PPAR\(\gamma\) Agonists Down-Regulate the Expression of \(Atp10c\) mRNA during Adipogenesis

Amanda L. Peretich\(^1\), Maria Cekanova\(^2\), Sarah Hurst\(^1\), Seung J. Baek\(^2\) and Madhu S. Dhar\(^*\)\(^1\)

\(^1\)Departments of Large Animal Clinical Sciences and \(^2\)Pathology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA

Abstract: We have shown that \(Atp10c\), a type 4 P-type ATPase, is a strong candidate affecting glucose and lipid metabolism in humans and mice. \(Atp10c\) is a putative phospholipid translocase associated with cell signaling and intracellular protein trafficking. In order to examine the biological role of \(Atp10c\), semiquantitative reverse transcriptase–polymerase chain reaction was carried out. \(Atp10c\) mRNA is expressed in 3T3-L1 cells and in primary preadipocytes and adipocytes generated from mice. \(Atp10c\) mRNA is regulated during fat cell differentiation and modulated by PPAR\(\gamma\) agonists and antagonists as well as by hormonal factors (insulin and dexamethasone). \(Atp10c\) expression is regulated by both the process of adipocyte differentiation and by effectors of fat and glucose metabolism. Taken together these data along with the published phenotype of the \(Atp10c\) heterozygote mice suggest that ATP10C is a newly identified protein with a possible biological role in the development of obesity and obesity-related metabolic disorders.

1. INTRODUCTION

Studies in yeast demonstrate that members of a novel type 4 subfamily of P-type ATPases are important in protein trafficking in the exocytic and endocytic pathways. These putative phospholipid translocases (PLTs), or flippases, presumably function to control cellular integrity and homeostasis by maintaining the lipid bilayer [1-4]. PLTs have been implicated in the translocation of phospholipids from the outer leaflet to the inner leaflet of lipid bilayers. Mutations in the sequences of mammalian flippases show their association with human disorders of intramembranous transport. For instance, mutations in human ATP8B1 are associated with Byler disease, which is a severe inherited disease of the liver. Studies in mice show that a deficiency of \(Atp8b1\) causes a loss of phospholipid asymmetry, which subsequently impairs bile salt transport and causes cholestasis [4-7]. Another member of this subfamily, the murine gene \(Atp10d\), has been mapped within a quantitative trait locus (QTL) associated with HDL-cholesterol levels in the C57BL/6 strain of mice [8]. Although there is consensus on the physiological function for PLTs, the molecular and cellular mechanisms by which these proteins function are poorly understood.

\(Atp10c\) is a putative PLT located on mouse chromosome 7 that has been associated with obesity, type 2 diabetes, and nonalcoholic fatty liver disease in mice. Heterozygous mice inheriting a maternal deletion of the \(Atp10c\) gene become obese and show hyperinsulinemia, glucose intolerance, and insulin resistance. Mutants show a 30-35% decrease in insulin-stimulated glucose uptake in the peripheral tissues. Gene expression profiling shows a dramatic, diet-induced loss of \(Atp10c\) expression in the soleus muscle of mutants along with subtle changes in adipocytes. Significant changes in the biochemical and molecular response of fat and muscle tissues in \(Atp10c\) mutants indicate an important role of ATP10C in the insulin signaling pathways that mediate glucose clearance by peripheral tissues [9-11]. We have also identified and characterized the human ortholog, \(ATP10C\), and have mapped it to the syntenic region on chromosome 15, \(HSA\) 15 [12]. A group of neurologically affected patients with the additional phenotype of increased body mass index who carry deletions in this region has also been reported, thus suggesting an association of \(Atp10c/ATP10C\) with some forms of glucose and lipid metabolism disorders in both mice and humans [13-15]. In general, data suggest a probable interaction between PLTs and obesity and insulin resistance, clearly emphasizing the importance of investigating their exact biological role(s).

In the study of metabolic diseases, the ability to distinguish between primary and secondary effects is a major problem. Given that \(Atp10c\) heterozygote mice are obese and exhibit metabolic abnormalities, it is difficult to understand if changes in glucose and lipid metabolism directly arise from alterations in ATP10C activity or if they are due to secondary complications. Thus, the present study was initiated to define the functional importance of \(Atp10c\) in a cell. Our objective was two-fold: to establish a cell system expressing \(Atp10c\), then to use this cell system to identify factors modulating \(Atp10c\) mRNA expression. 3T3-L1 cells, a commercially available mouse cell line [16], and purified preadipocytes and adipocytes from wild-type mouse fat tissue were used.

