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Research Article

ACTH Enhances Lipid Accumulation in Bone-marrow derived Mesenchymal stem cells undergoing adipogenesis

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ACTH is a major hormone of the stress axis or hypothalamic-pituitary-adrenal (HPA) axis. It is derived from pro-opiomelanocortin (POMC) the precursor to the melanocortin family of peptides. POMC produces the biologically active melanocortin peptides via a series of enzymatic steps in a tissue-specific manner, yielding the melanocyte-stimulating hormones (MSHs), corticotrophin (ACTH) and β-endorphin. The melanocortin system plays an imperative role in energy expenditure, insulin release and insulin sensitivity. Bone marrow derived mesenchymal stem cells circulate in the blood stream and as progenitor cells have the potential to differentiate into many cell types such as osteoblasts, chondrocytes and adipocytes. Here we examine the effects of ACTH on the mouse D1 bone marrow-derived MSC. ACTH significantly increased lipid accumulation during the adipogenic differentiation of D1 cells in a concentration- dependent manner. ACTH also shifts the temporal pattern of D1 adipogenic differentiation to the left i.e. differentiation occurs earlier with ACTH treatment. No significant differences in protein expression of peroxisome proliferator-activated receptor gamma (PPAR-γ2), a regulating transcription factor of adipogenesis were found. Therefore the effects of ACTH are suggested to be mediated by an alternative pathway. Overall the results indicate a connection between increased adipose deposition and the elevated circulating ACTH associated with stress.

Keywords: melanocortin; adrenocorticotropin; mesenchymal stem cells; glucocorticoid

Introduction

The melanocortin system has been extensively studied and has become one of the most defined neuronal pathways involved in the stress response and maintaining homeostasis [1]. During stress, pro-opiomelanocortin (POMC) production in the pituitary is increased along with adrenocorticotrophin (ACTH) which is then released into the blood stream. ACTH then elicits release of glucocorticoid from the adrenal cortex. Glucocorticoid in turn facilitates the body's response to stress by mobilizing glucose stores. It also feeds back on the pituitary and hypothalamus to turn off the production of ACTH. This endocrine negative feedback system is known as the hypothalamic-pituitary-adrenal (HPA) axis [2]. Overactivation of this axis is linked to obesity with the fat distributed primarily in the abdomen [3].

POMC is the precursor to ACTH as well as many other melanocortin peptides. The biologically active melanocortin peptides are produced from POMC through a series of enzymatic steps in a tissue-specific manner, producing the melanocyte-stimulating hormones (MSHs), ACTH and β-endorphin [4]. The effects of melanocortin peptides are regulated by the activation of specific melanocortin receptors which differ from each other in terms of their tissue distribution and affinity for the melanocortin agonists and antagonists. The melanocortin receptors (MC-R) are a family of G-protein coupled receptors (GPCR). There are five melanocortin receptors, termed MC1-5-R and all can be activated by ACTH [5]. GPCRs make up the largest and most diverse family of membrane receptors. GPCRs transduce varying stimuli from external signals and ligands such as neurotransmitters, peptides, lipids, nucleotides, ions, pheromones, tastes and odors. GPCRs have the ability to signal through G-proteins to regulate adenylate cyclase and modify cAMP production [6].

The melanocortin peptides and their receptors have many physiologic roles both inside and outside of the central nervous system. In the central nervous system they signal through MC-R expressed in the hypothalamus and are active in appetite regulation and energy expenditure [1]. Outside the central nervous system they play roles in skin pigmentation [7], in exocrine gland secretion [8] and the ostoechondrogenic differentiation of progenitor cells [9,10].

Our focus is on the role of the melanocortin peptide, ACTH, in the adipogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSC). BMSC are pluripotent progenitor cells that can differentiate into many different cell types such as chondrocytes, osteocytes and adipocytes. BMSC are precursors of connective tissue cells and will migrate to damaged and inflamed tissues to aid in repair [11]. The MC2-R, MC3-R and MC5-R are expressed by circulating BMSC [9] and all of these receptors can be activated by the ACTH ligand and result in elevations of cAMP [5]. Moderate increases in intracellular cAMP are linked to the adipogenic differentiation of BMSC. The cAMP-response element binding protein (CREB), activated by cAMP, has key functions in adipocyte differentiation. It binds to known cAMP response elements (CRE) located on multiple adipocyte gene promoters including the PPARγ2 adipocyte specific transcription factor leading to adipogenic differentiation [12]. The fact that ACTH by binding to MC-R located on the BMSC can result in elevations in cAMP led us to hypothesize that ACTH will enhance adipogenesis of bone-marrow derived mesenchymal stem cells.

