

Activation of estrogen receptor alpha induces beiging of adipocytes



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ABSTRACT

Objectives: Brown adipose tissue (BAT) and BAT-like adipose tissues, referred to as 'beige' adipose tissues uncouple respiration from ATP synthesis via uncoupling protein one (UCP-1). There is a sexual dimorphism with respect to beige and BAT tissues; pre-menopausal women have more BAT and are more sensitive to BAT activation than men or postmenopausal women. We hypothesized selective activation of adipose tissue estrogen receptor alpha (ER α) induces beiging of WAT through induction of lipolysis mediated by adipose tissue triglyceride lipase (ATGL).

Methods: 3T3-L1 and primary adipocytes were treated with the selective ER α agonist pyrazole triol (PPT), and selection deletion of ER α (using siRNA) was used to determine if selective ER α activation, or inhibition, influences the adipose tissue expression of genes associated with beiging. In a second series of experiments, ER α was selectively added back to adipose tissue of mice lacking total body ER α (ERKO) to determine if add back of ER α changed the morphology of adipose tissue to resemble beige tissues. Additionally, WT and ERKO mice were exposed to cold and FDG labeled glucose uptake was measured to determine the ability of cold to induce UCP-1 in ERKO mice. To begin to mechanistically probe *how* activation of ER α facilitates beiging, we tested the influence of PPT to activate the lipolytic pathway through ATGL. Finally, since ER α exerts its effects both at the genomic and non-genomic level depending on its cellular location, we determined *in vivo* if beiging occurs in mice expressing ER α only at the plasma membrane (MOER mice) or only at nucleus (NOER mice).

Results: Selective ER α activation by PPT increased markers of beiging *in vitro* in 3T3-L1 and primary adipocytes, whereas, knockdown of ER α with siRNA reduced the ability of PPT to induce beiging *in vitro*. ER α add back to the adipose tissue of ERKO mice resulted in multilocular adipose tissue resembling a beige phenotype. Following cold exposure, FDG labeled glucose in BAT tissues of ERKO mice was reduced when compared to weight-matched controls. Glycerol release and ATGL expression were increased after PPT treatment, while pre-treatment with the ATGL inhibitor prevented PPT's ability to increase UCP-1 expression. Finally, MOER mice were more sensitive to beiging of adipose tissues when compared to NOER mice.

Conclusion: Our results demonstrate for the first time that selective-activation of ER α in adipocytes increases markers of beiging and this is likely through induction of AMPK and ATGL-mediated lipolysis providing FFAs as a fuel to activate UCP-1.

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Keywords Beige adipocytes; Lipolysis; Estrogen receptor alpha (ER α); Obesity; Type II diabetes mellitus (T2DM)

1. INTRODUCTION

Obesity is characterized by an excessive accumulation of white adipose tissue (WAT), which is a predisposition for the development of insulin resistance and type 2 diabetes mellitus (T2DM) and causes an increased risk of morbidity and mortality [1]. Caloric restriction and physical exercise can reduce obesity-related comorbidities; however, these interventions require rigorous, long-term adherence for success, making compliance challenging. One possibility to reduce obesity and T2DM is to convert WAT storage tissues into metabolically active tissues analogous to brown adipose tissue (BAT), and this process is known as beiging of adipose tissues [2]. BAT and beige tissues are functionally characterized by their unique ability to uncouple mitochondrial respiration from ATP synthesis via uncoupling protein 1 (UCP-1), allowing for enhanced free fatty acid (FFA) oxidation and heat

production [3]. The recent discovery that BAT/beige tissues exist in adult humans has led to the concept that increasing their activity is a therapeutic strategy to reduce body weight and improve insulin sensitivity in humans [4–6]. Although several approaches have been proposed to induce beiging of WAT, most of them rely on activation of the sympathetic nervous system (SNS) [7–9]. Briefly, activated SNS releases norepinephrine, which binds to adrenergic receptors in BAT and beige tissues, promoting UCP-1 activation and heat production. However, adrenergic activation cannot be used therapeutically in humans because of its adverse effects on other tissues; therefore, there is a critical need to identify novel targets to safely and specifically activate BAT and beige tissues to reduce obesity and its comorbidities. Lipolysis is a key mechanism underlying activation of UCP-1 and heat production in BAT and beige tissues. There are data in humans and rodents indicating BAT and beige tissues utilize triglycerides stored in

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lipid droplets, and, following adrenergic stimulation, there is a release of FFAs, which are then used as a primary fuel for oxidation [6,10]. Perturbations of FFA mobilization are hallmarks of obesity that are associated with insulin resistance and T2DM. Specifically, adrenergic stimulation initiates the canonical adrenergic receptor-G_s-adenylyl cyclase-cAMP-PKA pathway [11], which in turn activates adipose tissue triglyceride lipase (ATGL) and other lipases such as hormone sensitive lipase (HSL) [12]. While whole body or adipose tissue specific ATGL knockout mice are unable to maintain body temperature upon cold exposure by activation of adrenergic signaling, HSL knockout mice are not cold insensitive, suggesting that ATGL is crucial for being of adipose tissues and cold tolerance [12]. Interestingly, the addition of FFAs to the culture medium of brown adipocytes stimulates thermogenesis in the absence of adrenergic stimulation [11], suggesting there might be non-adrenergic mechanisms which induce lipolysis and thereby, BAT/beige tissue stimulation.

There is a sexual dimorphism with respect to BAT and beige tissues, as demonstrated by the fact that premenopausal women have more BAT and are more sensitive to BAT/beige activation than men [13] or postmenopausal women. These data further suggest that sex hormones, and perhaps specifically estrogens and/or estrogen receptors, may be involved in mediating BAT and beige tissue activity. Estrogen receptor alpha (ER α) exists in numerous cell types to include the central nervous system (CNS) [14], as well as adipocytes [15], two tissues critical for the activation of BAT and beige tissues. We and others have demonstrated that ER α impacts whole body energy expenditure and glucose homeostasis in a tissue-specific manner [16,17]. Furthermore, glucose homeostasis is impaired in humans with polymorphisms in the ER α gene [15,18], while global ER α knockout mice (ERKO) are obese, insulin resistant, and dyslipidemic [19]. We and others have shown that ER α is involved in regulating adipose tissue function [14,20–22], and knockdown of ER α from adipocytes results in increased adipocyte size (hypertrophy) and number (hyperplasia), as well as increased adipose tissue inflammation and fibrosis [14,23]. Additionally, activation of ER α induces lipolysis in adipose tissues [24]. Due to the sexual dimorphism in BAT and beige tissues in humans and the critical role of ER α to modulate adipose tissue function, we hypothesize that activation of adipose tissue ER α induces being of WAT through induction of lipolysis which is mediated by the lipolytic factor ATGL.