2. MATERIALS AND METHODS

2.1 Materials

Cell culture reagents and oligonucleotides were obtained from Fisher Scientific (Fairlawn, NJ). Insulin, isobutyl-
methyllxanthine, isoproterenol, dexamethasone, all-trans retinoic acid, and fetal bovine serum were purchased from Sigma (St. Louis, MO). Thiazolidinediones MCC555 and troglitazone (TGZ) and the PPARγ inhibitor GW9662 were obtained from Cayman (Ann Arbor, MI).

2.2 Cell Culture

3T3-L1 preadipocytes obtained from ATCC (Manassas, VA) were grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (growth medium). At 70–80% confluency, adipocyte differentiation was induced by incubating the cells in growth medium supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin (differentiation medium) for two days. After 2 days, cells were maintained in growth medium supplemented with 1 μg/ml insulin (maintenance medium) and grown for 7–10 days; the medium was changed every two to three days. Cells were fully differentiated (>80%) by day 8–10 as assessed by Oil Red O staining and reverse transcriptase PCR (RT-PCR); these are 3T3-L1 adipocytes. Day 0 represents the day on which the differentiation media is replaced by maintenance media.

Confluent 3T3-L1 preadipocytes or adipocytes were incubated for 24 hours in serum-free medium before various effectors were added as described in the figure legends. PPARγ agonists and antagonists were replenished every time the media was changed.

Primary preadipocytes and adipocytes were isolated from adipose tissue of 4–6 month old adult female wild-type mice from ongoing crosses of Apo10c heterozygotes, using Rodbell’s procedure with modifications [17, 18]. Approximately 2 g of tissue was collected, minced into small pieces, and digested with collagenase (2 g/L) (type I, Worthington Biochemical Corp., Lakewood, NJ) at 37°C for 25 min in modified Krebs-Ringer bicarbonate albumin buffer pH 7.4, containing 1 g/L BSA and 4.4 mM glucose. Digested fat tissue was filtered through a 100 μm nylon mesh and primary adipocytes were isolated by a low speed centrifugation at 800 rpm for 10 min. These adipocytes were washed once with Hank’s balanced salt solution and collected. The stromal vascular fraction rich in primary preadipocytes was then pelleted by centrifugation at 1500 rpm for 15 min. Resulting stromal vascular fraction cell pellets were treated with red blood cell lysis buffer (eBioscience, San Diego, CA) for 5 min at room temperature, rinsed with phosphate buffered saline supplemented with 1% BSA, and centrifuged at 800 rpm for 10 min. Stromal vascular fraction cells were seeded in 6-well cell culture plates at a density of about 3x10⁵ cells/well.

At 70–80% confluency, primary preadipocytes in the stromal vascular fraction cells were induced to differentiate in growth medium supplemented with 10 μg/ml insulin (high insulin medium). Differentiation was complete in 8–10 days as assessed by Oil Red O staining and RT-PCR. These cells are the primary adipocytes obtained from primary preadipocytes differentiated in culture.

All animal procedures were in accordance with the University of Tennessee IACUC (protocol #1309-1206).

2.3 Oil Red O Staining

Adipocyte differentiation was assessed using a modified procedure of Oil Red O staining [19]. Cells were washed with Hank’s balanced salt solution and fixed with 1% paraformaldehyde for 5 min. Cells were rinsed with deionized water followed by the addition of two aliquots of 85% propylene glycol for 5 min each. Oil Red O stain was added (0.7% w/v in 85% propylene glycol) and cells were gently swirled for 20 min at room temperature. Following a final wash with 85% propylene glycol for 3 min, cells were counterstained with hematoxylin. They were visualized and photographed using a Zeiss Invertoskop 40C microscope under 10X magnification and captured by a Canon Powershot A620 digital camera.

2.4 Semiquantitative RT-PCR

These procedures are as previously described with a few modifications [10]. Total RNA was extracted from the harvested cells using RNeasy Mini RNA kit (Qiagen Inc. Valencia, CA) according to the manufacturer’s instructions. About 1x10⁶ cells yield 30–50 μg of total RNA. First strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Semiquantitative RT-PCR was performed for Apo10c (F–5′CCTGTCCTCCTTACGTG GC3′, R–5′CAGTCACTGTGTAATGTC3′), resis-tin (F–5′ACTGAGTGTCGCTTGCTGAAG3′, R–5′CACCGCT CACTTCCCGGACATC3′), and PPARγ (F–5′GGTGA ACTCTGGGAGATC3′, R–5′CAACCATTTGCTGGA CTTT3′) mRNAs using GoTaq Green Master Mix (Promega Inc. Madison, WI) with β-actin (F–5′ATGGTGCTAGAAGG ACTCCTA3′, R–5′CACCAATGACACGTTTCTT3′) as an internal control. RT-PCR was performed under the following conditions: 5 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, and finishing with 5 min at 72°C. RT-PCR products were analyzed on 1% agarose gels containing 1 μg/μl ethidium bromide and viewed under ultraviolet light.