Our first aim was to optimize the adipogenic induction protocol to a level below saturation to allow for the detection of any effect of ACTH. This was done by carrying out adipogenic induction of D1 bone-marrow derived MSC with one-quarter, one-half and at full strength of the standard induction agents. We also wanted to determine the effect of ACTH on lipid accumulation and PPARγ2 protein expression during the adipogenic differentiation. Using the protocol optimized in aim 1, MSC were induced to form adipocytes in the presence or absence of ACTH at a range of

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concentrations. Lipid accumulation and PPARγ2 protein expression were measured over a five day period.

Materials and Methods

Materials

Cell culture reagents were obtained from Invitrogen, unless otherwise specified. Porcine ACTH (1-39) was purchased from Sigma-Aldrich as well as all other chemicals. All primary and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

D1 ORL UVA (D1) cells, a bone marrow-derived mesenchymal stem cell line, were obtained from the American Type Culture Collection (ATCC #CRL-12424) and were maintained in culture with high glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin sodium, 100 U/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B. Cells were used at passage 5. The cells were initiated at a density of 6.25×10^3 cells/cm^2.

Adipogenic Induction

At confluence, culture medium was changed to experimental media (αMEM supplemented with 10% FBS, 100 U/mL penicillin sodium, 100 U/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B and treated with a full strength adipogenic induction cocktail (1 µM dexamethasone, 10 µg/mL insulin, 0.5 mM isobutyl methylxanthine (IBMX) and 15 mM glucose) to induce adipocyte differentiation. One half and one quarter induction cocktail concentrations were also used. Cells were exposed to induction medium for 2 days before being changed to experimental medium without induction cocktail. Medium was changed every two days until experiment completion (Day 5 or 7.) Final induction strength of one quarter of full strength was used for adrenocorticotropic hormone (ACTH) experimentation. ACTH was added on the day of adipogenic induction (day 0) at concentrations of 10 nM, 100 nM and 1000 nM.

Neutral Lipid Staining

Cell cultures were washed with PBS and fixed with 10% Formalin at room temperature for 30 minutes. Cells were then stained using Oil-red-O (0.5 g Oil-red-O in 100 mL isopropanol) for 15 minutes. Oil-red-O solution used contained 60% Oil-Red-O and 40% diH$_2$0 in mixture. After staining, cells were rinsed four times with distilled water and wells were cleaned to remove excess stain. Oil-red-O stain was re-solubilized with isopropanol. Samples from each culture well were taken and measured by spectrophotometry at a 490 nm wavelength.

Image Analysis

Initial adipogenic induction was analyzed by ImageJ retrieved online from The National Institutes of Health (http://rsb.info.nih.gov/ij/). Images were captured using an inverted phase-contrast microscope coupled to a camera. The program allowed for the quantification of lipid accumulation in stained cell cultures. The threshold in ImageJ was set to detect the red color of the neutral lipid and measured the area of red color in the images. Multiple random images were captured of each well and used in the comparative analysis.

Western Blot Analysis

Proteins were extracted from the cell layers using radio-immunoprecipitation assay (RIPA) buffer. Protein concentrations were determined by the bicinchoninic acid (BCA) assay. Protein separation was performed with SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Non-specific blocking of the membrane was done using blocking buffer (tris-buffered saline with Tween and 5% w/v non-fat dry milk) for an hour then incubated with PPARγ2 primary antibody (1:1000 dilution) overnight. Upon incubation completion, membranes were washed with TBST solution for a total of three times. To detect the primary antibody, a HRP-labeled secondary anti-rabbit IgG antibody was added to blocking buffer (1:2000 dilution) and incubated with the membrane for an hour. PPARγ detection was performed by visualizing bands using enhanced chemiluminescence (ECL).

