino-2-phenylindole; PPAR, peroxisome proliferator-activated receptor.

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adipocytes, and discuss its significance with respect to fat disposal in the BAT.

2. Materials and methods

All animals were treated according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocol of this study was approved by the Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Sciences.

2.1. Cell culture and treatment

Primary cultures of brown preadipocytes isolated from the interscapular BAT of Sprague–Dawley rats at 2–4 days after birth were purchased from Primary Cell (Hokkaido, Japan) [14,15]. Differentiation of these preadipocytes was induced according to the supplier's protocol. The proliferation medium was Dulbecco's modified Eagle's medium with 25 mM glucose, supplemented with 17 μM pantothenic acid, 33 μM biotin, 100 μM ascorbic acid, 1 μM octanoic acid, 50 nM triiodothyronine, 10% fetal calf serum, 10 units/mL penicillin, and 10 μg/mL streptomycin. The differentiation medium was the proliferation medium supplemented with 10 μg/mL insulin and 2.5 μM dexamethasone, while the maintenance medium was proliferation medium supplemented with 10 μg/mL insulin only. Briefly, the brown preadipocytes plated on collagen-coated plastic flasks (25 cm^2) were grown in the proliferation medium at 37 °C in a 5% CO_2 atmosphere. When the cell growth reached subconfluence (set as differentiation day 0), the medium was replaced with the differentiation medium and the cells were incubated for 48 h. Thereafter, the medium was replaced with the maintenance medium (day 2) and culture was continued for another 5 days (until day 7). The culture medium was changed every other day. The brown preadipocytes were either not passaged at all or used after one passage.

When the adipocytes were treated with nutrients or chemicals, these reagents were added to the maintenance medium, to which cells were exposed for 5 days from differentiation day 2. In the high glucose and lipid loading experiments, the glucose concentration of the maintenance medium was reduced to 5.5 mM, except for the high glucose cultures in which it remained at 25 mM. A lipid emulsion, Intralipos 20 (Otsuka, Tokushima, Japan) that contained 1.2 g lecithin, 2.2 g glycerol and 20 g soybean oil in a volume of 100 mL, was added to the medium at a 1/100 volume [16]. Palmitic acid was conjugated with BSA at a 6:1 M ratio [17]: palmitic acid was dissolved in ethanol at 60 °C and mixed with prewarmed maintenance medium containing 4% fatty acid-free BSA (Sigma–Aldrich, St. Louis, MO) to yield a concentration of 4 mM, which was used as 1 mM palmitic acid, 1% BSA and 0.3% ethanol. A BSA/ethanol vehicle was employed as a control. In experiments with PPAR ligands, Wy-14,643 (10 μM [18]; Tokyo Chemical Industry, Tokyo, Japan), GW501516 (0.1 μM [19]; Alexis, San Diego, CA), and troglitazone (10 μM [20]; Wako, Osaka, Japan) were dissolved in dimethyl sulfoxide (final 0.1%).

For microscopic examination, the preadipocytes were plated on collagen-coated glass coverslips at a density of 20,000 cells/cm^2 and cultured as described above. The cells were fixed with 4% paraformaldehyde dissolved in 80 mM sodium phosphate (pH 7.4). For western blotting, cells were lysed in 0.15 M NaCl containing 2 mM EDTA, 50 mM Tris–HCl (pH 7.5), 2% Triton ×-100 and Complete protease inhibitor cocktail (Roche, Mannheim, Germany), and the extract was used after centrifugation. The protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA) with BSA as the standard.

2.2. Immunofluorescent microscopy

Fixed cells were blocked with 5% skim milk, and were successively treated with anti-ACOT1 antibody (see below) for 1 h, biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) for 1.5 h, fluorescein-conjugated streptavidin (GE Healthcare, Little Chalfont, UK) for 1.5 h, mouse anti-cytochrome c oxidase subunit IV (COX IV) antibody (Invitrogen, Carlsbad, CA) for 1 h, and Cy3-conjugated goat anti-mouse IgG antibody (GE Healthcare) for 1 h. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Microscopic examination was performed using a BX-52 fluorescence microscope equipped with a mercury arc lamp (Olympus, Tokyo, Japan). For a negative control, the primary antibody was replaced by nonimmunized rabbit serum, in which the IgG concentration was adjusted to the same level as that in the primary antibody, and a lack of staining was confirmed (data not shown).

