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A N I N T E R N A T I O N A L J O U R N A L

Nuclear translocation of STAT3 by *in vitro* metreleptin administration causes lipolysis in human primary adipocytes

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ABSTRACT

We utilized subcutaneous (SC)- and omental (OM)-derived human primary adipocytes (hPA) from obese male, and investigated whether synthetic analog of leptin, metreleptin, may regulate lipolysis via translocation of STAT3 to the nucleus. We observed that 50 ng/mL of metreleptin increases STAT3 phosphorylation in both SC- and OM-derived hPA. Importantly, we found for the first time that metreleptin is capable of trans-locating STAT3 to the nucleus and STAT3 blockade inhibits metreleptin-induced lipolysis. Our initial data provide novel insights into the role of STAT3 as probable mediator of the action of metreleptin in regulating metabolism.

Key words: metreleptin, human primary adipocyte, STAT3, differentiation, lipolysis

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INTRODUCTION

Obesity is associated with a number of pathological disorders, such as type 2 diabetes, hypertension, hyperlipidemia, cardiovascular disease, respiratory complications, osteoarthritis, and cancer (Kopleman. 2000). Given the global epidemic and its association with many life-threatening chronic diseases, obesity induces a major health problem (James 2008; James. 2013). Adipose tissue mass is determined by processes governing adipocyte number and size (Greenwood et al. 1993; Shoelson et al. 2007). The number of adipocytes increases as a result of increased adipogenesis, i.e., proliferation and differentiation of pre-adipocytes, whereas adipocyte hypertrophy is determined by unbalanced lipogenesis and lipolysis (Greenwood et al. 1993).

Leptin, the adipocyte-secreted hormone, primarily regulates food intake and body weight by acting in the hypothalamus (Moon et al. 2013). Besides, the role of leptin in the brain for regulation of energy balance, it has also been indicated that leptin acts in peripheral tissues as a regulator of energy homeostasis, insulin action, lipid metabolism, and immune function (Carlton et al. 2012; Dardeno et al. 2010). Leptin receptors are found in diverse locations throughout the body supporting the central as well as peripheral role of leptin in metabolism (Lee et al. 2002; Seufert. 2004; Papathanassoglou et al. 2006). Administration of leptin has been observed to decrease insulin and glucose levels before a decrease in food intake or changes in body weight in leptin-deficient rodents (Schwartz et al. 1996). Also, peripheral leptin administration, but not food restriction, was able to completely correct severe insulin resistance in lipoatrophic mice (Ebihara et al. 2001). These results imply that leptin may act at the level of insulin-sensitive tissues independently from leptin's central action in regulating feeding behavior (Schwartz et al. 1996). Thus, it is important from the point of view of biology and human therapeutics to know which downstream signaling pathways are activated by leptin to mediate its effects in the peripheral tissues (Haque et al. 2002; Fiorenza et al. 2011).

Thus far, it has been determined only in animal models that leptin administration activates peripheral intracellular signaling pathways and plays a key role in the pathophysiology of insulin resistance (Margetic et al. 2002; Guo et al. 2007; Moon et al. 2012). However, whether metreleptin treatment has a direct effectiveness in metabolically important peripheral tissues, specifically adipose tissue, in humans remains unknown. Hence, in order to present detailed proof on metreleptin signaling in human primary adipocytes (hPA) metabolism, we extended previous observations by studying *in vitro* metreleptin signaling in cells from human adipose tissues. We also comparatively evaluated whether metreleptin may regulate differentiation and lipolysis in hPA from subcutaneous (SC)- vs. omental (OM)-derived hPA.