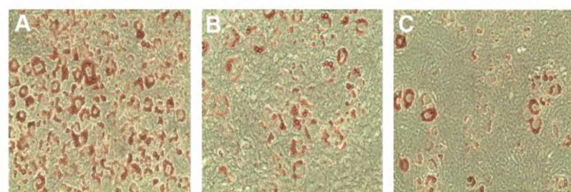
Statistical Analysis

Data were analyzed using two-way ANOVA with time and treatment as factors.

Results

Optimization of the adipogenic induction protocol

The first objective was to determine an optimal level of adipogenic induction; one that would induce adipogenesis of D1 cells at a sub-optimal level to allow for detection of ACTH effects. Confluent cultures were exposed to an adipogenic induction cocktail at full, one-half and one-quarter strengths. A cocktail consisting of 0.5mM isobutyl methylxanthine, 1 µM dexamethasone, and 10 µg/mL insulin was considered full strength. The full strength and one-half strength induction resulted in saturation by day 4 or day 7 of culture respectively. The induction cocktail at one-quarter strength did not reach saturation at 7 days making it the most effective concentration for our future experiments (Fig. 1).



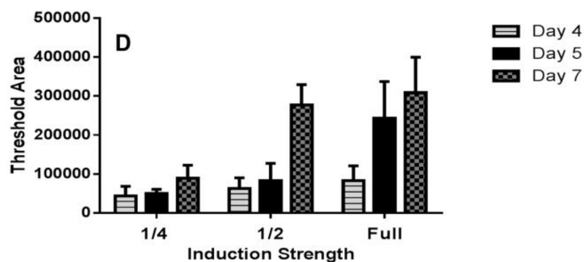


Figure 1: The effects of induction factors on lipid accumulation at a range of concentrations. Concentrations of 1µM DEX, 10µg/mL insulin, 0.5mM IBMX and 15mM glucose were used for full induction (A) one-half (B) and one-quarter (C) were adjusted accordingly. Images shown in A-C were from day 7. Images were captured using an inverted phase-contrast microcope coupled to a camera. Images were analyzed using ImageJ. The threshold was set to detect the red color of the neutral lipid after staining. The threshold area was determined for 3 images per well x 4 wells under each condition and data are presented as the mean ± SEM, n = 12 in (D).

ACTH increases adipogenesis of D1 MSC in a concentration dependent manner and shifts the peak of differentiation to an earlier time point

When D1 cells were induced to form adipocytes in the presence of ACTH there was a significant increase in adipogenesis at early time points; day 1 and day 2. This increase was dependent on concentration of ACTH; adipogenesis increased with increasing concentrations of ACTH (Fig. 2). ACTH treated cultures also reached peak lipid accumulation at day 3 whereas untreated cultures reached peak lipid accumulation at day 5 (Fig. 3).

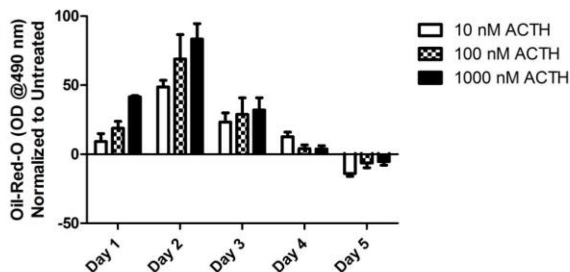


Figure 2: ACTH increases lipid accumulation early during adipogenic induction of D1 MSC. Absorbance of oil-red-o stain extracted from cell layers of ACTH treated cultures was measured and normalized to untreated cultures of corresponding time points. Data are presented as mean ± SEM, n = 4. Two-way ANOVA using ACTH treatment and time as factors indicates a significant effect of treatment, $P = 0.0116$ and time $P = 0.0001$ on lipid accumulation. Data are representative of 3 experiments with similar results.

