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β_3 -Adrenergic Signaling Acutely Down Regulates Adipose Triglyceride Lipase in Brown Adipocytes

Jeffrey A. Deiuliis,

Department of Animal Sciences, The Ohio State University, 2029 Fyffe Rd., Columbus, OH 43210, USA; The Ohio State University Interdisciplinary Human Nutrition Program, The Ohio State University, Columbus, OH 43210, USA

Li-Fen Liu,

The Ohio State University Interdisciplinary Human Nutrition Program, The Ohio State University, Columbus, OH 43210, USA; The Department of Human Nutrition, The Ohio State University, Columbus, OH 43210, USA

Martha A. Belury,

The Ohio State University Interdisciplinary Human Nutrition Program, The Ohio State University, Columbus, OH 43210, USA; The Department of Human Nutrition, The Ohio State University, Columbus, OH 43210, USA

Jong S. Rim,

Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA

Sangsu Shin, and

Department of Animal Sciences, The Ohio State University, 2029 Fyffe Rd., Columbus, OH 43210, USA

Kichoon Lee

Department of Animal Sciences, The Ohio State University, 2029 Fyffe Rd., Columbus, OH 43210, USA; The Ohio State University Interdisciplinary Human Nutrition Program, The Ohio State University, Columbus, OH 43210, USA

Kichoon Lee: lee.2626@osu.edu

Abstract

Mice exposed to cold rely upon brown adipose tissue (BAT)-mediated nonshivering thermogenesis to generate body heat using dietary glucose and lipids from the liver and white adipose tissue. In this report, we investigate how cold exposure affects the PI3 K/Akt signaling cascade and the expression of genes involved in lipid metabolism and trafficking in BAT. Cold exposure at an early time point led to the activation of the PI3 K/Akt, insulin-like signaling cascade followed by a transient decrease in adipose triglyceride lipase (ATGL) gene and protein expression in BAT. To further investigate how cold exposure-induced signaling altered ATGL expression, cultured primary brown adipocytes were treated with the β_3 -adrenergic receptor (β_3 AR) agonist CL

Correspondence to: Kichoon Lee, lee.2626@osu.edu.

Present Address: J. S. Rim, NuPotential, Inc., Louisiana Emerging Technology Center, Baton Rouge, LA 70808, USA

316,243 (CL) resulting in activation of PI3 K/Akt, ERK 1/2, and p38 signaling pathways and significantly decreased ATGL protein levels. ATGL protein levels decreased significantly 30 min post CL treatment suggesting protein degradation. Inhibition of PKA signaling by H89 rescued ATGL levels. The effects of PKA signaling on ATGL were shown to be independent of relevant pathways downstream of PKA such as PI3 K/Akt, ERK 1/2, and p38. However, CL treatment in 3T3-L1 adipocytes did not decrease ATGL protein and mRNA expression, suggesting a distinct response in WAT to β -adrenergic agonism. Transitory effects, possibly attributed to acute Akt activation during the early recruitment phase, were noted as well as stable changes in gene expression which may be attributed to β -adrenergic signaling in BAT.

Keywords

Nonshivering thermogenesis; Brown adipose tissue; Akt signaling; CL 316; 243; β -adrenergic receptor signaling

Introduction

Brown adipose tissue (BAT) in mammals is the site of nonshivering thermogenesis, which creates heat by actively oxidizing substrate via mitochondrial uncoupling protein (UCP1) without ATP production. Nonshivering thermogenesis, controlled by sympathetic neural-derived norepinephrine (NE) release [1], lowers metabolic efficiency while greatly increasing the thermogenic capacity of the animal. Application of our knowledge of BAT metabolism in mice to obesity-related health problems in humans is quite promising. β -adrenergic agonists, as well as cold exposure, have been shown to induce a brown fat phenotype in the white adipose tissue (WAT) of mice, resulting in metabolic inefficiency and prevention of diet and genetically induced obesity [1–3]. In addition, Tiraby et al. demonstrated that human white adipocytes can acquire [1, 2, 4] features of brown adipocytes [5]. Others have shown that β -adrenergic agonists and cold exposure results in the remodeling of some white fat depots with an induction of brown fat-like characteristics in a variety of mammals [4, 6–9]. The expression of UCP1 in WAT depots of adult humans has been shown to be variable [10]; a group of morbidly obese subjects were reported to have significantly lower UCP1 expression in the intraperitoneal adipose when compared to lean controls [11]. Cinti reported brown adipocytes were found dispersed among the WAT in 24% of individuals with the percentage increasing to 50% in adults under 50 years of age in 100 perirenal biopsies (32–87 years of age, $\bar{x} = 65$) [12]. Exposure to cool or cold ambient temperatures in humans may lead to increased BAT [13]. Adult humans with endocrine and non-endocrine pathologies have been shown to possess detectable UCP1 expression within periadrenal WAT depots. Presence of UCP1, a marker of brown adipose tissue, suggests certain hormonal signals may induce islets of brown adipocytes within WAT [3, 11]. For example, adult humans producing abnormally high amounts of adrenal catecholamines have been shown to regain BAT [3, 14, 15].

