Early intracellular signaling events induced by *in vitro* metreleptin administration in cardiac myocytes and uterine smooth muscle cells

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Abstract

Intracellular signaling pathways regulated by leptin have largely been studied in metabolically important organs such as adipose tissue and peripheral blood mononuclear cells, suggesting that leptin plays a key role in pathophysiology of insulin resistance. However, whether synthetic analog of leptin, metreleptin, has similar effects on cardiac myocytes (CM) and uterine smooth muscle cells (USMC) has not yet been studied. Hence, in order to address these questions, we extended previous observations and investigated *in vitro* signaling study whether metreleptin may activate key signaling pathways. We observed that metreleptin activates Jak2 and STAT3 signaling pathways in dose- and time-dependent manner in CM and USMC. Also, we found that metreleptin increases ERK1/2, JNK and/or p38 phosphorylation in CM. *In vitro* metreleptin administration also increased ERK1/2 and/or p38 phosphorylation in USMC. By contrast, JNK was not regulated by *in vitro* metreleptin administration in USMC. Moreover, metreleptin-activated all signaling pathways were blocked by pre-treatment of PD98095 (ERK inhibitor), SB203580 (p38 inhibitor) and/or SP600125 (JNK inhibitor), respectively. Finally, metreleptin increased cell size (hypertrophy) in both CM and USMC. Our data provide novel insights into the role of Jak2, STAT3, ERK1/2, JNK and/or p38 as probable mediators of the action of leptin in regulating hypertrophy in CM and USMC.

Key words: Cardiac myocytes, hypertrophy, metreleptin, signaling pathways, uterine smooth muscle cells.

Introduction

Metreleptin is a synthetic analog of the hormone leptin used to treat diabetes and various forms of dyslipidemia (1-3). It is has been approved in Japan for metabolic disorders including lipodystrophy and in the United States as replacement therapy to treat the complications of leptin deficiency, in addition to diet, in patients with congenital generalized or acquired generalized lipodystrophy (2-3). Also, metreleptin is currently being investigated for the treatment of diabetes and/or hypertriglyceridemia, in patients with rare forms of lipodystrophy, syndromes characterized by abnormalities in adipose tissue distribution, and severe metabolic abnormalities (3). In addition, it has been demonstrated that metreleptin activates key signaling pathways i.e. STAT3, Akt, ERK and AMPK which play a key role in pathophysiology of insulin resistance in metabolically important peripheral tissues such as adipose tissue and peripheral blood mononuclear cells (2,4). These results indicate that metreleptin may act at the level of insulin sensitive tissues independently from metreleptin's central action in regulating feeding behavior (2,5).

Cardiac muscle cells or cardiac myocytes (CM) are the muscle cells that make up the cardiac muscle (6). It has been shown that CM rapidly proliferates during fetal life but exit the cell cycle soon after birth in mammals (7). Although the extent to which adult cardiac myocytes are capable of cell cycle reentry is controversial and species-specific differences may exist, it appears that for the vast majority of adult CM the predominant form of growth postnatally is an increase in cell size (hypertrophy) not number (7,8). The uterus undergoes many changes during pregnancy and must achieve enormous expansion to accomodate the growing fetus, and to support the fetus through sustained muscle tone, without generating propagated contractions (9). Specifically, uterine smooth muscle cells (USMC) are supporting stromal and vascular tissue which induce uterine contractions (9,10), suggesting that the culture of USMC may identify potential regulatory pathways and study the action of a variety of signaling factors that modulate both normal and pathological uterine activity.

Recently, it has been shown that leptin receptor isoforms are expressed in CM and USMC, suggesting that leptin may play a role in CM and USMC (7,11). In fact, it has been suggested in *in vitro* studies that leptin induces hypertrophy in cultured neonatal rat ventricular myocytes in a concentration-dependent manner (12-13). However, no previous study has evaluated synthetic analog of the leptin, metreleptin, signaling nor investigated how metreleptin interacts with hypertrophy in human CM and USMC. In order to address these questions, we performed *in vitro* signaling studies to clarify the role of metreleptin in activating metabolically important signaling pathways as well as their potential interaction with hypertrophy in human CM and USMC.