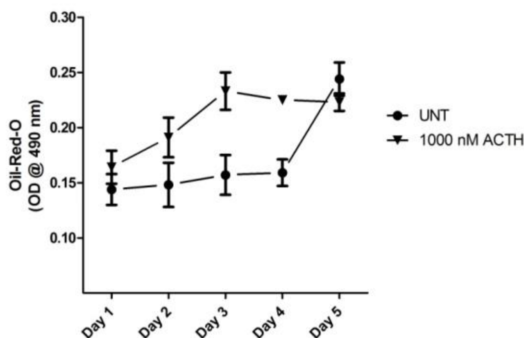


Figure 3: Induction with ACTH supplementation shifts peak lipid accumulation in D1 MSC. Absorbance of oil-red-o stain extracted from cell layers was plotted in a line graph to demonstrate the shift in peak lipid accumulation. Data presented as mean ± SEM, n = 4.

ACTH does not increase PPARγ protein expression

PPARγ2 is a transcription factor that is capable of activating the expression of many genes promoting adipogenesis [13]. We tested the hypothesis that ACTH exerts its effects through increasing the expression of PPARγ2. Using western blot we compared the expression of PPARγ2 in D1 cultures induced to form adipocytes in the presence or absence of ACTH at 100 nM. PPARγ2 expression increased with differentiation in both treated and untreated cultures confirming adipogenic differentiation. When comparing expression between ACTH treated and untreated cultures we found no significant difference in PPARγ2 expression levels. This shows that ACTH had no effect on the expression of PPARγ (Fig. 4 A&B).

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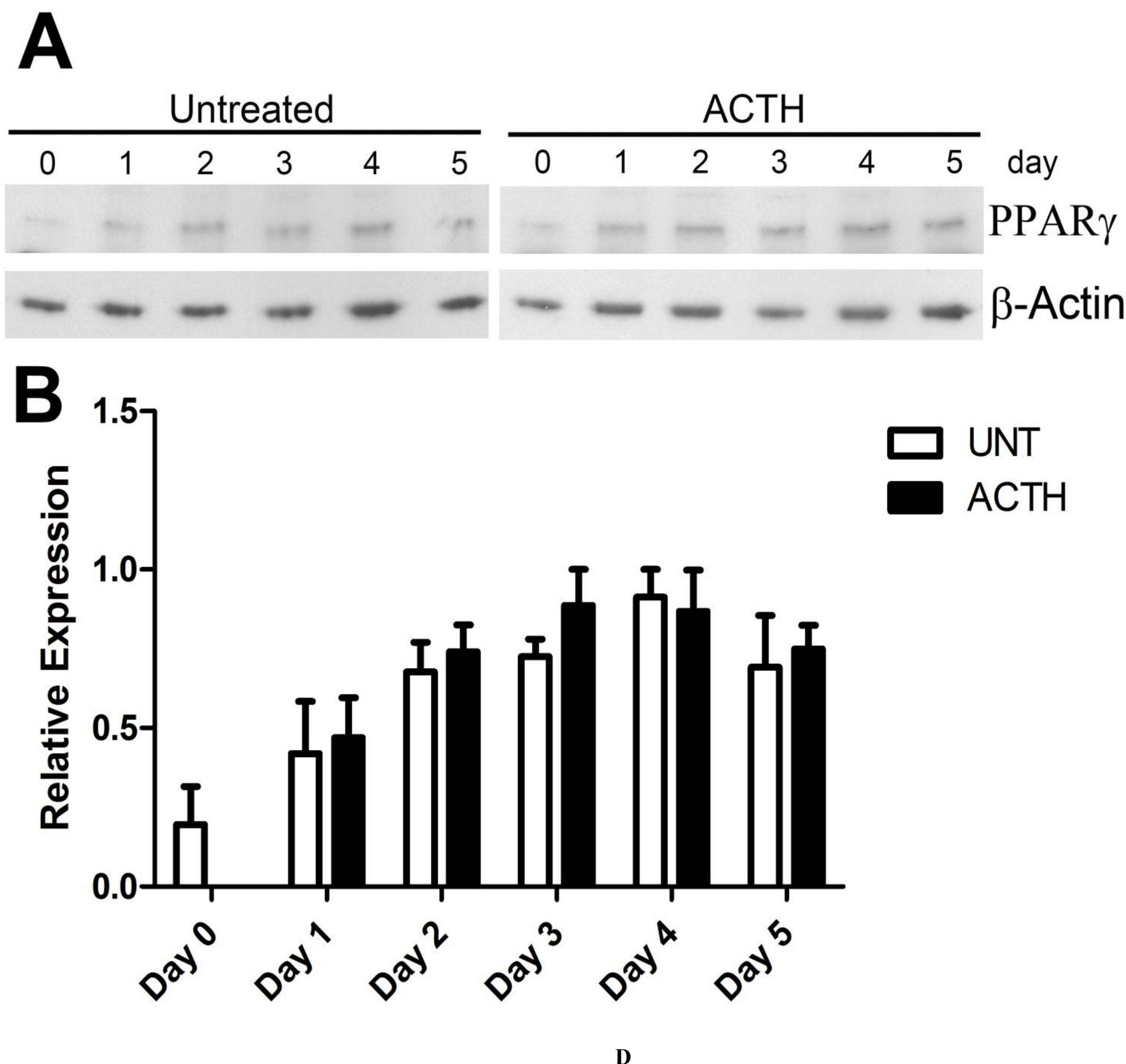