The process of BAT recruitment during chronic cold exposure occurs over weeks and requires major changes to cellular contents at a structural and enzymatic level in order to allow for maximal nonshivering thermogenesis. Much of this process is mediated by NE.

Each brown adipocyte is innervated by the ortho-sympathetic nervous system, which releases NE when stimulated [16]. NE binds to β_3 , α_1 , and α_2 -adrenergic receptors, thus affecting signaling events which effect lipolysis, thermogenesis, and apoptosis. Of the three β -adrenergic receptors, β_3 is the most common in rodents [17]. There is evidence that β_3 -adrenergic receptor (β_3 AR) couple to the G_s subtype of G proteins [18, 19]. This pathway has been suggested to be responsible for crosstalk between the β_3 AR and MAPK signaling pathways [20, 21]. The binding of NE or CL 316,243 (CL) to the β_3 AR results in the activation of adenylyl cyclase (AC), increased levels of cyclic AMP, and activated protein kinase A (PKA). In general, activated PKA phosphorylates many cellular protein substrates including signaling molecules (Src, ERK1/2, p38, and JNK). PKA activation leads to NE-induced lipolysis in brown adipocytes as demonstrated by the attenuation of lipolysis by H89, a PKA inhibitor [22]. Prolonged β -adrenergic stimulation and PKA activation in BAT leads to the processes necessary for sustained thermogenic activity. It is well known that transcription of lipoprotein lipase (LPL) is increased in BAT to mobilize fatty acid from the circulation during adaptation to chronic cold exposure. However, the roles of newly discovered lipolytic genes such as adipose triglyceride lipase (ATGL) and carboxylesterase 3 (Carb3) in BAT have not been studied.

Adipose triglyceride lipase (ATGL), a recently discovered triglyceride lipase that hydrolyzes the first ester bond of stored triacylglycerol (TAG) in adipocytes releasing non-esterified free fatty acids [23], has been shown to be the rate-limiting lipase in hormone-stimulated TAG hydrolysis [24]. ATGL and its homologues are associated with lipid droplets in eukaryotic cells and is highly upregulated in adipose tissue [25]. The product of ATGL TAG lipase activity, diacylglycerol, is hydrolyzed by activated hormone sensitive lipase (HSL). Thus, ATGL and HSL work in concert to mobilize free fatty acid stores from lipid storing tissues [23]. ATGL expression has been shown to be induced in mice by fasting over 12, 24, and 48 h with refeeding reducing ATGL expression [26]. Importantly, Smas and colleagues have shown that insulin activation of the PI3 K pathway leads to the down-regulation of ATGL gene expression with PI3 K inhibitors rescuing expression [27]. Dexamethasone upregulates ATGL gene expression; whereas, insulin, tumor necrosis factor alpha, and isoproterenol, downregulate ATGL gene expression in 3T3-L1 preadipocytes [26, 28]. Phosphorylation of ATGL has been demonstrated; however, it is not believed to be a substrate for phosphorylation by PKA [23, 29], leaving the mechanism by which ATGL is regulated undefined. The biochemical significance of the phosphorylation of ATGL remains to be shown. In this report, we investigate how cold stress and CL-mediated signaling affects ATGL and other lipolytic genes in BAT and primary brown adipocytes, respectively.

Materials and Methods

Animals and Cold Exposure

C57BL/6J mice (four males, 2 months old) were kept on a 12-h light/dark cycle and provided with food and water ad libitum. During cold exposure, 2–3 mice per pen were placed at 4 °C for 0.125, 0.25, 1, and 5–7 days. At the indicated time points, interscapular brown adipose tissue (four mice for each time point) was harvested. Animal experiments

were approved by the Pennington Biomedical Research Center Animal Care and Use Committee.