2.5 Data Analysis

RT-PCR products on the agarose gels were quantitated using Scion Image (http://www.scioncorp.com). Results are expressed as the ratio of the target gene expression to that of β-actin and are shown as means ± SD. Mean comparisons were tested by two-tailed paired Student’s t-test with P values <0.01 considered highly significant and <0.05 considered significant.

3. RESULTS

Using RT-PCR, an 886 bp product was detected in both the undifferentiated and differentiated 3T3-L1 cells. This product has been demonstrated and confirmed to be a part of Apo10c cDNA [11]. Quantitative analysis showed that Apo10c is 2-fold down-regulated in 3T3-L1 adipocytes after differentiation (Fig. 1). Apo10c mRNA is also expressed in purified preadipocytes and adipocytes from mouse adipose tissue and in primary preadipocytes differentiated in culture (Fig. 2). Compared to primary preadipocytes, Apo10c mRNA is significantly down-regulated in primary adipocytes, again confirming the regulation of Apo10c expression during
Adipogenesis. As expected, PPARγ (PCR product of size 268bp) and resistin (PCR product of size 386bp) are upregulated, serving as internal positive controls (Fig. 2A).

Adipose tissue is comprised of several types of cells such as fibroblasts, non-differentiated mesenchymal cells, preadipocytes, and adipocytes. Adipocytes develop from fibroblast-like preadipocytes within the stromal vascular fraction [20, 21]. Hence, to ensure that stromal vascular fraction cells in our preparations were comprised mainly of preadipocytes and that Atp10c expression was specific to the preadipocyte fraction, we differentiated the stromal vascular fraction cells to adipocytes, and then measured Atp10c expression. Interestingly, Atp10c expression was about 2-fold upregulated whereas, PPARγ and resistin expressions were...
significantly down-regulated (Fig. 2B). PPARγ and resistin mRNA expressions were very low in primary preadipocytes and after differentiation no PCR product was detected in primary adipocytes. As described below, we believe that this increase in Atp10c expression and the corresponding decrease in PPARγ and resistin expressions in primary adipocytes are due to high insulin present in the differentiation media used for primary cultures. Oil Red O staining was used to monitor differentiation in culture and to confirm that the harvested cells showed 80% differentiation (Fig. 2C).

The process of adipocyte differentiation is associated with a large number of cis - and trans- acting factors [22-24]. The regulation of different genes at distinct times after the exposure of cells to differentiation conditions suggests the existence of a regulatory hierarchy or cascade of events [25, 26]. PPARγ is considered to be the master regulator of adipogenesis, controlling the expression of adipocyte genes. Therefore, we investigated the effect of PPARγ agonists on Atp10c expression to assess whether Atp10c expression is also under PPARγ control. Two anti-diabetic drugs that activate PPARγ and thus promote adipogenesis, MCC555 and TGZ, were used [27, 28]. 3T3-L1 adipocytes treated with DMSO (vehicle), 10μM MCC555, 10μM TGZ, and 10μM GW9662 is shown (A), with β-actin serving as an internal control. The expression of Atp10c is denoted as arbitrary units (A.U.) and represented as the fold change normalized to β-actin. Oil Red O staining (B) was used to monitor morphological changes in adipocytes in the presence of PPARγ agonists and antagonists. Data from six independent experiments are shown. *P<0.05, ***P<0.001.

Fig. (3). Atp10c mRNA expression is regulated by PPARγ agonists and antagonists. RT-PCR analysis shows Atp10c expression in 3T3-L1 adipocytes harvested between days 8–10 of differentiation. MCC555, troglitazone (TGZ), and GW9662 were added to the media throughout the differentiation process and replenished whenever the media was changed. Expression of Atp10c mRNA in adipocytes treated with 10μM DMSO (vehicle), 10μM MCC555, 10μM TGZ, and 10μM GW9662 is shown (A), with β-actin serving as an internal control. The expression of Atp10c is denoted as arbitrary units (A.U.) and represented as the fold change normalized to β-actin. Oil Red O staining (B) was used to monitor morphological changes in adipocytes in the presence of PPARγ agonists and antagonists. Data from six independent experiments are shown. *P<0.05, ***P<0.001.