MATERIALS AND METHODS

Human primary adipocytes culture - Subcutaneous and omental human adipose tissue (hAT) samples were obtained from obese (aged 34–48 years, BMI 39–50 kg/m^2) male. The hAT was then digested with PBS/collagenase solution (3 mg collagenase/g tissue and 1 mL PBS/1 mg collagenase) + 3.5% fatty acid–free BSA and then filtered using a filter bottle unit (sterile funnel with double-layered gauze), and the solution was centrifuged at 1200 rpm for 10 min. The pellets were re-suspended in α-MEM supplemented with 15 mmol/l $NaHCO_3$, 15 mmol/l HEPES, 33 μmol/l biotin, 17 μmol/l pantothenate, 10 mg/ml human transferrin, 0.05 mg/mL gentamicin, and 10% FBS and then were plated overnight. After confluence, α-MEM was removed, and the cells were washed once or twice with Hanks' balanced salt solution. To induce adipocyte differentiation, the cells were exposed to differentiation medium containing 66 nmol/l insulin, 100 nmol/l cortisol, 0.2 nmol/l triiodothyronine, and 1 μg/mL ciglitazone. The medium was changed every 2 days, and cells were kept in culture for 28 days. All subjects provided written informed consent to participate, and the study was approved by the institutional review board at Beth Israel Deaconess Medical Center/Harvard Medical School and Korea University.

Lipolysis assay - Cells were seeded at 4 × 10^4 cells/cm^2 on 12-well culture plates and allowed to differentiate for 30 days. After cultures were serum-starved, the cells were treated with metreleptin for 24 h in the absence or presence of STAT3 inhibitor (AG549, 0.1% in media), and glycerol in the medium was measured by using a glycerol-3-phosphate oxidase (GPO)-Trinder kit (Sigma-

Aldrich). Protein content was determined using the BCA protein assay.

Glycerol-3-phosphate dehydrogenase (GPDH) activity - The GPDH assay was performed by a spectrophotometric method for determination of the disappearance of NADH during GPDH-catalyzed reduction of DHAP under zero-order condition.

Triacylglycerol (TG) content – The TG content was measured by using TG human ELISA kit (Sigma-Aldrich). Protein content was determined using the BCA protein assay.

The total protein extraction and Western Blotting - Cells were suspended in a lysis buffer containing 20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 0.1% Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 14,240 *g*, and the supernatant was saved as the total extract. 50 μg of tissue lysate protein per lane was resolved by SDS-PAGE (8% gel) and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose membranes were blocked with 5% nonfat dry milk for 1 h at room temperature, incubated with primary antibody (1:500 dilution in 1% nonfat dry milk overnight at 4°C. The membranes were washed with Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min, incubated with horseradish peroxidase secondary antibody (1:1000 dilution; Amersham Pharmacia Biotech, Arlington Heights, IL) for 2 h and washed with TBST for 30 min. The bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and quantified using Image J (http://rsbweb.nih.gov/ij/).

Nuclear fraction - For nuclear extracts, collected cells were suspended in a lysis buffer containing 10 mm Hepes-NaOH (pH 7.8), 10 mm KCl, 2mm $MgCl_2$, 1 mm dithiothreitol (DTT), 0.1 mm EDTA, and 0.1 mm PMSF and then incubated for 15 min at 4°C. After addition of 10% (v/v) Nonidet P-40, the cell suspension was mixed, incubated for 30 min at 4°C and centrifuged for 15 min at 890*g*. Next, the pellet was re-suspended in a lysis buffer containing 50 mm Hepes-NaOH (pH 7.8), 50 mm KCl, 300 mm NaCl, 0.1 mm EDTA, 1 mm DTT, 0.1 mm PMSF, and 10% (v/v) glycerol. The suspension was mixed and centrifuged, and the supernatant was saved as the nuclear extract.

Lipid staining - Cells were stained with ORO. Briefly, dishes were washed three times with PBS and fixed with 10% formalin for 1 hr at room temperature. After fixation, cells were washed once with PBS and stained with a filtered ORO stock solution (0.5 g ORO in 100 ml of isopropyl alcohol) for 30 min at room temperature. Then, the cells were washed twice with water for 15 min and visualized.

Statistical analysis - All data were analyzed using student t-test and/or one-way ANOVA followed by post-hoc test for multiple comparisons using SPSS (version 11.5).