In Vitro and Ex Vivo Experiments

Interscapular brown fat was dissected from weanling mice. Primary cell culture was performed by mincing the pooled tissue of two mice, followed by digestion with 3 mg/mL type II collagenase (Sigma–Aldrich, St. Louis, MO) in DMEM media (10% FBS) with shaking (140 rpm) at 37 °C. Digesta was strained with a 100 µM cell strainer and spun at 400 × g for 5 min. The pellet (stromal vascular fraction—SVF) was re-suspended in growth media [Dulbecco's modified Eagle's medium (DMEM), 15% fetal bovine serum (FBS), antibiotics (50 units/mL penicillin, 50 µg/mL streptomycin)], seeded in a 12-well plate, and cultured in a humidified 5% CO₂ incubator. If greater numbers of wells were needed to accomplish an experiment more mice were pooled proportional to the additional wells needed to assure consistency of cells within an independent experiment. Cells were grown to 2 days post-confluence at which time growth media was replaced with differentiation media (DMEM, 15% FBS, antibiotics, insulin 5 µg/mL, dexamethasone 0.4 µg/mL, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 500 nM rosiglitazone) for 3 days. The cells were maintained in growth media (lacking differentiation factors) for another 4 days. At this point, the average well was 70–80% differentiated and showed obvious and abundant lipid accumulation. The adipose cell line, 3T3-L1, was purchased from American type culture collection (ATCC). Low passage number cells were grown to confluence in DMEM 10% FBS and differentiated 2 days post confluence with the same cocktail as the primary brown adipocytes. Primary and 3T3-L1 cells were treated on the same day of differentiation after 3 h of serum starvation. CL 316,243 (Sigma–Aldrich) was used at 1 µM for all experiments according to a dose response curve and the concentrations used in the literature. Inhibitors [H 89 (cAMP-dependent protein kinase inhibitor—Sigma–Aldrich), LY 294002 (PI3 K gamma inhibitor—Cell Signaling technology, Denver, MA), PD 98059 (MEK inhibitor—Cell Signaling Technology), SB 203580 (p38 inhibitor—Santa Cruz Biotechnology, Santa Cruz, CA)] were added 45 min before CL treatments at the concentration indicated in the figure legends.

Brown adipose tissue and white adipose tissue for ex vivo experiments was minced in the same manner as for digestion. The minced tissue was suspended in DMEM and aliquoted at equal volumes into 12-well plates. CL (1 µM) was added and the tissue was collected at the time points indicated, spun down, media removed, and frozen for analysis.

Quantitative-Real-Time PCR Detection of Total Gene Expression

RNA was isolated from BAT of mice using Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis. cDNA was reverse transcribed using approximately 1 µg of total RNA according the manufacturer's instructions (Invitrogen Life Technologies—M-MLV reverse transcriptase). Real-time PCR was performed using SYBR green I nucleic acid dye (Molecular Probes, Invitrogen detection technologies) on an ABI 7300. AmpliTaq Gold™ (Applied Biosystems, Foster City, CA) was used in all real-time reactions as was the following thermal profile: 95 °C 10 m, 40 cycles of 94 °C 30 s, 60 °C 60 s, 82 °C 30 s. The C_T values for the internal

control (cyclophilin) and target genes as determined by the ABI software were used to calculate gene expression. All target genes were normalized to cyclophilin and displayed as a fold increase in proportion to the zero hour time points. Randomly selected samples from all real-time runs were resolved by agarose gel electrophoresis to ensure the production of one product. In addition, dissociation curves were performed. Real time primers were designed to span genomic introns, thus avoiding amplification of genomic DNA possibly present in the RNA samples. Primer sequences can be provided upon request. “No template” negative controls were included in all PCR reactions to detect possible contamination.

Protein Isolation and Immunoblotting

Approximately 80 mg of BAT was cut to weight and homogenized in 800 μ L of lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 1 mM EDTA, 100 mM NaF, 100 μ M sodium orthovanadate, 1 mM PMSF, and 10 μ L/mL commercial protease inhibitor cocktail). The protein content of cell lysate was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL). Samples were separated by SDS-PAGE using the mini-Protean system (Bio-Rad, Hercules, CA). The protein was wet-transferred to a PVDF membrane (Amersham Biosciences, Uppsala, Sweden) and blocked in 5% nonfat dry milk (NFD) in 1x-TBST (0.1% Tween 20) and incubated with primary antibody (5% NFD) overnight at 4 °C specific to ATGL, P-Akt, P-ERK1/2, total ERK1/2, P-p38 (Cell Signaling Technologies, 1:1,000); β -actin (Santa Cruz Biotechnology, 1:2,000); A-FABP (R&D systems, Minneapolis, MN, 1:2,000); and H-FABP (Abcam, Cambridge, MA, 1:1,000). After washing in 1x-TBST, blots were incubated with the appropriated HRP-conjugated secondary antibody for 1 h at room temperature. Blots were washed before addition of ECL plusTM (Amersham Biosciences) and detection of bands with Kodak BiomaxTM film. Some immunoblotting used a fluorescent luminescence detection system. After washing in 1x-TBST, blots were incubated with fluorescence-labeled secondary antibodies (Irdye800 anti-rabbit IgG and Alexa680 anti-mouse IgG). Bands were visualized using the Odyssey imaging system (LI-COR Bioscience, Lincoln, NE).

Glycerol Release Assay

Differentiated primary mouse brown adipocytes were serum starved for 3 h in phenol red-free, serum-free DMEM. Those cells receiving PD 98059 (50 μ M) and CL (1 μ M) were pretreated with the inhibitor 45 min before stimulation. The media was collected after 3 h and centrifuged to remove cells and cellular debris. Glycerol content of conditioned medium was determined using the “free glycerol reagent” kit (Sigma-Aldrich). The final concentration of free glycerol was determined according to the kit instructions using a free glycerol standard and normalized to the protein content of cell lysates.