Materials and methods

Materials

Primary and secondary antibodies such as mouse monoclonal STAT3, goat polyclonal phosphorylatedS-TAT3, rabbit polyclonal ERK1/2, goat polyclonal phosphorylated ERK1/2, mouse polyclonal JNK, goat polyclonal phosphorylated JNK, mouse monoclonal p38, rabbit polyclonal phosphorylated p38, anti-rabbit IgG horseradish peroxidase (HRP), anti-mouse IgG HRP and anti-rabbit IgG HRP were purchased from Santa Cruz Biotech (San Francisco, CA, USA). Metreleptin recombinant was purchased from ProSpec (East Brunswick, NJ, USA). PD98095 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) were purchased from Sigma-Aldrich (Carlsbad, CA, USA).

CM and USMC cell culture

The CM from normal human ventricle tissue of the adult and USMC from normal human coronary artery of the adult were purchased from Promo Cell GmbH (Heidelberg, Germany). The cells were cultured using CM's and/or USMC's basal and growth medium according to the manufacture's protocol.

The 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/lphenyl methyl sulfonyl 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 30min, 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/**).

Hypertrophy

Hypertrophy assay was performed as described in detail previously (14). Briefly, the cells grown in 48-well plates, fixed with 4% paraformaldehyde in PBS for 15 min, followed by 0.5% Triton-100 treatment for 5 min. After incubated with 0.1% rhodamine-phalloidin for 30min, cells were washed in PBS for further interaction with DAPI. The images of cell surface are from
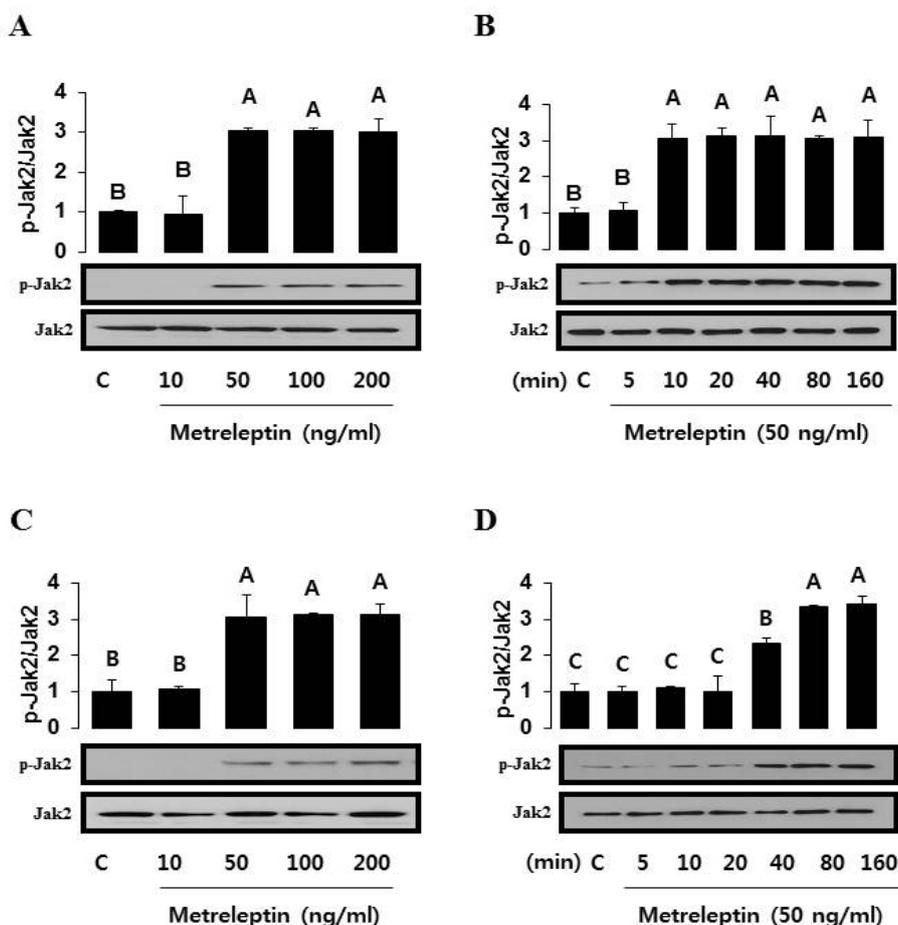