2.3. Oil Red O staining

Fixed cells were washed with water, and placed in 60% isopropanol for 1 min, after which they were stained for 15 min at 37 °C in freshly diluted Oil Red O solution (0.3% stock in isopropanol by water at 6:4), followed by color separation with 60% isopropanol for 1 min. The cells were then counterstained with hematoxylin.

2.4. Western blotting

Proteins were resolved on 10% SDS–polyacrylamide gels and detected using ECL Advance Western Blotting Detection Reagents (GE Healthcare) as described previously [6]. Rabbit polyclonal antibodies against ACOT1 and carnitine palmitoyltransferase 2 (CPT2) were generated in-house and have been described in our previous studies [6,7,9]. Mouse anti-α-tubulin antibody was obtained from Sigma–Aldrich. For quantitative analysis, the signal intensities of the bands detected on the blots were measured and transformed into relative values using a calibration curve generated with known amounts of protein.

2.5. Statistical analysis

The statistical significance of differences among values was examined by one-way analysis of variance followed by Fisher's protected least significant difference multiple comparison test. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results and discussion

We examined changes in the expression of ACOT isoform proteins during brown adipocytes' acquisition of the capability to store and combust fat. Rat brown preadipocytes were cultured and differentiated, then analyzed using an anti-ACOT1 antibody (Figs. 1 and 2). As shown by Oil Red O staining, the preadipocytes that had almost no lipid droplets within the cell (day 0) accumulated small amounts of lipid just after differentiation (day 2), whereas mature adipocytes contained multilocular, large lipid droplets in the cytoplasm (day 7) (Fig. 1A–C). During this process, the pyramidal preadipocytes became increasingly rounded. Immunoreactivity to the anti-ACOT1 antibody was diffuse throughout the cytosol at all time-points examined (Fig. 1D–F). However, double-labeling immunofluorescence using antibodies against ACOT1 and COX IV (a mitochondrial marker) showed overlapping immunoreactivities, thus localizing ACOT to mitochondria as well as the cytosol in mature brown adipocytes (day 7) (Fig. 1G–I).