Statistical Analysis

All statistical analysis was performed using SASTM. Gene expression was analyzed using one-way ANOVA analysis followed by Fisher’s Protected LSD. Densitometry for immunoblotting was analyzed as a randomized complete block design in order to measure how dependent the effect of CL was on the presence of the inhibitor. Treatments were compared via Fisher’s Protected LSD. The minimum level of significance was set at $P < 0.05$. All statistical values using primary cultured brown adipocytes are derived from three

or greater independent experiments cultured from separate isolations/differentiations of primary mouse preadipocytes isolated from interscapular brown adipose (2 mice per 12 well dish—see “Materials and Methods”) unless otherwise specified.

Results

Cold Stress Modulates Fatty Acid Binding Protein and Fatty Acid Transport Expression Levels in BAT

Fatty acids are the main substrate oxidized during BAT-mediated nonshivering thermogenesis. Among several fatty acid binding proteins, A-FABP, H-FABP and mal1 (FABP5) have been shown to be expressed in BAT tissue of mice [30]. The increased expression levels of specific FABPs are putatively explained by a greater need for fatty acid metabolism and trafficking in BAT than other bodily tissues. We tested the response of these genes within BAT to cold exposure. The level of FABP5 was not significantly changed during 1 week of cold exposure (data not shown). The levels of A-FABP gene expression decreased gradually, reaching a significantly lower level (60% reduction) at day 1 and returned to pre-cold exposure control levels (Fig. 1c). In contrast, the levels of H-FABP gene expression linearly increased with increasing time achieving statistical significance in 6 h and a fivefold induction at day 5–7 in BAT of mice (Fig. 1a). It has been shown that FABP concentrations are largely controlled at the transcriptional level [31]. In order to confirm the upregulation, we performed immunoblotting demonstrating H-FABP gene expression corresponded with an increase in protein levels in vivo and decreases in A-FABP gene expression corresponded with decreases in protein levels (Fig. 1b). Since transmembrane transport of free fatty acids is actively facilitated by transporter proteins, we assayed the gene expression of the fatty acid transporter, FAT/CD36. FAT/CD36 gene expression increased dramatically at 6 h, returning to normal by 5–7 days (Fig. 1d). The opposing trends in A- and H-FABP expression during cold exposure suggest they play different roles in fatty acid mobilization during cold stress.

Cold Induces Down-Regulation of Lipolytic Genes in BAT

In addition to fatty acid transport, lipolysis plays an important role in the utilization of fatty acid in BAT. In order to better understand lipolysis in BAT during cold exposure, we analyzed the expression of the major lipolytic genes. Real-time gene expression analysis of three lipolytic enzymes, i.e., ATGL, carboxylesterase 3, and HSL, revealed ATGL levels decreased initially and gradually increased; whereas, carboxylesterase 3 (EC 3.1.1.1) and HSL expression levels were consistently depressed in BAT of mice during cold exposure (Fig. 1e, g, h). In addition, ATGL protein levels were transiently decreased during the early period of cold exposure but returned to normal (Fig. 1f). The rapid changes in gene and protein levels in BAT in vivo indicated there was an acute response to cold exposure.

Cold Exposure Affects BAT Intracellular Signaling Events

To better understand the acute response to cold exposure, we focused on the main intracellular signaling pathway controlling insulin and fatty acid utilization, PI3 K/Akt. We found cold exposure increased phosphorylation of Akt at serine 473 with an increase obvious at 30 min and maximal induction at 1 h (Fig. 2). Activated Akt was maintained

through day 2 and returned to control levels by day 7. Detection of Akt phosphorylation at threonine 308 was increased steadily with maximal phosphorylation on day 1. Phosphorylated GSK-3 β levels, a downstream target of pAkt, increased in parallel with Akt signaling as expected. Antagonists of Akt signaling, such as pPTEN, lipid phosphatase and tensin homologue slightly decreased during cold exposure when Akt activation was maximal. Total Akt, as well as β -actin, remained consistent during treatment. ATGL levels were decreased due to cold exposure. Previous reports demonstrated that insulin treatment/insulin receptor substrate signaling (IRS) decreased ATGL expression and that LY294002 rescued the down-regulation of ATGL by blocking PI3 K [27, 28, 32]. Analyzing our findings in the context of the literature on ATGL and lipolysis, we hypothesized that PI3 K/Akt signaling antagonized the lipolytic system by downregulating ATGL levels. In order to test this hypothesis mechanistically, we used ex vivo mouse brown adipose, primary mouse brown pre-adipocytes grown and differentiated in culture and a mouse adipocyte cell line.