2. MATERIAL AND METHODS

2.1. Cell culture

2.1.1. 3T3-L1 cells

Mouse 3T3-L1 pre-adipocytes (Cat. number CL-173TM) were obtained from American Type Culture Collection (ATCC[®], Manassas, VA, USA), and their growth and differentiation were performed according to ATCC[®]'s protocol [25]. Briefly, cells were grown in DMEM high glucose (ATCC[®], Cat. number 30-2002) supplemented with 10% Bovine Calf Serum (BCS) (ATCC[®], Cat. number 30-2031), and after 48 h they reached confluency. Cells were induced to differentiation in DMEM containing 10% Fetal Bovine Serum (FBS) (GibcoTM, Thermo Fisher ScientificTM, Waltham, MA, USA, Cat. number 26140-079) and supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma[®], St Louis, MO, USA, Cat. number 858455), 1 μ M dexamethasone (DEXA) (Sigma[®], Cat. number D1756), and 1 μ g/ml insulin (Sigma[®], I0516). 48 hrs after induction of differentiation, cells were maintained in DMEM supplemented with 10% FBS and 1 μ g/ml insulin for 8–10 days until they reached full differentiation and were ready to be used in

experiments. Cells were then exposed to DMEM phenol-free medium (GibcoTM, Cat. number 21063-029) supplemented with 10% FBS charcoal:dextran stripped (Gemini-Bio Products, West Sacramento, CA, USA, Cat. number 100-119) and 1% sodium-pyruvate (GibcoTM, Cat. number 11360-070) for 24 h prior to treatments. To begin to determine if there is a correlation between ER α and UCP-1 expression, we assayed ER α and UCP-1 mRNA at the same time point across a time course during the differentiation process (days 0, 4 and 10) of 3T3-L1 cells. To test if ER α activation in mature adipocytes promotes markers of being, differentiated 3T3-L1 adipocytes were treated with the ER α agonist propyl-pyrazole (PPT) (Tocris Bio-Techne Corporation, Minneapolis, MN, USA, Cat. number 1426) in the concentration of 10 nM, according to our previous publication [21] for the duration of 5 min to 5 h. Gene expression for makers of being to include: *Ucp1*, *Ppargc1 α* , *Pdk4*, *Tnfrsf9*, and *Tbx1* was determined by qPCR, and UCP-1 protein was measured by WB, as described below.

2.1.2. Primary adipocytes

Pre-adipocytes from female WT C57BL/6J mice were obtained and differentiated into adipocytes according to published protocols [26,27]. Briefly, dissected inguinal WAT (iWAT) from mice was minced in PBS and then digested for 2 h in buffer containing 100 mM HEPES (pH 7.4), 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 M CaCl₂, 1.5% BSA, and 1 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN, USA, Cat. number 11088866001). Digested tissues were then diluted in Stromal Vascular Fraction (SVF) media composed of DMEM/F12 (GibcoTM, Cat. number 10565-018) and 10% FBS, filtered through a 100 μ m cell strainer, and spun down for 5 min at 600 g to pellet the SVF and separate the adipocytes. The SVF pellet was resuspended in SVF medium, filtered through a 40 μ m cell strainer and centrifuged again. At the end, the final pellet was resuspended in SVF media and plated. Cells were grown in SVF media until confluency, and 48 h later, they were differentiated in SVF media containing 0.5 mM IBMX, 1 μ M DEXA, 5 μ g/ml insulin, and 1 μ M rosiglitazone (Sigma[®], Cat. number R2408). Cells were then maintained in SVF media supplemented with 5 μ g/ml insulin for 8–10 days. Differentiated adipocytes were exposed to DMEM/F12 phenol-red free medium (GibcoTM, Cat. number 21041-025) and supplemented with 10% stripped FBS for 24 h. In order to test the role of ER α activation to induce markers of being in primary adipocytes, differentiated cells were treated with PPT in the concentration of 1 nM. For this series of experiments we used 1 nM of PPT because we saw no effect using 10 nM of PPT to induce markers of being in primary adipocytes. Primary cells were treated for 5 h and probed for the following being markers: *Ucp1*, *Ppargc1 α* , *Pdk4*, *Tnfrsf9*, and *Tbx1* for UCP-1 protein by WB.

2.1.3. Small interfering RNA (siRNA) knockdown

In order to determine if ER α is necessary to induce markers of being in adipocytes, we used a small interfering RNA (siRNA) to knockdown the ER α gene (*Esr1*) in mature 3T3-L1 cells according to our previously published protocol [21,28]. Briefly, 3T3-L1 differentiated adipocytes were transferred to 12-well plates and transfected with siRNA targeting murine *Esr1* (siGENOME mouse *Esr1* siRNA, GE Dharmacon, Lafayette, LA, USA, Cat. number M-058688-01-0005), at the concentration of 100 nM, using Opti-MEM non-phenol medium (GibcoTM, Cat. number 11058-021). As a negative control, cells were treated with a scrambled sequence siRNA at the same concentration (siGENOME non-targeting siRNA, GE Dharmacon, Cat. number D-001206-13-05). LipofectamineTM was used as the transfection reagent (RNA iMAX, InvitrogenTM, ThermoTM, Cat. number 13778-030), at the concentration of 20 μ M/ml, according to the manufacturer's instructions. 72 hrs

after siRNA transfection, cells were collected and assayed to test the efficacy of the ER α knockdown by ER α mRNA. Using this protocol, we were able to achieve approximately 40% knockdown of ER α consistent with our previous publications [21,28]. Transfected cells were then treated with PPT (10 nM), or the β 3-adrenergic receptor agonist CL316,243 (Sigma®, Cat. number C5976) at a dose of 100 nM, according to previous reports [29] and cells were collected for UCP-1 mRNA measurement.