To clarify and confirm that the decrease in Atp10c is indeed due to the process of adipogenesis controlled by PPARγ, Atp10c expression was also assessed in cells treated with a PPARγ antagonist, GW9662 [29]. Atp10c expression in adipocytes treated with GW9662 alone did not decrease significantly and was similar to that in control cells treated with the vehicle DMSO. However, when GW9662 was added to the cells along with MCC555 or TGZ, there was only about a 2-fold decrease in Atp10c expression. This suggests that GW9662 inhibited adipogenesis and rescued some of the decrease in Atp10c expression due to MCC555 or TGZ alone. Similarly, Atp10c expression did not decrease
following treatment of adipocytes with 1 μM all-trans retinoic acid (data not shown). 1 μM all-trans retinoic acid also inhibits adipocyte differentiation thus, confirming that Atp10c is highly expressed in undifferentiated cells and negatively regulated during adipocyte differentiation.

To gain further insight into the transcriptional control of Atp10c during adipogenesis Atp10c expression was compared to that of PPARγ in cells treated with MCC555 or a combination of GW9662 and MCC555. Expression of Atp10c and PPARγmRNA is denoted as arbitrary units (A.U.) and represented as the fold change normalized to β-actin and compared to day 1 DMSO. Oil Red O staining (C) was used to assess the corresponding morphological changes. Data from five independent experiments are shown. *P<0.05, **P<0.005, ***P<0.001.

4. DISCUSSION

Protein trafficking and intracellular signaling processes are important in controlling cellular homeostasis. Defects in

Fig. (4). Atp10c and PPARγmRNAs are oppositely regulated in 3T3-L1 cells during adipogenesis. RT-PCR analysis was used to determine Atp10c (A) and PPARγ (B) mRNA expression in 3T3-L1 cells at days 1, 2, 4, and 7 post-induction; β-actin served as an internal control. Cells were treated throughout differentiation with DMSO, MCC555, or a combination of GW9662 and MCC555. Expression of Atp10c and PPARγmRNA is denoted as arbitrary units (A.U.) and represented as the fold change normalized to β-actin and compared to day 1 DMSO. Oil Red O staining (C) was used to assess the corresponding morphological changes. Data from five independent experiments are shown.
these processes can lead to changes in cellular function resulting in serious pathological conditions like obesity and type 2 diabetes [30, 31]. Although numerous target genes and their cognate protein factors have been identified and their roles characterized, our understanding of these processes is still limited. It is probable that many components of the system are still unidentified. Hence, novel factors are continually being identified and strategies to further our understanding of mechanisms underlying the above processes are being developed.

Type 4 P-type ATPases belong to a newly identified subfamily of PLTs in eukaryotes. They are suggested to maintain the asymmetrical nature of the lipid bilayer and are proposed to be important in protein trafficking [1,3,5]. These ATPases are thought to catalyze the transbilayer transport of amphipathic molecules such as phospholipids, rather than being pumps for the transfer of cations [32-35]. Based on published reports from our laboratory, it is reasonable to suggest that Atp10c/ATP10C, one member of this subfamily, may play an important role in obesity of multiple etiologies in mice and humans.

Mice heterozygous for Atp10c, a putative PLT, represent a diet-induced genetic model of obesity associated with insulin resistance. Prior studies suggest that misexpression of Atp10c promotes obesity by modulating glucose and lipid metabolism [9, 10]. To gain mechanistic insight into the link between the translocase activities of PLTs and metabolic diseases, in the current report we have successfully established cell culture systems which express Atp10c and thus, will be used to study its functional role(s) in insulin signaling and adipogenesis.

We show for the first time here that Atp10c mRNA is expressed in the undifferentiated and differentiated adipocytes both in vitro and ex vivo. We also demonstrate changes in Atp10c expression during adipose cell differentiation. Finally, we report that Atp10c expression in 3T3-L1 adipocytes is significantly altered by peroxisome proliferator-activated receptor-γ (PPARγ) agonists and antagonists as well as by the hormonal factors insulin and dexamethasone. A solid understanding of Atp10c mRNA will now assist in the future studies to understand the regulation of ATP10C protein.

Adipocyte differentiation involves a temporally regulated set of gene expression events, and understanding the underlying transcriptional networks is of fundamental importance to adipocyte biology. PPARγ, a member of the nuclear-receptor superfamily, is considered as a master regulator of adipogenesis. It is both necessary and sufficient for adipogenesis. Numerous pro- and anti-adipogenic factors work via PPARγ to activate or repress adipocyte differentiation [36, 37]. Almost every important cellular signaling pathway exerts a positive or negative effect on adipocyte development via factors that are still poorly understood. As a result, the identification and characterization of novel factors affecting these processes is ongoing. 3T3-L1 cells are being used extensively as an in vitro model and primary cultures derived from mouse adipose tissue as an ex vivo model to study the cellular and molecular aspects of adipocyte differentiation.