RESULTS

Metreleptin regulates differentiation and lipolysis in human primary adipocytes - First, we checked whether *in vitro* metreleptin administration may regulate differentiation in hPA. As shown in Figure 1A and 1B, metreleptin did not regulate cell differentiation in dose- and time-dependent manner. In order to confirm this result, we measured GPDH activity (Supplementary data 1) and/or TG content (Supplementary data 2), showing that *in vitro* metreleptin administration did not regulate GPDH activity and TG content. Also, we could not find any major differences in differentiation activities at each time-point between SC- and OM-derived hPA in response to *in vitro* metreleptin administration. By contrast, we observed that *in vitro* metreleptin administration increases lipolysis in a time-dependent manner in both SC- and OM-derived hPA (Fig.1C and 1D). Also, despite the minor differences in magnitude of the results between SC- and OM-derived hPA, we did not find any major differences in both hPAs in terms of *in vitro* metreleptin-regulated lipolysis.

Fig. 1

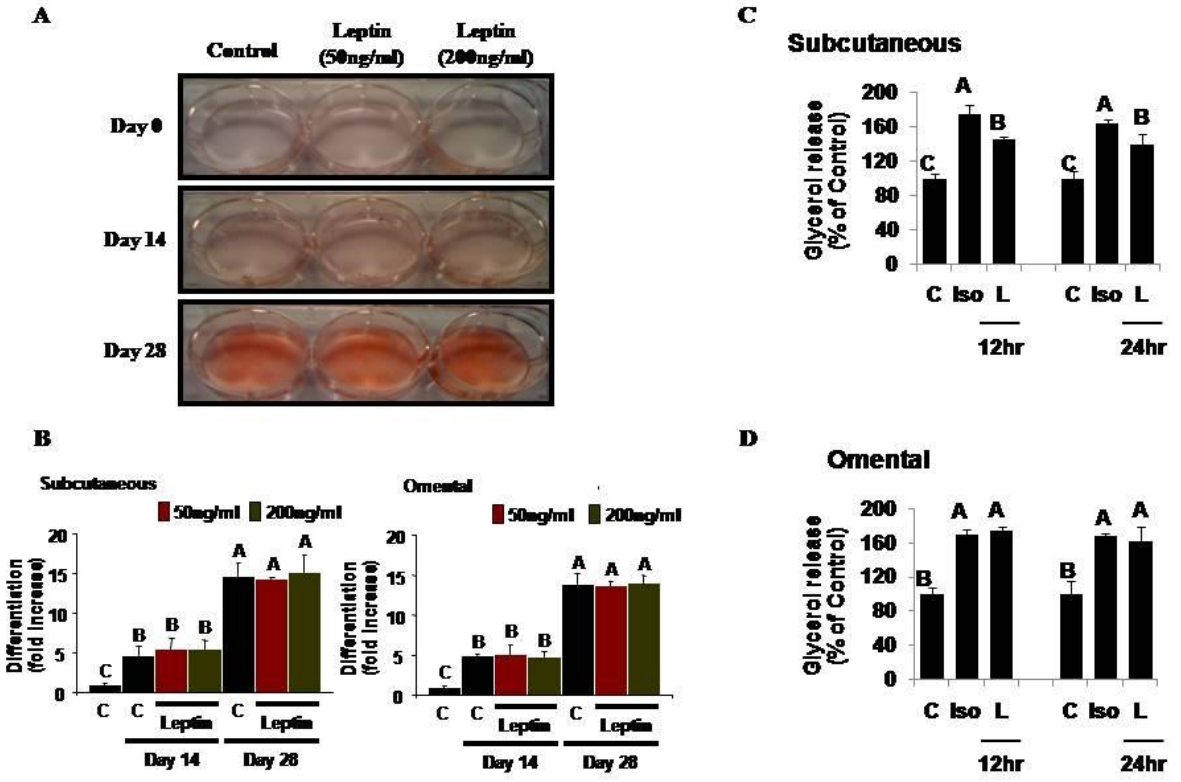

Figure 1- Regulation of cell differentiation and lipolysis by *in vitro* metreleptin administration in hPA. Cell culture was performed as described in Methods. (A-B) Cells were treated with metreleptin at indicated concentration for 14 and 28 days, respectively, and cell differentiation was measured by Oil-Red O staining as described in Methods. (C-D) Cells were treated with metreleptin (50 ng/mL) for 12 h and 24 h, respectively, and lipolysis assay was performed as described in Methods. Isoproterenol (10 µM for 2 h) was used as a positive control. All data were analyzed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; n=3; $p<0.05$ for any given superscript letter vs all others. C, control.