2.1.4. Glycerol release in the culture medium and lipolysis markers

To determine if ER α is necessary for mediating lipolysis, 3T3-L1 differentiated cells were exposed to media supplemented with 2% fatty-acid free BSA for 4 h according to previously published protocols [29–31]. Cells were then pre-treated for 1 h with the ATGL lipolysis blocker Atglinstatin (Sigma®, Cat. number SML 1075) at the concentration of 20 μ M, or Isoproterenol (Tocris, Cat. number 10009951), which was used as a positive control at the dose of 10 μ M [30]. Another set of cells were pre-treated with a blocker of HSL, CAY 10499 (Cayman, Ann Arbor, MI, USA, Cat. number 359714-55-9) at the dose of 10 μ M, followed by which cells were treated with PPT (10 nM) for 5 h and glycerol release was measured in the media by the Adipolysis Assay kit (Cayman, Cat. number 10009381). ATGL protein and AMPK phosphorylation were assayed by WB as described below.

2.2. *In vivo* studies

2.2.1. Total body ER α knockout mice (ERKO)

To begin to determine the necessity of ER α to mediate BAT/beige thermogenesis *in vivo*, weight matched C57BL/6J WT and ERKO male mice were used. Mice were housed in normal vivarium conditions (22 °C), exposed to a 12 h light/dark cycle, fed a standard chow diet and provided *ad libitum* water. Animal care and procedures were approved by the University of Texas Southwestern Medical Center (UTSW, Dallas, TX), and were performed in accordance to approved IACUC protocols. For the first experiment, male mice were maintained at room temperature (RT, 22 °C) for 16 h, and fasted for 12 h following which they were sacrificed and the interscapular adipose tissue (iBAT) was collected for ER α and UCP-1 protein measurement by WB. In a second experiment, weight-matched WT and ERKO male mice were exposed to PET/CT scan performed using Siemens Inveon PET/CT Multi Modality System (Siemens Medical Solutions, Knoxville, TN). Briefly, mice were exposed to thermoneutrality (TN 28 °C) for 16 h; 3 days later, they were exposed to room temperature (RT 22 °C) for 16 h; and 3 days later, to cold temperature (4 °C) for 16 h. At the end of 16 h in each temperature, mice received 150 μ Ci of 18F fluorodeoxyglucose (FDG) in 150 μ l of saline intravenously via tail vein injection. PET images were acquired 1 h post-injection for each temperature. PET and CT images were co-registered in Inveon Acquisition Workplace (Siemens Medical Solutions, Knoxville, TN). Regions of interest (ROIs) were drawn around the iBAT and standardized uptake values (SUV) were established within this region and was recorded. SUV was defined by the FDG uptake in BAT and normalized by total FDG uptake and body weight of the animal.

2.2.2. ER α added back to ERKO mice (Ad-ER α)

To determine the role of adipose tissue ER α to mediate beiging, ER α was added back to iWAT of female ERKO mice. Briefly, C57BL6 WT and ERKO female mice, housed in normal vivarium conditions (22 °C), were injected with an ER α overexpression vector (Ad-ER α) in one iWAT fat pad and a control vector (Ad-GFP) in the contra-lateral iWAT using the methods previously reported [26]. iWAT was collected 6 days after

injections and samples were fixed in 10% neutral buffered formalin for 48 h, then embedded in paraffin wax for histology performed by the Cedars-Sinai Biobank and Translational Medicine Core (Los Angeles, CA). Slide images were acquired using Apeiro AT Turbo (Leica Biosystems Inc., U.S.). The average fluorescence intensity of 10 randomly selected iWAT regions of interest (ROI) were measured in each sample using StrataQuest software (TissueGnostics, Austria). Results are expressed as arbitrary units comparing the Ad-ER α iWAT to the contralateral Ad-GFP fat pad.

2.2.3. Adipocyte-specific knockdown of ER α (AdipoER α) *in vivo*

In order to determine the influence of ER α activation on markers of lipolysis *in vivo*, we used mice with selective knockdown of ER α in adipocytes as previously published [23]. Briefly, adiponectin promoter driven-Cre transgenic mice were crossed with mice carrying the lox-P-flanked ER α allele (ER $\alpha^{lox/lox}$), producing ER $\alpha^{lox/lox}$ /Adiponectin-Cre (AdipoER α) littermates on a C57BL/6 background. Mice were housed in vivarium standard temperatures (around 22 °C), exposed to a 12 h light/dark cycle, and were fed standard chow with *ad libitum* water. Animal care and procedures were approved by the UTSW Medical Center in accordance to IACUC approved protocols. For markers of lipolysis analysis samples of iWAT and visceral adipose tissue of female AdipoER α mice and weight-matched WT controls were processed for perilipin, HSL and β 3-AR mRNA. In another set of experiments, female and male AdipoER α and weight-matched WT controls were treated with CL316-243 for 5 days at the concentration of 1 mg/kg/d i.p. [32–34], and fasting glycerol was determined.

2.2.4. Membrane (MOER) or nuclear (NOER) only estrogen receptor alpha

In order to begin to differentiate the genomic vs nongenomic ability of ER α to mediate adrenergic-stimulated beiging of adipose tissues *in vivo* we used mice expressing ER α only in the membrane (MOER), or only in the nucleus (NOER), and compared their response to CL316,243 to that of age matched WT mice (a gift by Dr. Ellis Levin, generated as previously published) [35,36]. Mice were housed in standard vivarium temperatures (22 °C), fed with a standard chow diet, and were then treated with saline (vehicle) or CL316,243 for 5 days in the concentration of 1 mg/kg/d i.p. [32–34]. The Animal Studies and the Research and Development Committees at the Department of Veterans Affairs Long Beach Healthcare Facility, CA approved the experimental procedures. On the final and 5th day of injections, mice were sacrificed following a 4 h fast and iWAT was collected and processed at Cedars-Sinai Medical Center for UCP-1 immunofluorescence and mRNA expression of UCP-1 and ATGL.