The β_3 AR Agonist, CL 316,243, Down-Regulates ATGL Protein and Gene Transcript Levels in Mouse Brown Adipocytes, but not White

It is well documented that NE signaling via the β_3 -adrenergic receptor controls the thermogenic process; thus, we utilized a β_3 AR agonist in ex vivo and in vitro analysis of brown adipose/adipocyte signaling. Treatment of small segments of BAT ex vivo with CL showed activation of Akt at 3 and 6 h and a decrease of ATGL protein (Fig. 3a). We continued this line of investigation in primary mouse brown adipocytes that were differentiated in vitro. In order to validate the system, we tested the effects of CL (1 μ M) on UCP1 gene expression in differentiated brown adipocytes. CL administration resulted in a ~20 fold increase ($P < 0.01$) in UCP1 gene expression in 3 h (Fig. 3b). In addition, CL (1 μ M) treatment of primary brown adipocytes induced lipolysis as indicated by an approximate three-fold increase in glycerol release ($P < 0.01$). In order to determine the most effective dose of CL on ATGL expression in our in vitro system, we performed a dose response curve, measuring ATGL protein levels at 3 h post treatment (Fig. 3c). The levels of ATGL protein were decreased by CL in a dose-dependent manner. One μ M showed consistent efficacy in reducing ATGL protein levels and was the dose used in the majority of the literature on β_3 -adrenergic signaling. WAT, like brown, expresses the β_3 AR and is sensitive to CL-activated PKA signaling. We show, however, that CL administration did not result in a significant change in ATGL protein levels in 3T3-L1 cells and WAT ex vivo (Fig. 3d, e).

CL 316,243 Activates Akt, However, Blockade of PI3 K Does not Rescue ATGL Protein Levels

CL 316,243 (1 μ M) administration down-regulated ATGL protein and potently activated Akt ex vivo and in vitro. Activation of Akt by the β_3 AR signaling is non-classical and involves crosstalk between G-protein coupled receptors (GPCR) and the PI3 K signaling pathway. In order to determine if PI3 K signaling caused the decrease in ATGL protein levels, we blocked Akt activation with the PI3 K inhibitor LY294002. However, blocking Akt activation did not prevent ATGL down-regulation in vitro (Fig. 4b, c). In addition, insulin treatment potently activated PI3 K but did not result in decreased ATGL protein levels in

differentiated primary brown adipocytes (Fig. 4b). These findings led us to conclude that the PI3 K/Akt signaling pathway does not antagonize PKA signaling as it does in other tissues. From this point, we focused on classical β_3 AR-signaling as a putative ATGL regulatory mechanism in BAT.

β_3 AR Signaling-Mediated Degradation of ATGL is PKA Dependent in Brown but not White Adipocytes

We show that inhibition of PKA signaling by H89 rescues the down-regulation of ATGL (Fig. 4a, b). An H89 dose curve with CL (1 μ M) shows that 10 μ M H89 was most effective at rescuing ATGL (Fig. 4a). This is also a commonly used dose in the literature for inhibition of PKA signaling. Interestingly, CL and H89 treatment did not modulate ATGL protein levels in differentiated 3T3-L1 adipocytes at 3 h (data not shown).

Adipose triglyceride lipase can exist as a phosphoprotein, although it has been reported that ATGL is *not* a direct substrate of PKA [23, 29]. Due to the rapid decrease in ATGL levels after β_3 AR agonism, we hypothesized that ATGL phosphorylation leads to degradation via an intermediate downstream of PKA. In order to identify this intermediate, we focused on known signaling pathways downstream of β_3 AR-mediated lipolysis, including ERK 1/2 and p38 signaling [33]. CL administration potently activated ERK1/2 (Fig. 5a). The inhibition of ERK 1/2 by PD 98059, however, did not rescue ATGL protein levels. We do show that inhibition of ERK 1/2 by PD 98059 led to a significant inhibition of CL-mediated lipolysis. This shows that ERK1/2 signaling is important in β_3 AR-mediated BAT lipolysis. Also, brown adipocytes are capable of increased lipolysis concurrent with significant decreases in ATGL protein levels. H89 attenuated CL-mediated upregulation of UCP1 gene expression by blocking PKA-mediated phosphorylation of p38 [34]. Therefore, we assessed the role of p38 signaling on ATGL expression. CL treatment results in p38 activation; however, the p38 blockade by 10 and 25 μ M of SB 203580 did not rescue ATGL protein expression in cultured primary brown adipocytes (data not shown).

Adipose triglyceride lipase protein levels decreased significantly after only 30 min of CL treatment and reached maximal downregulation by 1 h which was maintained to the 3 h time point (Fig. 6a). H89 pretreatment rescued ATGL protein levels 1 and 3 h after CL treatment. In order to determine if the decrease in ATGL protein was due to decreased transcription of ATGL gene expression, we treated the primary mouse brown adipocytes with 1 μ M of CL over a time course. ATGL gene expression showed a downward trend which became significant after 3 h as in the *in vivo* study (Fig. 6b). CL administration results in decreased ATGL transcription, however, ATGL protein levels decrease at a greater rate than can be attributed to transcriptional effects. Our data suggest that the decrease in ATGL protein is partially due to the protein degradation via PKA signaling. Thus, we hypothesized that ATGL was being degraded by 26S proteasome hydrolysis. In order to test this hypothesis, we pretreated primary brown adipocytes with the proteasome inhibitor MG 132 (25 μ M) for 1 h then stimulated the cells with CL. The cells were collected at 3 h. However, blockade of the proteasome did not prevent ATGL degradation (Fig. 7), suggesting degradation by an alternative pathway.