Figure 1. Metreleptin activates Jak2 in dose- and time-dependent manner in CM and USMC. Cell culture was performed as described in Method section. (A) CM and (C) USMC were treated for 80 min with metreleptin at the indicated concentrations. (B) CM and (D) USMC were treated at the indicated times with 50 ng/ml metreleptin. All density values for each protein band of interest are expressed as a fold increase. 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.

randomly selected fields (50 for each group, at least 200 cells) were determined by Image J.

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). $p < 0.05$ for any given superscript letter vs all others.

Results

Metreleptin activates Jak2 and STAT3 in dose- and time-dependent manner in CM and USMC

Treatment of CM and USMC with 10 ng/ml metreleptin had no effect on Jak2 (Fig. 1A and 1C) and STAT3 (Fig. 2A and 2C) activation when compared to control. By contrast, administration of 50-200 ng/ml metreleptin in CM and USMC cells increased Jak2 (Fig. 1A and 1C) and STAT3 phosphorylation (Fig. 2A and 2C) by ~3-fold when compared to control. Importantly, metreleptin signaling was saturable at metreleptin concentrations of 50 ng/ml (Fig. 1A, 1C, 2A and 2C). Metreleptin administration also significantly induced phosphorylation of Jak2 and STAT3 in a time-dependent manner, with phosphorylation increased by ~3-fold when compared to control at 20 min in CM (Fig. 1B and 2B) and at 80 min in USMC (Fig. 1D and 2D).

Metreleptin activates ERK1/2, JNK and p38 in CM and USMC

Based on the above (Fig. 1 and 2), we chose a representative administration time (80 min) and metreleptin (50 ng/ml) concentration, and performed metreleptin signaling studies. We observed that *in vitro* metreleptin administration increases ERK1/2, JNK and/or p38 phosphorylation in CM (Fig. 3A, 3B and 3C). *In vitro* metreleptin administration also increased ERK1/2 and/ or p38 phosphorylation in USMC (Fig. 3D and 3F). By contrast, JNK was not regulated by *in vitro* metreleptin administration in USMC (Fig. 3E). Also, we observed that increased ERK1/2, JNK and/or p38 phosphorylation by metreleptin administration was abolished by ERK, JNK and/or p38 inhibitors, respectively (Fig. 3A-3F).

Metreleptin increases hypertrophy in CM and USMC

Hypertrophy was increased by *in vitro* metreleptin administration in both CM (Fig. 4A) and USMC (Fig. 4B).