2.2.5. RT-qPCR

mRNA expression of markers of beiging and lipolysis following PPT or CL316,243 treatments was determined by RT-qPCR. Adipose tissue samples were lysed in TRIzol® reagent (Invitrogen™, Thermo™, Cat. number 15596026) and RNA was extracted and isolated using phase separation reagent (BCP) (MRC®, Cincinnati, OH, USA, Cat. number BP151) and RNeasy kit (Qiagen®, Hilden, Germany, Cat. number 74104), according to the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometric analysis (NanoDrop ND-1000, Thermo™), and all samples had an A₂₆₀/A₂₈₀ ratio around 2. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Thermo™, Cat. number 4387-406). Real time qPCR was performed using TaqMan® Universal MasterMix II, with UNG (Applied Biosystems™, Cat. number 4440038), and TaqMan® specific primers

(Applied Biosystems™) (Table 1), on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems™). Mouse β -actin gene (*Actb*) was used as the reference gene, and data was normalized and relative expression determined from the threshold cycle (Ct) following the $2^{-\Delta\Delta Ct}$ method.

2.2.6. Western blotting (WB)

Protein levels of UCP-1, ATGL and phosphorylation of AMPK were determined by WB in cell and adipose tissue lysates. To do this, cells and adipose tissues were lysed in Pierce™ RIPA® buffer (Thermo™, Cat. number 89900) supplemented with anti-phosphatase cocktail (PhosSTOP EASY pack, Roche, Cat. number 04-906-837-001) and anti-protease cocktail (cOmplete™ mini, Roche, Cat. number 11836153001). After sample centrifugation, the supernatant was collected and protein concentration was determined using Pierce® BCA sample assay (Thermo™, Cat. number 23228). Proteins (30 μ g) from lysates were separated by electrophoresis and electrotransferred to nitrocellulose membranes. Later, membranes were stained with Ponceau-S (Sigma®, Cat. number P7170) to be used as the loading control. Membranes were blocked in TBS-T containing 5% non-fat milk powder, followed by overnight incubation with primary antibodies: anti-UCP1 (Abcam, Cambridge, MA, USA, Cat. number ab10983, 1:500), anti-ER α (Sta Cruz Biotechnologies, Dallas, TX, Cat. number sc-542), anti-AMPK (Cell-Signaling, Danvers, MA, USA, Cat. number 2532S), anti-phospho-AMPK^{Thr172} (Cell, Cat. number 2535S), and anti-ATGL (Cell, Cat. number 2138S). Blot's intensity was quantified by densitometry (ImageQuant LAS 4000, GE Healthcare, Pittsburg, PA, USA). Results were expressed in arbitrary units in comparison to controls.

2.2.7. Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Statistical significance between groups was determined by one-way ANOVA followed by Tukey post-test. Two-tailed paired Student's test was used as appropriate. *P* values $<$ 0.05 were considered statistically significant. Statistical analyses and graphs were generated using GraphPad Prism 6 for Windows software (GraphPad software, San Diego, CA, USA).

3. RESULTS

3.1. Activation of ER α induces markers of beiging in mature adipocytes

To begin to determine if there is correlation between ER α and UCP-1, we first characterized the expression level of ER α and UCP-1 in adipocytes throughout the differentiation process. Both ER α and UCP-1 mRNA expression were increased in parallel throughout differentiation, with the highest expression of both at day 10 (Suppl. Figure 1). Based on these results, only fully differentiated adipocytes were used for all future experiments. In 3T3-L1 cells treated with PPT there was a

significant increase in UCP-1 protein following 1 h of PPT treatment which remained elevated for 2 h (Figure 1A). Following 5 h of PPT exposure, UCP-1 mRNA, as well as *Ppargc1 α* , *Pdk4*, *Tnfrsf9*, and *Pbx1*, were elevated (Figure 1B). Importantly, where there is a trend for PPT to increase all markers of beige adipose tissue, the effect was most robust for UCP-1. Primary adipocytes extracted from iWAT of WT C57BL/6J female mice also had increased UCP-1 protein and mRNA levels following PPT treatment (Suppl. Figure 2).

3.2. ER α expression in mature adipocytes facilitates PPT and CL316,243 induced UCP-1 upregulation

Suppressing the expression of ER α by 40% using siRNA in mature adipocytes reduced the ability of PPT to induce *Ucp1* expression (Figure 2A). Additionally, adrenergic stimulation of β 3-AR by CL316,243 increases UCP-1 expression in adipocytes [7–9], and in our model, treatment of mature 3T3-L1 adipocytes with CL316,243 increased UCP-1 expression (Figure 2B) and knockdown of ER α reduced CL316,243's ability to upregulate *Ucp1* (Figure 2B) further suggesting that ER α in adipocytes is critical to mediate UCP-1 transcription, either following direct ER α stimulation or through activation of adrenergic receptors.

Exposure to cold induces thermogenesis of BAT and beige tissues [32–34]; thus, we tested the role of ER α to mediate this process by exposing weight matched WT and ERKO male mice to cold. Mice were injected with 18F-fluorodeoxyglucose (FDG) after cold exposure which was followed by a PET/CT scan to measure glucose uptake in adipose tissues. ERKO mice had less FDG uptake in BAT upon cold exposure when compared to WT mice (Suppl. Figure 3A, B), and lower UCP-1 expression in BAT (Suppl. Figure 3C), further indicating ER α is necessary to mediate thermogenesis. To validate our model, we measured ER α levels in iBAT, and as expected, ER α was less expressed in iBAT of ERKO compared to WT mice (Suppl. Figure 3D). To extend our knowledge on the role of ER α in adipocytes to mediate beiging, we overexpressed ER α (Ad-ER α) selectively in one iWAT fat pad while the contralateral iWAT pad served as the control (GFP control). This was done in WT and ERKO female mice. We observed that in the iWAT that received the control vector, the adipocyte morphology was different between ERKO and WT mice consistent with our previous reports [26]. Specifically, ERKO mice have significantly larger adipocytes than WT mice. ER α overexpression in the WT and ERKO iWAT reduced the size of the adipocytes. Notably, upon histological examination of the adipose tissue in the WT and ERKO Ad-ER α we observed multilocular cells resembling beige adipose tissues (Suppl. Figure 4A) [37], further suggesting ER α expression in adipose tissues is important to facilitate beiging of adipose tissue.