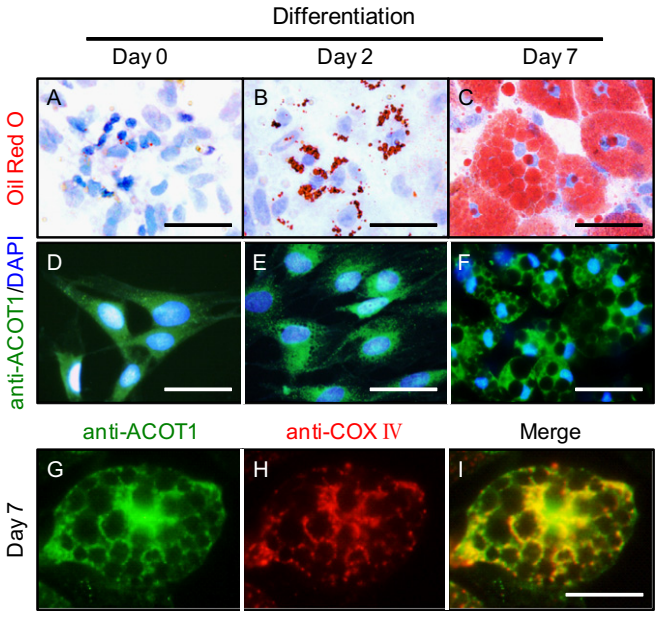

Fig. 1. Immunocytochemistry of ACOT isoforms in cultured brown adipocytes. The brown preadipocytes grown to subconfluence (set as day 0) were induced to differentiate for 2 days, and maintained for another 5 days. Cells were subjected to Oil Red O/hematoxylin staining (A–C) or double-labeling immunofluorescence using anti-ACOT1 and anti-COX IV antibodies with DAPI counterstaining (D–I). In A–C, lipid droplets are shown in red and nuclei are in blue. In D–I, immunoreactivities to the anti-ACOT1 and anti-COX IV antibodies are shown in green and red, respectively, and the merged signals are yellow (I), while the nuclei are blue. It should be noted that the anti-ACOT1 (cytosolic) antibody cross-reacts with ACOT2 (mitochondrial). A and D, differentiation day 0; B and E, day 2; C, F, G–I, day 7. Scale bars, 50 μm in A–F, 20 μm in I. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Because the amino acid sequences of ACOT1 and ACOT2 are 91% identical [21], the anti-ACOT1 antibody cross-reacts with ACOT2 [4,6,9]. However, on western blots, almost no immunoreactivities other than those to ACOT1 (46 kDa) and ACOT2 (45 kDa) were observed (Fig. 2A). Taking account of the known subcellular localization of ACOT1 and ACOT2, the immunocytochemistry suggested that ACOT1 was present in the cytosol of the brown preadipocytes and ACOT2 was additionally expressed in the mitochondria after differentiation.

This finding was confirmed by western blotting (Fig. 2B). On days 0 and 2 of brown preadipocyte differentiation, considerable levels of ACOT1 were expressed in the adipocytes, whereas ACOT2 was at very low levels. On day 7, however, the ACOT1 level fell to one-half of the baseline level, and ACOT2 increased 18-fold, to a level similar to that of ACOT1 on day 0 (Fig. 2B, top panel). When CPT2 was analyzed as a mitochondrial β-oxidation enzyme, its expression level was very low on days 0 and 2. However, it markedly increased by day 7 to reach a level 10-fold higher than the baseline (Fig. 2B, middle panel). This change in CPT2 level correlated well with the change in ACOT2, underscoring the functional relationship between fatty acid oxidation and ACOT2 in this cell type. In contrast, the CPT2 levels inversely correlated with ACOT1 levels, although the magnitudes of their changes were not equivalent. In this context, lipid accumulation in the adipocytes also inversely correlated with the ACOT1 level, which could be estimated by the number and volume of lipid droplets (Fig. 1A–C). Taken together, in the differentiating brown adipocytes, cytosolic ACOT1 was downregulated as cellular use of acyl-CoA (fat storage and combustion) increased, while mitochondrial ACOT2 was upregulated as the β-oxidation capacity increased.

Next, we examined the effects of high glucose, lipid loading or PPAR ligands on the expression levels of ACOT1 and ACOT2 proteins (Fig. 3). The brown adipocytes just after differentiation were exposed to nutrients or chemicals for 5 days. While high glucose had no effect, the lipid emulsion and palmitic acid induced ACOT2 expression 1.4- and 1.7-fold, respectively, compared with the corresponding controls (Fig. 3A). These were also accompanied by 1.4- and 2.6-fold inductions of CPT2, respectively. When the cultures were treated with synthetic ligands for the PPAR subtypes α, β/δ or γ, troglitazone, a PPARγ-specific ligand, increased ACOT2 levels