Discussion

Leptin is an adipocyte-derived hormone, showing that it is increased in patients with obesity (15). Also, metreleptin is a synthetic analog of the leptin and has been approved for metabolic disorders as replacement
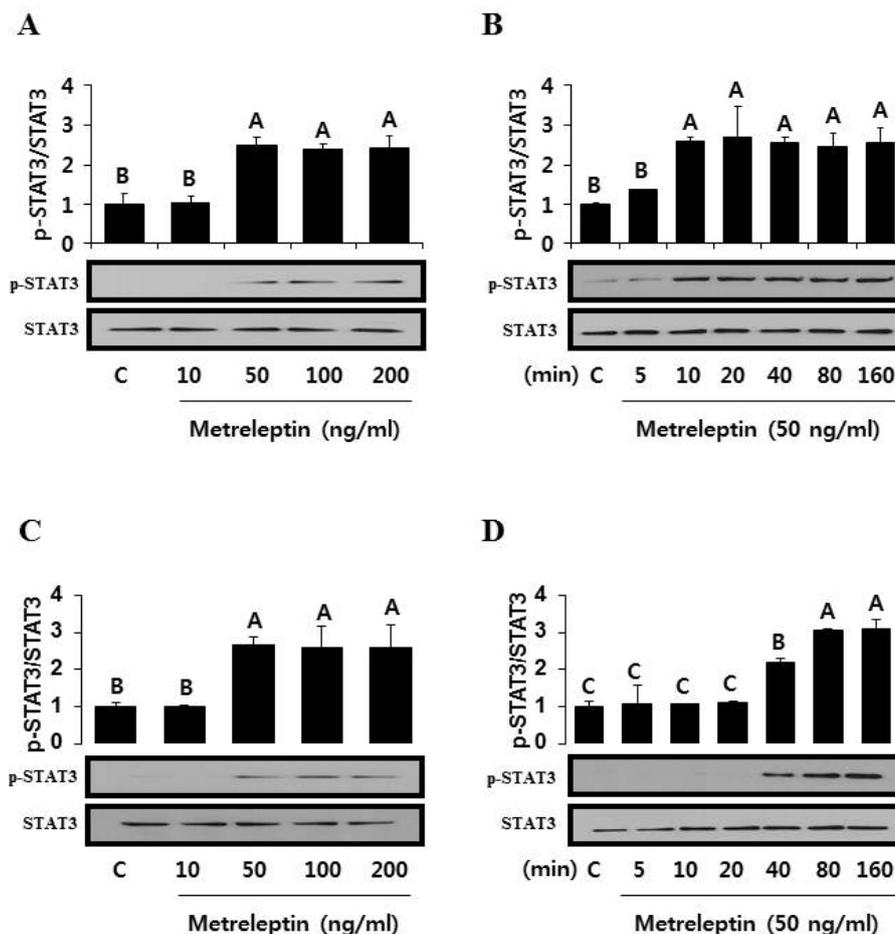