3.3. ER α activation induces beiging in adipocytes via ATGL-dependent lipolysis

Adrenergic stimulation of mature adipocytes directly upregulates lipolysis [38,39]. Liberation of FFAs from intracellular lipids has been shown to increase UCP-1 expression and activation. Activation of ER α has also been shown to increase lipolysis [24], although this is species and tissue specific. We hypothesized that selective ER α activation would enhance adipose tissue lipolysis and thereby promote UCP-1 expression. To test this, we determined glycerol release, an indicator of lipolysis, from adipocytes following PPT, isoproterenol (used as a positive control) or vehicle. Our data demonstrate increased glycerol release following PPT and isoproterenol in differentiated 3T3-L1 cells (Figure 3A). We next assayed ATGL protein levels in 3T3-L1 cells pretreated with Atglinstatin, an ATGL inhibitor, prior to PPT treatment. We found that PPT increases ATGL protein rapidly (after 5 min of PPT

Table 1 — List of TaqMan® primers.

<i>Ucp1</i>	Mm01244861_m1
<i>Actb</i>	Mm02619580_g1
<i>Ppargc1a</i>	Mm01208835_m1
<i>Pdk4</i>	Mm01166879_m1
<i>Tnfrsf9</i>	Mm00441899_m1
<i>Tbx1</i>	Mm00448949_m1
<i>Esr1</i>	Mm00433149_m1
<i>Lipe</i>	Mm00495359_m1
<i>Adgrb3</i>	Mm00657451_m1
<i>Pnpla2</i>	Mm00503040_m1

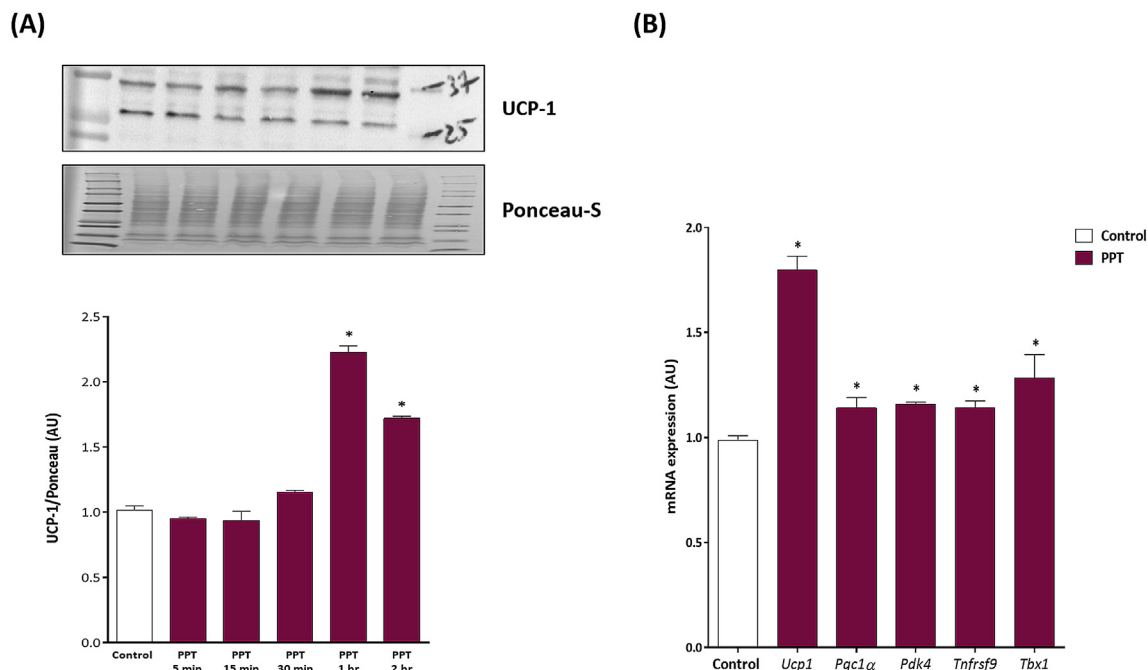


Figure 1: ER α activation increases markers of beige in 3T3-L1 adipocytes. (A) 3T3-L1 differentiated cells were treated with the ER α agonist PPT at 5 min, 15 min, 30 min, 1 h, and 2 h for UCP-1 protein. (B) 3T3-L1 adipocytes were treated with PPT for 5 h to probe mRNA expression of beige markers. Data on graphs represent mean \pm SEM of 3 independent rounds of cells. * $P < 0.05$ vs control.

treatment) and this was sustained over the course of 2 h, and the ability of PPT to increase ATGL protein was reduced by pre-treatment with Atglistatin (Figure 3B). Furthermore, we tested if UCP-1 mRNA was upregulated by PPT following pretreatment of Atglistatin, and found that Atglistatin reduced the ability of PPT to increase UCP-1 expression (Figure 3C). Finally, we measured phosphorylation of AMPK, a key element in the lipolytic pathway that activates ATGL [12], and we found that PPT rapidly increased AMPK phosphorylation (after 5 min of PPT treatment) (Figure 3D), suggesting that rapid activation of ER α initiates ATGL-mediated lipolysis to induce markers of beige in mature adipocytes.