Metreleptin regulates lipolysis in human primary adipocytes via STAT3 signaling pathway - *In vitro* metreleptin administration activates STAT3 signaling in a time-dependent manner, showing that 50 ng/mL of metreleptin for 10 to 160 min significantly induces phosphorylation of STAT3 in both hPAs (Fig. 2A and 2B). Also, highest phosphorylation status of STAT3 (~5.5-fold higher than control) was observed at 20 min of *in vitro* metreleptin administration. Moreover, metreleptin showed a biphasic response in STAT3 activation and there was no difference in STAT3 activation in response to *in vitro* metreleptin administration between SC- and OM-derived hPAs. Importantly, we observed that *in vitro* metreleptin administration increases nuclear translocation of STAT3 in a dose-dependent manner in both hPAs (Fig. 2C). However, when challenged with STAT3 inhibitor, the metreleptin-stimulated lipolysis was abolished (Fig. 2D), suggesting that metreleptin-increased lipolysis is regulated by STAT3 signaling pathway.

Fig. 2

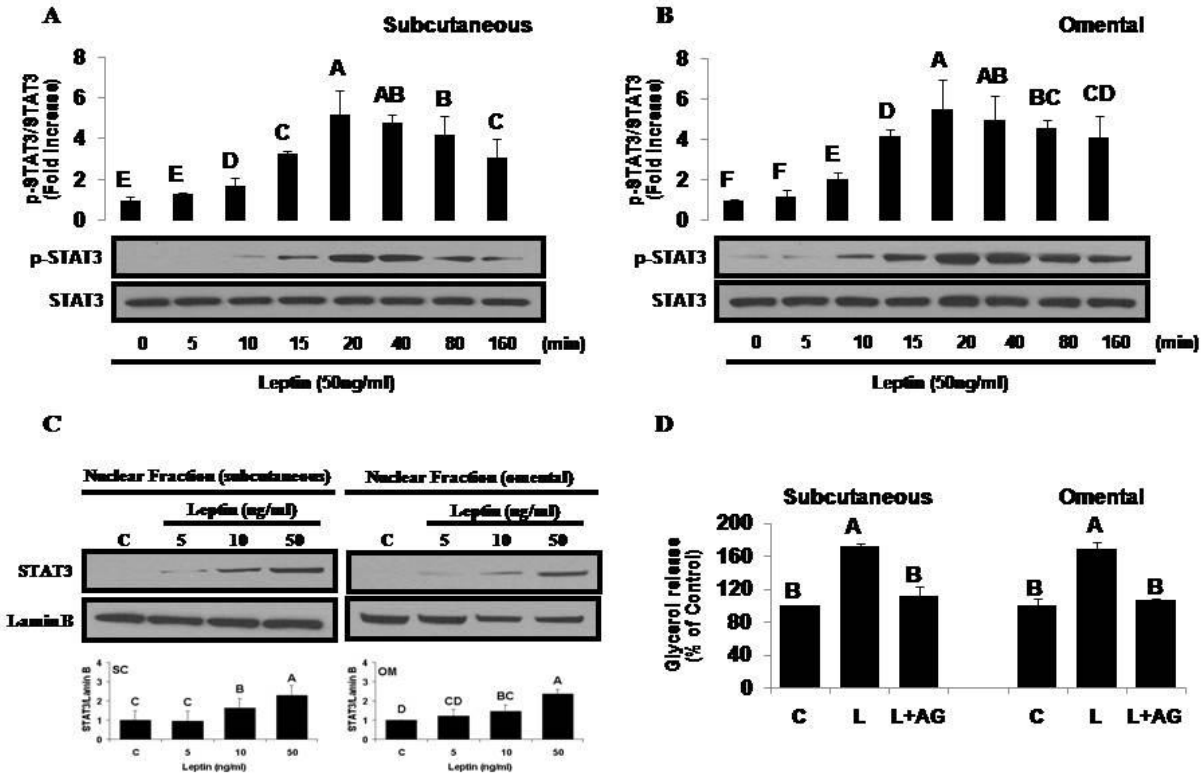

Figure 2- Regulation of STAT3 activation by *in vitro* metreleptin administration in hPA. Cell culture was performed as described in Methods. (A-C) Cells were treated with metreleptin (50 ng/mL) at indicated time period, and Western Blotting was performed as described in Methods. (D) Cells were treated with metreleptin (50 ng/mL) for 24 h in the absence or presence of STAT3 inhibitor (AG490 for 4 h), and lipolysis assay was performed as described in Methods. All data were analyzed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means ± SD; n=3; $p<0.05$ for any given superscript letter vs all others. C, control.