Figure 2. Metreleptin activates STAT3 in dose- and time-dependent manner in CM and USMC. Cell culture was performed as described in Method section. (A) CM and (C) USMC were treated for 80 min with metreleptin at the indicated concentrations. (B) CM and (D) USMC were treated at the indicated times with 50 ng/ml metreleptin. All density values for each protein band of interest are expressed as a fold increase. 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.
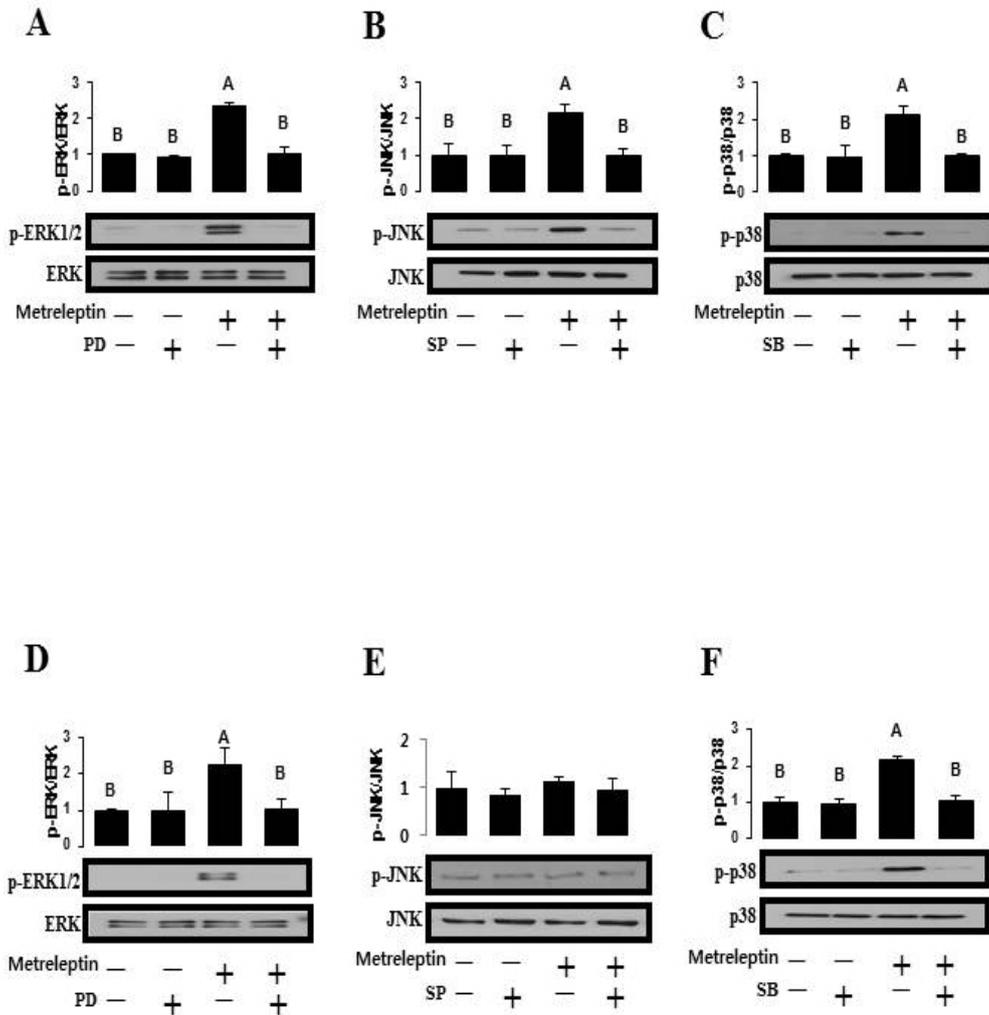
Figure 3. Metreleptin activates ERK1/2, JNK and/or p38 in CM and USMC. Cell culture was performed as described in Method section. (A-C) CM and USMC (D-F) were pre-treated with PD98095 (ERK inhibitor), SB203580 (p38 inhibitor) and/or SP600125 (JNK inhibitor), and then stimulated for 80 min with metreleptin. All density values for each protein band of interest are expressed as a fold increase. All data were analyzed using one-way ANOVA followed by a post hoc test for multiple comparisons. Values are means $\pm$ SD; n=3; $p<0.05$ for any given superscript letter vs all others. C, control.
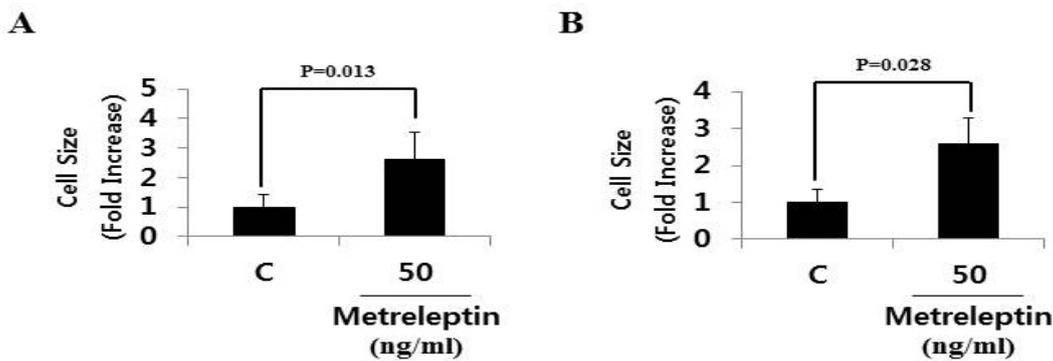

Figure 4. Metreleptin increases hypertrophy in CM and USMC. Cell culture was performed as described in Method section. (A) CM and (B) USMC were treated with metreleptin for 3 days and hypertrophy was then measured as described in Method section. All data were analyzed using student t-test. Values are means $\pm$ SD; n=3; C, control.

therapy to treat the complications of leptin deficiency (2-3). While it has become clear that leptin 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, it has to be elucidated whether synthetic analog of the leptin, metreleptin, may have similar effects on CM and USMC. Also, it has been shown that increased plasma leptin level is associated with coronary heart disease and heart failure (15). Moreover, it has been suggested that leptin may play a role in uterine contractility (16-17). Since leptin is obesity-related peptide (3), it has become increasingly important to understand the effect of obesity-associated hyperleptinemia on cardiovascular system and/or uterine contraction (17). However, it is unclear whether synthetic analog of leptin, metreleptin, may regulate uterine and/or cardiovascular hypertrophy. In order to address these questions, we conducted *in vitro* signaling studies and checked whether metreleptin may regulate key signaling pathways i.e. Jak2, STAT3,

ERK1/2, JNK and/or p38 in CM and USMC. Next, we directly measured the hypertrophy of CM and USMC in response to metreleptin administration.