Discussion

The nine currently identified cytoplasmic fatty acid binding proteins (FABP_C) belong to the superfamily of lipid binding proteins. FABP_C all possess similar protein structures but show tissue specific expression [35]. This tissue specific expression pattern suggests unique or specialized functions for the various FABP_C. H- and A-FABP proteins are FABP_C that function to increase cytoplasmic diffusion and transport of FFA from the plasma membrane [36, 37]. A-FABP is exclusively expressed in adipose tissue where it has been shown to be involved in increasing the solubility and aiding in trafficking of free fatty acids between cellular compartments. Whereas, H-FABP is mainly expressed in cardiac and skeletal muscles where fatty acid oxidization is a major source of energy. For example, H-FABP increased in rat heart and skeletal muscle tissues in conditions of increased fatty acid oxidation, such as diabetes and fasting, respectively [38, 39]. In humans, increased H-FABP expression is correlated to increased fatty acid oxidation in type two diabetic individuals undergoing dietary and exercise interventions [40]. In the current studies, the dramatic increase in H-FABP transcription contrasted by the decrease in A-FABP transcription suggests that cold shock alters the fatty acid trafficking within the brown adipocytes. We propose that the FFA flux into the BAT has a direct or indirect affect upon the transcription of A- and H-FABP. While H-FABP's precise role in intracellular trafficking and fat metabolism is undefined, our study suggests that H-FABP may function to transport/direct the influx of intracellular FFAs to the mitochondrial FFA transport system (CPT1) for oxidation thus fueling non-shivering thermogenesis. Finally, the FFA influx and trafficking in BAT has a direct/indirect affect upon the transcription of A- and H-FABP.

Nonshivering thermogenesis requires the up-regulation of β -oxidation and uncoupled oxidative phosphorylation in BAT. Cold stress-induced NE release stimulates the β_3 and α_2 -adrenergic receptors and has profound effects on BAT including upregulation of lipolysis and UCP1 expression, promotion of proliferation and differentiation, and inhibition of apoptosis [17]. It has been shown recently that in vitro stimulation of the β_3 -adrenergic receptor by NE, isoprenaline, and CL induced glucose uptake in brown adipocytes in culture by increasing GLUT1 gene expression with decreasing GLUT4 expression [41]. Adams and colleagues showed that cold exposure from 1 to 48 h increased glucose uptake and de novo lipogenesis while increasing β -oxidation [42]. Our in vivo cold exposure data show phosphorylation of GSK-3 β , suggesting an increase in glycogen synthesis in BAT during cold exposure. This finding complements the work of others [41, 42] showing increased glucose uptake in brown adipocytes upon NE or cold exposure. PI3 K/Akt signaling is the main regulator of GLUT4 translocation to the plasma membrane due to the effects of insulin [43]. Thus, β_3 AR mediated activation of PI3 k/Akt may function to increase glucose uptake in the tissue as well as increase sensitivity to circulating insulin levels. This response appears to be specific to BAT, as PI3 K/Akt signaling normally antagonizes the lipolytic pathway. In addition, muscle and WAT exhibit reduced sensitivity of PI3 K/Akt signaling to insulin in the rat during cold exposure [44].

Current studies show in cold exposure that ATGL is transiently down-regulated with a corresponding increase in Akt signaling in vivo. During cold exposure, changes in ATGL expression were more sensitive and occurred before the decrease in the other lipases, HSL

and Carb3. Also, supporting the sensitivity of ATGL to metabolic stress are data from mouse fasting and refeeding studies where ATGL expression changes in the WAT according to positive or negative energy balances [26]. A recently published article shows that noradrenaline-induced lipolysis was positively correlated with HSL protein levels, but not with ATGL protein levels in the WAT of women [45]. In agreement with this, CL treatment did not affect ATGL protein levels in 3T3-L1 adipocytes or in WAT treated ex vivo. However, defective cold adaptation of ATGL knockout mice clearly indicates an important role of ATGL-mediated lipolysis in WAT to supply BAT with free fatty acids. Considering no change in expression of ATGL protein in response to CL, ATGL activity can be regulated by other interacting proteins. The recent discovery of two proteins, comparative gene identification-58 (CGI-58) and the G0/G1 switch gene 2 (G0S2) that physically interact with ATGL protein, increases the complexity of ATGL regulation. CGI-58 functions as an activator of ATGL without affecting HSL activity, increasing lipolysis [46]. In contrast to CGI-58, interaction of G0S2 with ATGL decreased ATGL-mediated lipolysis, acting as a negative regulator of ATGL [47]. Up-regulation of G0S2 by anti-lipolytic hormone insulin and drastic down-regulation by β -adrenergic agonist isoproterenol or another lipolysis-inducing hormone, tumor necrosis factor α , demonstrated the importance of G0S2 as a regulator of ATGL activity in response to metabolic hormones [47]. This implicates possible mechanisms by which alteration of G0S2 amounts affect ATGL activity without changing ATGL protein levels in WAT in response to CL.