D

Figure 4: Protein expression of the PPARγ2 adipogenic transcription factor is not increased by ACTH treatment. (**A**) A representative western blot demonstrating PPARγ expression in untreated and ACTH treated (100 nM) cultures. (**B**) A bar graph of the relative band intensities in A. Data are presented as the mean ± SEM of 3 different experiments. Two-way ANOVA analysis with ACTH treatment and time as factors show no significant effect of treatment, $P = 0.44$ but a significant effect of time, $P = 0.0119$ on PPARγ expression.

Discussion

Many previous works have used the 3T3-L1 pre-adipogenic cell line to examine the effects of melanocortin peptides in adipogenesis [14]. The melanocortin peptides induce interleukin 6 (IL-6) production from this cell line as well as induce lypolysis or the breakdown of these fat cells [15, 16]. IL-6 is a pleiotropic cytokine secreted by many cell types and plays an active role in tissue inflammation [16]. The current study focuses on the response of bone-marrow derived mesenchymal stem cells to ACTH during adipogenic differentiation. The D1 ORL UVA cell line, capable of osteogenic and adipogenic differentiation, was first described by Diduch et.al. [17]. This cell line represents an earlier progenitor than the 3T3-L1 cell line which is already committed to the adipocyte lineage. The difference in lineage commitment between these two cell lines may explain why others did not find an enhancement of adipogenesis by ACTH using 3T3-L1 cultures [14] and the current studies find ACTH enhances adipogenesis in D1 cells.

3T3-L1 cultures express the MC2-R and MC5-R after differentiation and signaling through these receptors enhances lipolysis in a cAMP dependent manner [14]. Conversely, bone marrow mesenchymal stem cells express the MC2-R and MC5-R prior to differentiation and signaling through receptors can enhance osteochondrogenic differentiation [9]. Here we also demonstrate that under adipogenic conditions, ACTH can enhance lipid accumulation in the D1 cell line and

this is presumably through MC-R signaling and the enhancement of cAMP accumulation. More studies are needed to make these determinations.

It was found that PPAR-γ2 was not involved in adipogenesis when induced by DEX in D1 cells [13]. We found similar results, showing that ACTH-induced elevated lipid accumulation in D1 cells was not mediated through increased expression of PPAR-γ2. This suggests that the effects of ACTH are mediated by an alternative transcriptional pathway.

Glucocorticoid is another primary hormone of the HPA stress-axis has been implicated as the primary hormone involved in stress-related obesity [3]. The synthetic glucocorticoid, dexamethasone, is a component of the standard induction cocktail. Therefore our data suggest that increased stress with the subsequent increase in circulating ACTH and glucocorticoid may prime circulating bone-marrow derived mesenchymal stem cells for adipogenic differentiation and contribute to adipose. This may in part account for stress-related redistribution of fat stores.

Future Research

The mechanisms of ACTH and dexamethasone effects on adipogenic differentiation of BMSCs remain unclear and require further investigation. Therefore studies using specific MC-R receptor agonist and antagonists to determine which melanocortin receptor expressed on the BMSC surface is responding to ACTH treatment are warranted. Additional investigations aimed at finding other transcription factors, activated by cAMP, that may be involved in ACTH-induced adipogenic differentiation such as the CAAT-enhancer binding proteins (C/EBP) would also provide further insight into the mechanisms behind ACTH-enhanced adipogenesis in this cell line.

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