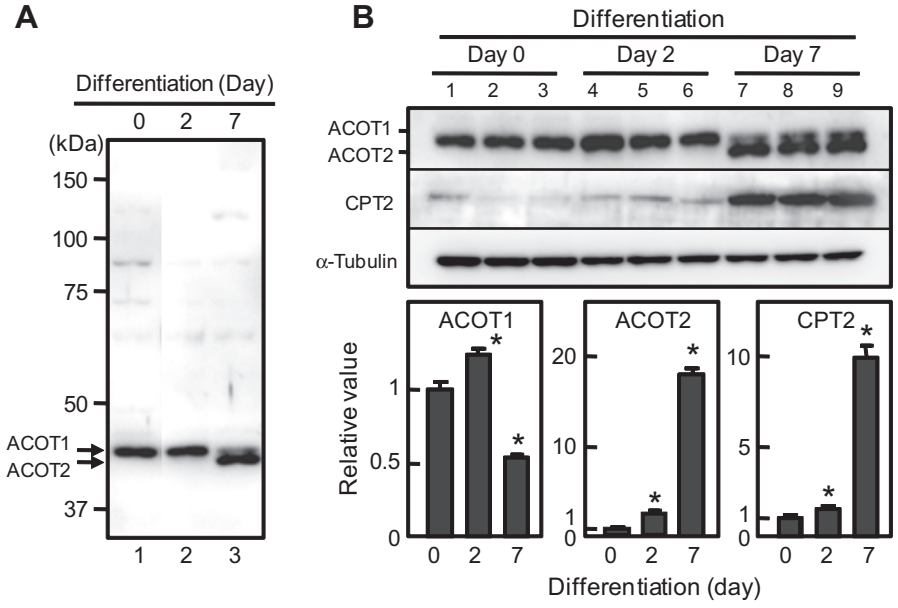

Fig. 2. Changes in the expression levels of ACOT isoforms and CPT2 during differentiation of cultured brown adipocytes. The brown preadipocytes were cultured as described in the legend for Fig. 1, from which cell lysates were prepared and western blotted using anti-ACOT1 and anti-CPT2 antibodies. (A) Western blots (10 μg protein/lane) using the anti-ACOT1 antibody. The positions of ACOT1 (46 kDa) and ACOT2 (45 kDa) are indicated by arrows on the left, with the sizes of the molecular mass markers. It should be noted that the anti-ACOT1 antibody cross-reacts with ACOT2. Lanes 1–3, differentiation days 0, 2 and 7, respectively. (B) The signal intensities of the bands corresponding to ACOT1, ACOT2 and CPT2 were measured and are expressed relative to the values for day 0, which were set as 1 (mean ± SEM of three cultures). Lanes 1–3, differentiation day 0; lanes 4–6, day 2; lanes 7–9, day 7. $*p < 0.05$ *vs.* day 0. The blots for all three cultures are presented for each time point (10 μg protein/lane). Alpha tubulin was used as a loading control.