It has been reported that the ATGL gene is a target for PPAR γ transactivation [27]. Decreases in PPAR γ levels have been shown in primary brown adipocytes treated with NE as well as in white adipocytes treated with β_3 AR agonists, leading to its degradation and lowered transcription of PPAR γ mediated genes [48, 49]. Thus, the decreases in ATGL levels during cold-stress in vivo may be due to decreases in PPAR γ levels. ATGL gene expression has been shown to be down-regulated in 3T3-L1 cells, a mouse adipocyte cell line, by insulin and isoproterenol administration [27, 28]. Smas et al. [27] went on to show that insulin activation of the PI3 K pathway leads to the down-regulation of ATGL with PI3 K inhibitors rescuing transcript levels. Rapid down-regulation of ATGL by both anabolic and catabolic hormones is intriguing and is as of yet unexplained. Hormones that increase lipolysis such as CL and isoproterenol paradoxically decrease ATGL gene and protein levels. This would suggest that catecholamine-induced lipolysis is partially independent of ATGL protein levels and most probably relies on regulation of ATGL activation. Zimmerman et al. [23] showed that ATGL is a phosphoprotein. Phosphorylation is a key signal for ubiquitination and 26S proteasomal catabolism of proteins [50]. However, ATGL was demonstrated *not* to be a substrate for PKA [23]; however, our data suggests ATGL is an indirect target of PKA signaling in brown adipocytes. In addition, our data show the proteasome inhibitor MG 132 does not rescue ATGL protein levels indicating β_3 AR signaling causes ATGL protein level decreases due to non-proteasomal degradation. ERK 1/2 plays a role in MAPK signaling mediated lipolysis and can be activated by PKA. However, while blocking ERK 1/2 decreases lipolysis in primary brown adipocytes by ~20%, it does not rescue ATGL protein levels. Similarly, the p38 signaling pathway does not mediate PKA-dependent ATGL degradation. Importantly, current studies show that CL

activates Akt in primary mouse brown adipocytes and decreases ATGL protein levels via a PKA dependent, ERK 1/2, p38, and Akt-independent mechanism.

The current study showed that cold exposure at early time points elicits β_3 AR and insulin-like signaling in BAT and primary brown adipocytes, including transient decreases in ATGL gene and protein levels. In this situation, fatty acids from the diet and mobilization of fatty acids from WAT can be a primary source for nonshivering thermogenesis in BAT rather than the break down of TAG stored in BAT. Also, appetite and glucose uptake in BAT increases during the early stage of cold exposure [42] providing metabolic substrates for nonshivering thermogenesis. Under conditions of long-term cold exposure with limited dietary glucose and circulating fatty acids, the TAG in BAT will most likely be hydrolyzed by ATGL to produce an energy substrate for nonshivering thermogenesis. H-FABP expression increases purportedly to shuttle fatty acids within the cell to the mitochondria as an oxidative substrate for nonshivering thermogenesis. Further investigation into the crosstalk between the PI3 K/Akt and the β_3 -adrenergic pathway and how they affect lipid storage, fatty acid mobilization, transport, and utilization within BAT is warranted. A better understanding of the distinct differences between PI3 K and β_3 AR crosstalk in WAT and BAT is important to endocrinology, adipose physiology, and metabolism. Finally, β_3 AR agonists are exciting potential targets for pharmaceutical therapy of obesity; however, a better understanding of the mechanisms underlying this process is needed and will advance clinical management of obesity.

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Abbreviations

AC	Adenylyl cyclase
A-FABP	Adipocyte-type fatty acid-binding protein
ATCC	American type culture collection
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
β_3AR	β_3 -adrenergic receptor
Carb3	Carboxylesterase 3
CL	CL 316,243
CPT1	Carnitine palmitoyltransferase I
DMEM	Dulbecco's modified Eagle's medium

FABP5	Fatty acid binding protein 5
FAT/CD36	Fatty acid transporter/Cluster of Differentiation 36
FBS	Fetal bovine serum
GLUT	Glucose transporter
GPCR	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase 3 β
H 89	cAMP-dependent protein kinase inhibitor
H-FABP	Heart-type fatty acid binding protein
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
IRS	Insulin receptor substrate
LPL	Lipoprotein lipase
LY 294002	PI3 K gamma inhibitor
NE	Norepinephrine
PI3 K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PTEN	Phosphatase and tensin homolog
SB 203580	p38 inhibitor
TBST	Tris-buffered saline with Tween 20
TAG	Triacylglycerol
UCP1	Uncoupling protein 1
WAT	White adipose tissue

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Fig. 1.