Interestingly, our data also shows that Atp10c is a novel factor negatively regulated during adipogenesis. We have shown that Atp10c mRNA is significantly down-regulated by the process of adipocyte differentiation and that these changes are not due to any experimental manipulation of the
cells in culture. The differential expression of *Atp10c* in preadipocytes and adipocytes is an example of cell type-specific expression similar to that of *pref-1*, where the gene is active in preadipocytes and is repressed in adipocytes [38]. *Atp10c* may be an important component of preadipocytes, functioning to maintain a preadipose state or to prepare the cells for the process of differentiation. The exact role of *Atp10c* in adipogenesis is not known at this time; however, our data clearly establishes a pattern completely opposite to that of PPARγ and some other well-characterized obesity genes like resistin. Our data suggests a regulatory control of *Atp10c* transcription during adipocyte development and lends further support to the use of the 3T3-L1 cell line. Specific experiments to study translational control and the effect of ATP10C - silencing or - overexpression on the other obesity/diabetes genes can now be accomplished.

PPARγ agonists, such as thiazolidinediones, promote adipocyte differentiation and have insulin-sensitizing effects in animals and diabetic patients [25-27]. Conversely, the PPARγ-specific antagonist GW9662 has been shown to inhibit adipogenesis and has been successfully used to study the regulation of gene expression during 3T3-L1 adipocyte differentiation [29]. Similarly, retinoic acid prevents and antagonizes PPARγ gene expression and is shown to be an inhibitor of adipocyte differentiation [39]. Thus, down-regulation of *Atp10c* expression by the PPARγ agonists MCC555 and TGZ along with restoration of *Atp10c* expression by PPARγ antagonists GW9662 and retinoic acid, suggest a possible relationship between PPARγ-mediated adipogenesis and *Atp10c* transcription. Whether a similar association exists between adipogenesis and ATP10C translation is to be determined.

Finally, our results also suggest transcriptional control of *Atp10c* expression by the hormonal factors insulin and dexamethasone during differentiation. Insulin is already known to regulate a number of mRNAs coding for obesity and diabetes genes [39, 40]. Hence, in this study, we prove that insulin regulates yet another novel gene, *Atp10c*, again suggesting it to be important in insulin signaling processes. Dexamethasone induces insulin resistance and stimulates *Atp10c* production, which is similar to its effect on *ob* gene expression and leptin production [41]. In contrast, lack of a significant effect by isoproterenol suggests that *Atp10c* expression is not controlled by adrenoceptors in 3T3-L1 adipocytes [42].

In summary, the results of this study strengthen our earlier hypotheses that *Atp10c* has a putative role in glucose and lipid metabolism. However, the exact mechanism is still unknown. Even though we have not yet proved our hypotheses we have certain clues to demonstrate that when the adipocytes are treated with dexamethasone, which is known to induce insulin resistance, *Atp10c* expression increases (Fig. 5), suggesting that an increased *Atp10c* expression might result in a loss of insulin sensitivity. If this is proved correct then conversely, a decrease in *Atp10c* expression should decrease insulin resistance thereby improve insulin sensitivity? This is supported by the expression patterns observed in presence of TZDs (Fig. 4).

In all types of obesity and type 2 diabetes, the major abnormality lies in the glucose uptake system. In peripheral tissues, insulin-stimulated glucose uptake is dependent upon the translocation of insulin-responsive glucose transporter GLUT4 from intracellular storage compartments, the trans-Golg network to the plasma membrane. Membrane trafficking of GLUT4 is a complex phenomenon involving three major steps — insulin signaling to GLUT4 vesicles, trafficking of GLUT4 vesicles to the plasma membrane and finally docking and fusion of GLUT4 vesicles with the plasma membrane [43, 44]. In view of our data, it is conceivable that the translocase activities of ATP10C protein mediate GLUT4 translocation at any one of these steps. Changes in *Atp10c* gene expression might affect the surface properties, membrane curvature, vesicle formation, fusion, or bilayer thickness/fluidity of the plasma membrane thereby affecting the trafficking and/or biogenesis of glucose transporters. Focused, hypothesis-driven experiments to address these questions can now be initiated in our cell systems.

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**REFERENCES**