In order to better characterize if this is an adipocyte mediated phenomenon, we used iWAT from mice lacking ER α specifically from adipocytes (AdipoER α mice) and assayed perilipin, which regulates ATGL [40], and we found that perilipin mRNA was decreased in AdipoER α compared to controls (Suppl. Figure 5A). Additionally we found a reduction in β 3-AR mRNA in AdipoER α iWAT further suggesting the importance of ER α in adipocytes in facilitating the programming of thermogenic adipose tissues. In contrast to iWAT, there were no differences in the beige markers in visceral adipose tissue comparing WT and AdipoER α mice (Suppl. Figure 5A). Interestingly, AdipoER α male and female mice treated with CL316,243 presented lower

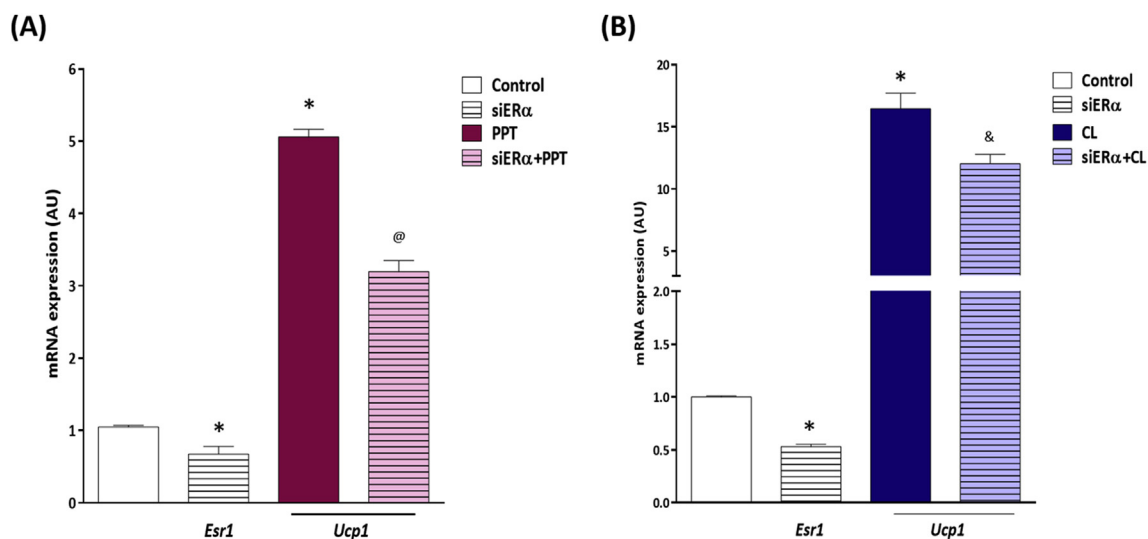


Figure 2: ER α is necessary to induce *Ucp1* expression in mature adipocytes. *Esr1* and *Ucp1* mRNA expression following siRNA knockdown (siER α) in mature 3T3-L1 adipocytes and treatment with (A) PPT or (B) CL316,243. * $P < 0.05$ vs control. @ $P < 0.05$ vs PPT. & $P < 0.05$ vs CL.