Lipase expression in BAT of mice with cold exposure. All graphs represent real-time PCR analysis ($n = 4$). Target genes as a ratio to the housekeeping gene cyclophilin are expressed in fold difference to control. **a** Gene expression levels of H-FABP increased in a linear fashion with time of exposure. **b** The corresponding protein levels of H-FABP and A-FABP were measured by immunoblotting, confirming the gene expression pattern. **c** Real time PCR analysis of A-FABP gene expression decreased with cold exposure and was significantly ($P < 0.05$) lower at day 1 when compared to control mice (0 h). **d** FAT/CD36 fatty acid transporter gene expression increased acutely (6 h) in the BAT during cold

exposure only to return to control levels by 5–7 days. **e** ATGL gene expression in the brown adipose of mice dropped significantly after 3, 6, and 24 h of cold exposure. However, expression rebounded reaching near normal levels after 7 days. **f** The corresponding protein levels of ATGL were measured by immunoblotting, confirming an initial decrease followed by normalization and an increase in ATGL protein levels as shown in this representative blot from three independent experiments ($n = 3$). **g** HSL gene expression was significantly decreased at all times during cold exposure. **h** Carboxylesterase 3.0 gene expression was significantly decreased at 6 h, 1 day, and 5–7 days when compared to 0 h control as measured by real-time PCR. The letters (*a*, *b*, *c*, *d*) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$

Fig. 2.

Akt and related signaling in the BAT of cold exposed-mice. Phosphorylation and total proteins in BAT of mice during cold exposure are presented by immunoblotting. Phosphorylation of Akt, GSK, PTEN are reported as well as the loading control, β -actin. Data represent three independent experiments ($n = 3$)

Fig. 3.

ATGL and phosphorylated Akt in BAT ex vivo and in differentiated primary brown adipocytes. **a** CL (1 μ M) decreases ATGL protein levels after 3 h in BAT ex vivo at 3 and 6 h. CL activated Akt in BAT ex vivo similar to the results obtained from in vivo cold exposure. **b** CL increased UCP1 gene expression in primary mouse brown adipocytes and significantly (**, $P < 0.01$) induced lipolysis as measured by glycerol release ($n = 3$). **c** CL treatment lowered ATGL protein levels in this representative immunoblot after 3 h at even nanomolar concentrations with maximal effects being observed at 1 μ M. **d, e** Immunoblotting results ($n = 3$) show that β_3 AR signaling in 3T3-L1 adipocytes ($n = 3$) and WAT ex vivo (two independent experiments with pooled adipose tissues from two mice per experiment) does not result in the same decrease in ATGL as observed in brown adipocytes. Both BAT and WAT express the β_3 AR, however, β_3 AR agonism results in depot-specific regulation of ATGL expression levels

Fig. 4.

Inhibition of PKA signaling, not Akt signaling, rescues ATGL protein levels in a dose-dependent manner. **a** The PKA inhibitor, H89, rescues the CL-mediated decrease in ATGL protein in a dose-dependent manner at 3 h in primary brown adipocytes as shown in this immunoblot. **b, c** CL as well as 100 nM insulin potently activated Akt, however, blockage of Akt signaling did not rescue ATGL protein levels. The letters (*a, b*) represent a significant difference among groups by using one-way ANOVA at $P < 0.05$. Statistical values are derived from three independent experiments ($n = 3$) using primary brown adipocytes cultured from separate isolations/differentiations of primary mouse preadipocytes isolated from interscapular brown adipose (2 mice per 12 well dish—see “Materials and Methods”)

Fig. 5.

Activation of ERK 1/2 in brown adipocytes by CL; ERK 1/2 are not downstream of CL-PKA-mediated ATGL downregulation. **a** CL potently activated ERK 1/2, which correlates with a decrease in ATGL protein levels; however, blocking ERK 1/2 with PD 98059 (50 μ M) does not rescue ATGL expression ($n = 3$). **b** CL potently induces lipolysis in brown adipocytes as measured by glycerol release. ERK 1/2 inhibition resulting in a significant ($P < 0.01$) decrease, approximately 20%, in β_3 AR-stimulated lipolysis ($n = 3$). The letters (a, b, c) represent a significant difference among groups by using one-way ANOVA at $P < 0.05$

Fig. 6.

CL-PKA-mediated ATGL protein degradation occurs rapidly while effects on transcript lag.

a Immunoblotting ($n = 3$) shows that CL administration results in a significant decrease in ATGL protein levels in as little as 30 min ($P < 0.05$). H89 pretreatment rescues ATGL protein. **b** ATGL gene expression is significantly reduced only at 3 h post CL administration and is rescued by H89 at 1 and 3 h ($n = 3$). The letters (*a*, *b*, *c*, *d*) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$

Fig. 7.

ATGL protein level decrease in CL 316,243-treated cells is not due to 26S proteasomal degradation. **a** Inhibition of the proteasome by MG 132 (25 μ M) does not rescue CL-mediated ATGL protein degradation at 3 h as shown in this representative immunoblot ($n = 3$). The letters (*a*, *b*) represent a significant difference among groups by using one-way ANOVA at $P < 0.05$