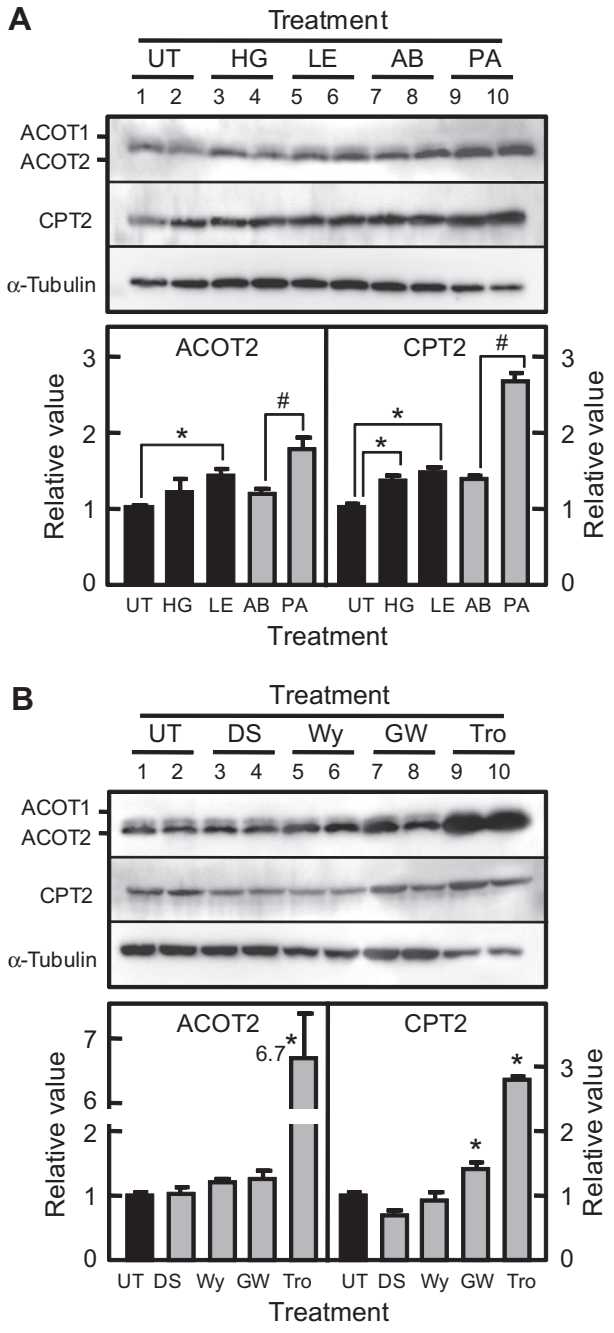

Fig. 3. Effects of high glucose, lipid loading and synthetic PPAR ligands on the expression levels of the ACOT isoforms and CPT2 in cultured brown adipocytes. (A) The brown adipocytes (differentiation day 2) were untreated (UT) or treated with high glucose, 25 mM (HG), 1% lipid emulsion (LE), BSA/ethanol vehicle (control for palmitic acid) (AB) or 1 mM palmitic acid conjugated with 1% BSA in 0.3% ethanol (PA) for 5 days. Cells were lysed and western blotted using anti-ACOT1 and anti-CPT2 antibodies. The signal intensities of the bands corresponding to ACOT2 and CPT2 were measured and are expressed relative to the values for UT, which were set as 1 (mean ± SEM of three cultures). It should be noted that the anti-ACOT1 antibody cross-reacts with ACOT2. $*p < 0.05$ *vs.* UT; $\#P < 0.05$ *vs.* AB. (B) The adipocytes were similarly untreated (UT) or treated with 0.1% dimethyl sulfoxide (DS) as a vehicle, 10 μM Wy-14,643 (Wy), 0.1 μM GW501516 (GW) or 10 μM troglitazone (Tro) for 5 days. Cells were lysed and analyzed, and the results are expressed as described above. $*p < 0.05$ *vs.* DS. The blots for two cultures are presented for each treatment (10 μg protein/lane). Alpha tubulin was used as a loading control.

6.7-fold, with a concomitant 4.1-fold induction of CPT2, compared with the vehicle-treated cultures (Fig. 3B). The PPARα (Wy-14,643) and PPARβδ (GW501516) ligands had no effect on ACOT2 levels. In all the cases, the ACOT1 level was relatively low and seemed unchanged, although precise quantification was difficult, especially for the troglitazone treatment in which the vastly increased ACOT2 levels interfered with detection of ACOT1 on the blots (Fig. 3B, top panel, lanes 9 and 10).

While these increases in ACOT2 and CPT2 represented direct effects of the reagents applied to the adipocytes, it is possible that they resulted from cellular maturation that could be promoted by these reagents. To investigate this, fully matured adipocytes were required, but the cells used in this study were not suited to longer culture periods. Therefore, we examined these effects in the BAT of adult animals, which is dominated by fully differentiated mature brown adipocytes. In rats fed a high fat diet, ACOT2 and CPT2 tended to be co-induced in the BAT (Supplementary Fig. S1). However, such an induction did not occur when male C57BL/6 J mice aged 10 weeks were orally administered troglitazone at 200 mg/kg body weight, once a day for 14 days (data not shown). These results suggest that the lipid-induced upregulation of ACOT2 and CPT2 observed in the cultures involves a direct effect of fatty acids on the mechanism of enzyme induction, whereas the troglitazone induction largely reflects its PPARγ-mediated stimulatory effect on the differentiation and maturation of adipocytes [22,23].