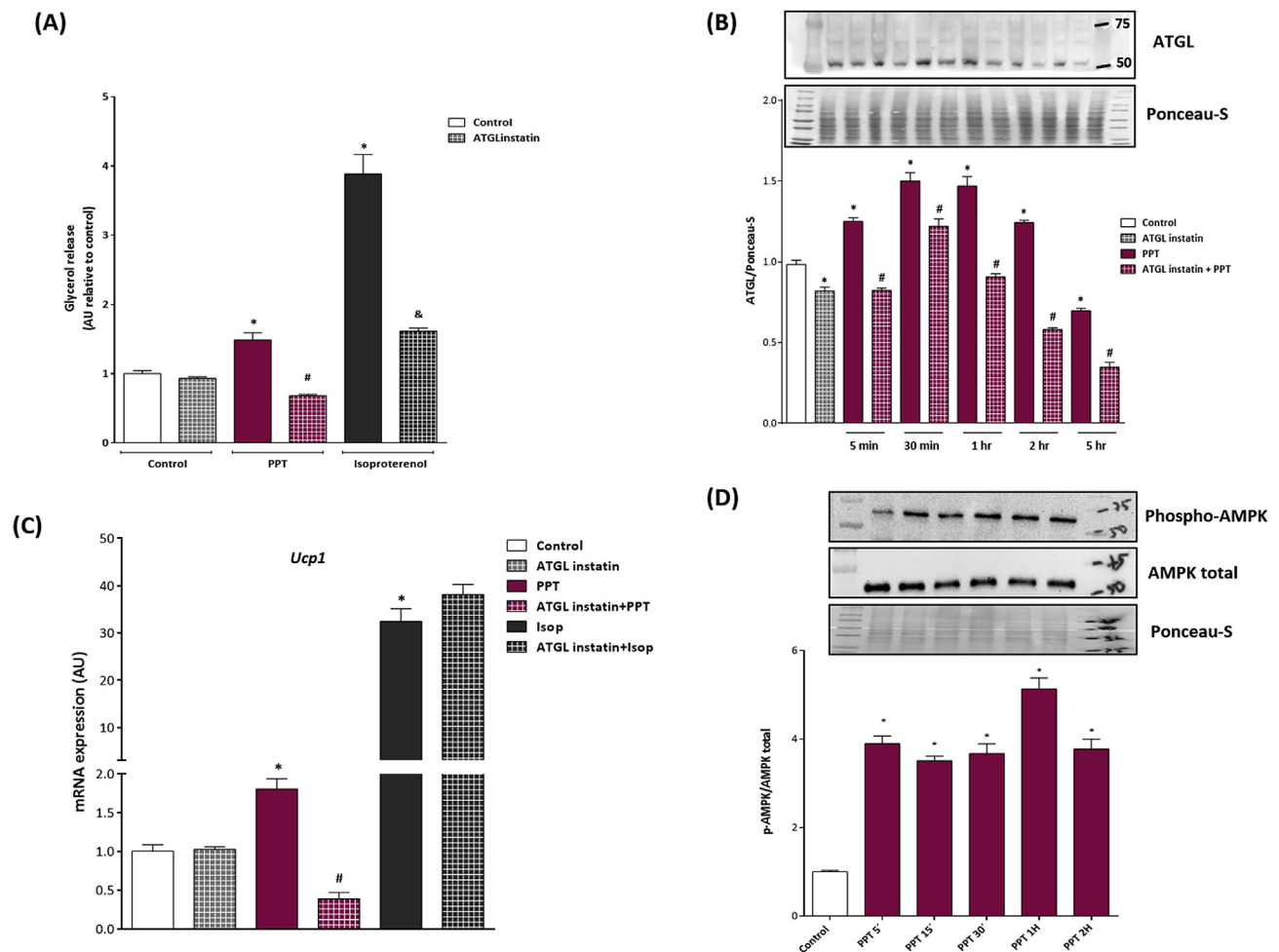


Figure 3: ER α activation increases ATGL-mediated lipolysis of adipocytes. 3T3-L1 adipocytes were pre-treated with the ATGL blocker, Atglistatin, for 1 h prior to PPT or isoproterenol treatments. **(A)** PPT treatment increased glycerol release in the medium while pre-treatment with Atglistatin prevented it. **(B)** PPT treatment increased ATGL protein while pre-treatment with Atglistatin prevented its ability. **(C)** Atglistatin prevented PPT's ability to increase *Ucp1* mRNA expression. **(D)** PPT increased phosphorylation levels of AMPK, which activates ATGL, suggesting an involvement of membrane-initiated ER α signaling. Data on graphs represent mean \pm SEM of 3 independent rounds of cells. * $P < 0.05$ vs control. # $P < 0.05$ vs PPT. & $P < 0.05$ vs isoproterenol.

plasma glycerol concentration compared to WT mice treated with CL, although it was only statistically significant in male mice (Suppl. Figure 5B).

3.4. Mice expressing ER α in the plasma membrane (MOER) have increased UCP-1 upregulation following CL316,243 when compared to mice expressing ER α in the nucleus (NOER)

Based on our observation of a rapid increase in UCP-1 protein and phosphorylation of AMPK following 1 h of PPT treatment (Figures 2B and 3D), we sought to determine if these effects were mediated by membrane or nuclear ER α . We conducted these experiments *in vivo* using mice that express ER α predominantly in the cell membrane (MOER) or in the nucleus (NOER) [35,36]. We treated these female MOER and NOER mice with CL316,243 for 5 days using the same protocol previously discussed. Upon analysis of the adipose tissue following the treatment protocol, we found large patches of UCP-1 positive cells in both WT and MOER mice but to a lesser degree in NOER mice (Figure 4A,B). These data suggest membrane ER α activation induces beiging of adipose tissues. This was supported by the fact that MOER mice had higher expression of UCP-1 (Suppl. Figure 6A) and ATGL mRNA (Suppl. Figure 6B) following CL316,243 treatment

when compared to WT or NOER mice, further indicating the method by which ER α facilitates beiging of adipose tissues is through membrane-initiated lipolysis.

4. DISCUSSION

Our results demonstrate selective activation of ER α in mature 3T3-L1 adipocytes by the ER α agonist PPT, increased UCP-1 expression and markers of beiging. To our knowledge, this is the first-time selective activation of ER α in mature adipocytes has been used to induce beiging of adipose tissues. Our data further suggest that one of the mechanisms by which ER α mediates beiging of adipose tissues is through rapid activation of adipocyte AMPK, which promotes ATGL-dependent lipolysis, followed by increases in UCP-1 protein. Early observational studies in humans demonstrated an inverse relationship between human brown adipose tissues (hBAT) mass and BMI [34] suggesting that hBAT may defend against weight gain. Moreover, cold exposure was shown to increase the amount and activity of detectable hBAT [34] as well as improve glucose uptake in hBAT [37]. Taken together, these data suggest that, in addition to its role as a heat generating organ, hBAT exerts a beneficial effect on glucose

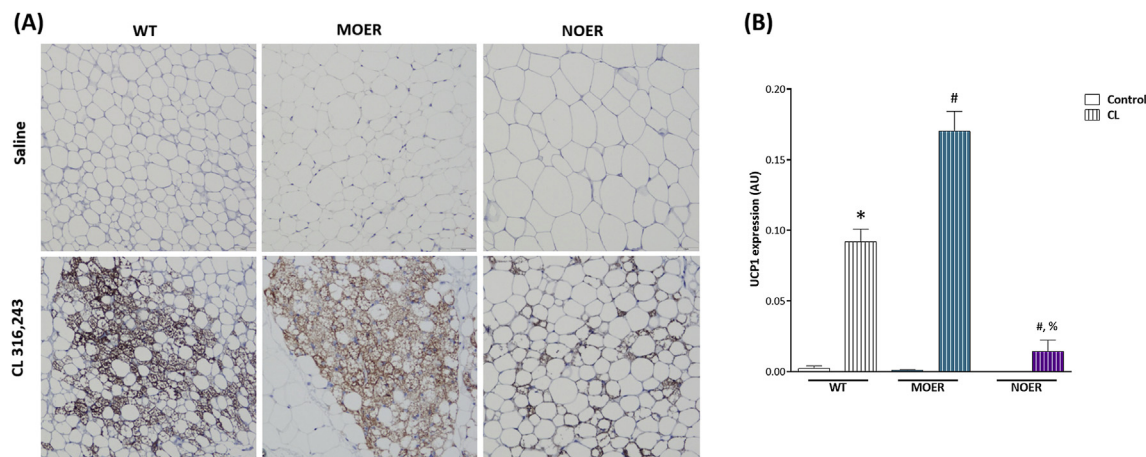


Figure 4: UCP-1 expression is higher after CL treatment in ER α membrane only (MOER) compared to nucleus only ER α mice (NOER). (A) Representative images of UCP-1+ protein staining in female 1) WT mice, 2) mice with functional ER α only in the plasma membrane (MOER), or 3) only in the nucleus (NOER). (B) quantification of UCP-1 positive signal in WT, MOER, and NOER mice. Data on graphs represent mean \pm SEM of 3–4 mice/group. * P < 0.05 vs WT control. # P < 0.05 vs WT CL. % P < 0.05 vs MOER CL.

metabolism and could be harnessed as a novel obesity therapy. Pharmacological and genetic studies of classical BAT and inducible beige adipose tissues in rodents support this notion; however, a definitive hBAT-specific intervention for obesity or dysregulated glucose metabolism in humans has not yet been developed. Furthermore, studies have also demonstrated that hBAT generally resembles rodent beige adipose in terms of its molecular signature [9,38]. As such, there is an intense effort to characterize the molecular pathways that guide beige adipose formation, activation, and function in human and rodent models, with the goal of their eventual translation to human obesity and metabolic therapies.