DISCUSSION

It has been demonstrated that leptin, adipocyte-derived hormone, plays many roles as a growth factor in several cell types, including adipose tissue and peripheral blood mononuclear cells which play a key role in pathophysiology of insulin resistance (Miyazaki et al. 2008; Asiamah et al. 2009). However, it has to be elucidated whether synthetic analog of the leptin, metreleptin, may have similar effects on hPA in terms of regulating differentiation and lipolysis. Also, since STAT3 signaling pathway is considered to be primary target of leptin in adipocytes, it has to be confirmed whether metreleptin may stimulate STAT3 activation in hPA. In order to address these questions, we conducted *in vitro* signaling studies and checked whether metreleptin-regulated differentiation and lipolysis is modulated by STAT3 signaling in hPA.

It has been shown that overexpression of the leptin gene in adipocytes and increased circulating levels of leptin may contribute to enhance lipolysis in obesity (Sadri et al. 2011). Similarly, we observed that *in vitro* administration of metreleptin induces lipolysis in both SC- and OM-derived hPA. Also, we found that metreleptin increases phosphorylation level of STAT3 in both SC- and OM-derived hPA. STAT3 is the prototype signaling molecule related

to the leptin receptor, which modulates food intake and body weight primarily in the hypothalamus (Tanida et al. 2015). STAT3 mediates the expression of a variety of genes in response to cells stimuli (Moon et al. 2012; Moon et al. 2011) and, therefore, STAT3 plays a central role in cellular processes such as cell growth and apoptosis (Moon et al. 2011). In fact, interrupting the ability of the leptin receptor to activate the STAT3 pathway causes to severe obesity and several other neuroendocrine abnormalities (van den Brink et al. 2000). Also, in response to cytokines and growth factors, STAT family members are phosphorylated by receptor-related kinases and then form homo- or hetero-dimers that translocate to the cell nucleus, where they act as transcription activators (Qin et al. 2008). This protein is activated by phosphorylation of tyrosine 705 in response to diverse cytokines and growth factors including leptin (Qin et al. 2008). Based on these results, we performed whether *in vitro* metreleptin-regulated lipolysis is modulated by STAT3 signaling, demonstrating that metreleptin increases lipolysis via STAT3 signaling in hPA. Importantly, we found for the first time that metreleptin is capable of trans-locating STAT3 to the nucleus and STAT3 blockade inhibits metreleptin-induced lipolysis. These results are consistent with rodent studies previously demonstrating that leptin-treated rats tend to have a greater number of p-STAT3-positive cells than vehicle controls in hypothalami (Turek et al. 2010). Although it is important to study the effects of metreleptin in human hypothalamic or other central nervous system cells, our data suggest that metreleptin-increased translocation of STAT3 to the nucleus in hPA may have important clinical implications.

In summary, we observed for the first time that metreleptin-modulated lipolysis is regulated by translocation of STAT3 to the nucleus in both SC- and OM-derived hPA. Also, despite minor differences in the timing of signaling activation in hPA studied, we did not observe major differences in the magnitude of STAT3 activation in response to metreleptin administration between SC- and OM-derived hPA. Although we studied the signaling pathway that is considered to be primary target of leptin, on the basis of current evidence deriving mainly from rodent studies (Moon et al. 2012), we did not look at all possible signaling pathways. Thus, much more work needs to be done (additional tissues/cells, other signaling pathways) in the future. Despite these limitations, our initial data suggest that metreleptin may provide novel insights into the role of STAT3 as probable mediators of the action of metreleptin in regulating hypertrophy in hAT.

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DECLARATION OF INTEREST

The authors have declared no conflicts of interest.

AUTHOR CONTRIBUTION

All authors participated in the study design, performance and coordination. All authors contributed to writing the manuscript. All authors read and approved the final manuscript.

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