The significance of our findings is illustrated in Fig. 4, based on the hypotheses so far presented regarding the functions of ACOT1 and ACOT2 [1,6,24]. Briefly, in the brown preadipocytes, ACOT1 may maintain cytosolic acyl-CoA below a certain level to prevent its excessive use. However, as the adipocytes differentiate and mature, this restriction may be lifted due to ACOT1 downregulation,

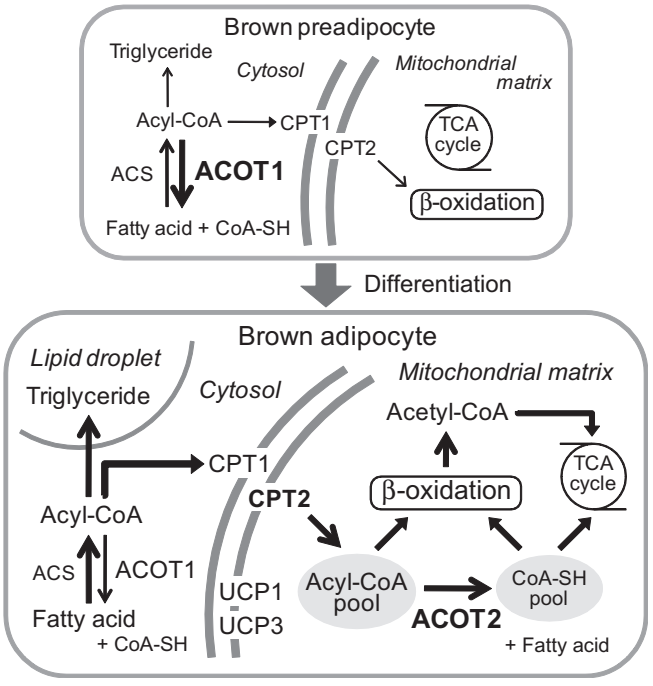

Fig. 4. Proposed role for the regulated expression of ACOT isoforms in differentiating brown adipocytes. In brown preadipocytes, highly expressed ACOT1 restricts cytosolic acyl-CoA use. However, as the preadipocytes differentiate and mature, the ACOT1 expression is downregulated, resulting in increased flux of acyl-CoA substrates into lipid synthesis and fatty acid oxidation. In the mitochondrial matrix, ACOT2 is induced along with β-oxidation enzymes, and hydrolyzes some of acyl-CoA transported *via* CPT to regenerate CoA-SH. Thus, upregulation of ACOT2 serves to maintain an adequate rate of β-oxidation by modulating the substrate supply and retaining the level of coenzymes used in the TCA cycle and β-oxidation itself. This contributes to efficient oxidation of fatty acids. Therefore, ACOT isoform switching supports the fat storage and combustion in this cell type. ACS, acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; TCA, tricarboxylic acid; UCP, uncoupling protein.

resulting in an increased flux of acyl-CoA substrates into lipid synthesis and fatty acid oxidation. In the mitochondrial matrix, ACOT2 co-induced with β-oxidation enzymes may support efficient oxidation of fatty acids. In this model, it is unclear why acyl-CoA utilization is restricted in the preadipocytes, but that may be advantageous to proliferation or differentiation of the preadipocytes. Similar changes in ACOT1 expression have been reported in the mammary gland of mice [25]. In the mammary epithelium, ACOT1 was significantly upregulated during the secretory differentiation phase of pregnancy, and the elevated level of ACOT1 fell precipitously during the secretory activation phase at parturition when the milk (fat) production and secretion are induced. It is likely that the fall in ACOT1 facilitates the switch to lipogenesis also in this tissue. We speculate that the regulated expression of the ACOT isoforms is part of the program of brown adipocyte differentiation to support the fat storage and combustion characteristics of this cell type, based on the concept that ACOT acts to regulate the level of acyl-CoA substrate for its effective cellular metabolism [1,6,24].

To elicit the anti-obesity potential of the BAT, it is important to sustain this tissue against regression with increasing age, and activate it independently of cold exposure [26,27]. In addition, it is necessary to maintain its robust metabolic capacity and adequately regulated ACOT expression. This point will also be of importance when characterizing human BAT to take advantage of its function as a fat consumer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.066.

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