Interestingly, women have more hBAT mass and higher hBAT activity than similarly aged men [4], indicative of a critical link between sex and brown/beige adipose tissue development and function. Indeed, sex hormones, in particular estrogens, have already been shown to mediate a central mechanism of classical BAT activation in rodents, specifically, estradiol treatment was shown to increase BAT activity via hypothalamic ER α [14]. Though this study was conducted in rodents, the authors speculate that a similar mechanism may operate in humans and explain, at least partly, the reductions in energy expenditure and increases in adipose mass typical of the loss of ovarian estrogens during menopause. By contrast, our study reveals a direct, peripheral effect of ER α activation to promote UCP-1 expression in mature white adipocytes; therefore, it stands as a separate mechanism from centrally mediated estradiol brown/beige tissues activation.

Our findings suggesting that ER α activation increases UCP-1 protein in both mature 3T3-L1 and primary inguinal adipocytes from mice, and that knockdown of ER α reduces this response, indicates that activation of ER α can promote beiging of adipocytes. Admittedly, we were only able to achieve a 40% reduction in ER α using siRNA technology, this was still significant enough to reduce the efficacy of PPT and CL to induce beiging. The lack of complete knockdown of ER α may be due to the fact that mature adipocytes are difficult to efficiently transfect due to their large lipid droplets. PPT treatment throughout differentiation of adipocytes increased UCP-1 expression, further evidence that ER α activation has a clear ability to promote markers of beiging in adipocytes. We confirmed our cell culture data *in vivo* using mice lacking whole-body ER α (ERKO) and found that following cold exposure, ERKO mice had significantly lower glucose uptake when compared to WT

mice. ER α added back to iWAT of ERKO mice enhanced the presentation of multilocular lipid droplets in iWAT suggestive of beiging [37]. Our findings also suggest the mechanistic pathway by which ER α promotes beiging of adipocytes is through rapid/membrane-initiated ER α signaling which induces lipolysis. While both extracellular FFAs taken up from the circulation and intracellular FFAs released by lipolytic enzymes fuel uncoupled respiration via UCP-1, it is known that intracellular FFAs both increase the expression and activation of UCP-1 [39,41]. ATGL is a major intracellular lipase that functions in adipocytes to hydrolyze TGs to FFAs and diacylglycerides (DAGs). *In vivo* studies support a regulatory relationship between ATGL and adipose tissue thermogenesis. For example, adipocyte-specific overexpression of ATGL increased lipolysis and thermogenesis [42]. Adipocyte specific ATGL-KO mice have increased expression of WAT signature genes in BAT, and reduced UCP-1 expression [43]. Additionally, AMPK has been shown to activate ATGL, thereby increasing TG lipolysis [43,44] and UCP-1 thermogenesis [45]. Our findings are consistent with these data in that, following selective ER α activation by PPT, AMPK phosphorylation increased, followed by increases in ATGL and UCP-1 proteins. Blockade of ATGL- using the ATGL-specific inhibitor Atglistatin-blunted PPT-induced UCP-1 expression. Importantly, we found that blocking hormone sensitive lipase (HSL)- another critical modulator of lipolysis- did not prevent the ability of PPT to induce UCP-1 expression (Suppl. Figure 7), supporting the concept of an ER α -ATGL signaling pathway to promote beiging of adipocytes. We supported these findings *in vivo* using mice lacking ER α specifically in WAT (AdipoER α), by demonstrating that these mice had lower levels of fasting plasma glycerol and markers of lipolysis in iWAT when compared to WT mice. Using the MOER and NOER mice, we were able to demonstrate that membrane ER α appears to be necessary to induce lipolysis and facilitate beiging. This is supported by our findings that treatment with CL316,243, a β -adrenergic receptor agonist known to induce beiging of WAT [46], increased UCP-1 expression in MOER mice to a level comparable to WT but not in NOER mice, suggesting that nuclear ER α signaling may be less important for the beiging response to adrenergic stimulation. Membrane ER α has been shown to participate in extra-nuclear signaling cascades [15] and has been implicated in various aspects of metabolism, and our data constitute evidence of a connection between membrane-initiated ER α signaling and UCP-1 induction. In a recent study of Ueda et al. (2018) [47], membrane-

initiated ER α pathway was blocked in female mice and this led to excessive weight gain, glucose intolerance and impaired adaptive thermogenesis which is in accordance to our study. Activation of central protein phosphatase (PP) 2A improves metabolic function suggesting an association between membrane-initiated ER α and PP-2A protein activation to promote metabolic homeostasis.

While our data are suggestive of a role for ER α to induce beiging, we admit there are limitations in our findings. First, we were unable to achieve a complete knockout of ER α using the siRNA approach; however, even with lack of a complete knockdown, we were still able to determine reductions in ER α reduce the ability of both PPT and CL to induce beiging. Additionally, all of our *in vivo* studies were conducted in vivarium's which do not house mice at thermoneutrality and therefore, introduce a 'cold challenge' to the mice even at baseline and within their normal housing conditions. Lastly, not all experiments were performed in all mouse models and therefore there are some gaps in knowledge gained which future studies will need to correct.

5. CONCLUSION

Our results suggest that selective-activation of adipocyte ER α increases markers of beiging and this appears to be through induction of AMPK and ATGL-mediated lipolysis providing free fatty acids as a fuel to activate UCP-1. Thus, selective activation of ER α represents a promising novel therapeutic target to promote beiging of adipose tissues to improve metabolic homeostasis.

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CONFLICT OF INTEREST

Authors declare there are no conflicts of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2018.09.002>.

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