STAT3 is the prototype signaling molecule associated with the leptin receptor, which regulates food intake and body weight primarily in the hypothalamus (18). We have previously shown that metreleptin activates Jak2 and STAT3 signaling in adipose tissue, peripheral blood mononuclear cells, GT1-7 hypothalamic cells, AML12 liver cells and/or C_2C_{12} muscle cells (1,2,4). Our data are consistent with previous human and rodent studies that metreleptin increases Jak2 and STAT3 phosphorylation in dose- and time-dependent manner in CM and USMC. Importantly, metreleptin signaling pathway was saturable at a level of ~50 ng/ml, suggesting that no additional signaling effect can be observed at higher doses of metreleptin. Since Jak2 and STAT3 mediate the expression of various genes in response to cell stimuli and thus plays a key role in many cellular processes such as cell growth and apoptosis (1,4), our data suggest that metreleptin-activated Jak2 and STAT3 signaling in CM and USMC has important clinical implications.

Since mitogen-activated protein kinase (MAPK) is the major kinase responsible for cell proliferation, differentiation and survival, and is also a significant downstream target for leptin's physiological effects (19,20), we next investigated whether metreleptin-increased hypertrophy in CM and USMC is associated with MAPK signaling pathway. The ERK1/2 pathway is a chain of proteins in the cell that communicate a signal from a receptor on the surface to the DNA in the nucleus (21). It has been shown that ERK is an additional pathway downstream of the leptin receptor (4,22). Also, metreleptin has been shown to activate ERK1/2 in a time- and dose-dependent manner in several cultured mouse cells *in vitro*, as well as in rodent tissues *in vivo* (23,24).The JNK and p38, the MAPK family, are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock (25.26). It has been demonstrated that JNK is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions and/or cytokine production (27). Leptin has been shown to increase Kupffer cell function via JNK signaling pathway (28). Also, it has been demonstrated that leptin induces hypertrophy via p38 signaling in rat vascular smooth muscle cells (29). In agreement with these previous results, we observed herein that synthetic analog of leptin, metreleptin, also activates ERK1/2, JNK and p38 signaling in CM, and stimulate ERK1/2 and p38 signaling in USMC. Interestingly, we found that metreleptin did not regulate JNK signaling in USMC, suggesting that metreleptin-increased hypertrophy in USMC is not regulated by JNK signaling pathway.

In conclusion, we observed for the first time that metreleptin activates metabolically important signaling pathways such as Jak2, STAT3, ERK1/2, JNK and/or p38 in CM and USMC. Also, we found that despite minor differences in the timing of signaling activation, we did not observe major differences in the magnitude of all signaling activation in response to metreleptin administration in CM and USMC studied. Importantly, activations of all signaling pathways were saturable at a metreleptin concentration of ~50 ng/ml, suggesting that

above that level, i.e., the level clinically seen in obese subjects at baseline, no additional signaling effect can be observed. Since *in vitro* metreleptin signaling may differ from those in mouse *in vivo*, much more work needs to be dome in the future. Also, since we did not look at all possible signaling pathways, *ex vivo* and/or *in vivo* animal studies are needed to confirm the *in vitro* metreleptin signaling data. Despite these limitations, our initial data from *in vitro* metreleptin signaling studies provide novel insights into the role ofJak2, STAT3, ERK1/2, JNK and/or p38 as probable mediators of the action of metreleptin in regulating hypertrophy in CM and USMC.

Acknowledgments

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