

Early signaling interactions between the insulin and leptin pathways in bovine myogenic cells

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Abstract

Cross-talk between hormone signaling pathways provides mechanisms to facilitate flexibility in the cellular response to extracellular conditions. One function of insulin is to signal high extracellular glucose, while leptin may signal the abundance of extracellular lipid, both energy sources being readily utilized by muscle. The present study reports early signaling events in the insulin and leptin cascades in primary bovine myogenic cells (BMC). BMC were treated with insulin, or leptin for 1, 10, 30 and 120 min, or pretreated with leptin for 10 min followed by insulin for 1, 10, 30 and 120 min. BMC were insulin resistant, showing a significant inhibition of IRS-1 association with the insulin receptor (IR) following insulin stimulation, a corresponding increase in PI 3-kinase association with the IR, and a slow and modest increase in GLUT4 recruitment to the plasma membrane. Pretreatment of BMC for 10 min leptin, followed by insulin time-course, caused IRS-1 recruitment to be unresponsive, but evoked a rapid, phasic response of PI 3-kinase recruitment to the IR and abrogated the response of GLUT4 translocation to the plasma membrane evoked by insulin alone. The lack of insulin response was independent of IR abundance or affinity. JAK-2 association with the ObR and JAK-2 tyrosine phosphorylation were responsive to all three treatments. Insulin alone down-regulated the leptin signaling pathway, JAK-2 association with ObR decreased at all time-points, and JAK-2 phosphorylation decreased similarly. Leptin alone also appeared to down-regulate JAK-2 association with the ObR, but stimulated the down-regulated pathway to signal, JAK-2 tyrosine phosphorylation being increased at later time-points. Pretreatment with leptin followed by insulin time-course showed marked up-regulation of the early leptin signaling pathway, JAK-2 association with the ObR being increased by insulin while JAK-2 tyrosine phosphorylation was also increased. The contrasting responses of BMC to insulin alone, leptin alone and the sequential leptin–insulin treatment may point to the ability of these cells to respond to energy substrate availability, as bovine muscle has evolved to utilize lipids and fatty acids in response to a metabolism which provides only limited glucose. This cross-talk between insulin and leptin signaling pathways points to a better understanding of the mechanisms driving energy substrate utilization in ruminant muscle and may provide a useful model for greater understanding of the molecular mechanisms underlying the development of insulin resistance and Type 2 diabetes in man.

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Abbreviations: IR, insulin receptor kinase; IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; GLUT4, glucose transporter 4; ObR, leptin receptor; JAK-2, Janus kinase-2; BMC, bovine myogenic cells

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1. Introduction

Muscle is an important tissue in the study of the development of peripheral insulin resistance. Recent studies have begun to use cultured myogenic cells isolated from patients with type 2 diabetes [1,2] to investigate possible defects in the insulin signaling pathways in these cells. We

have recently described early insulin signaling events in the mouse Sol8 myogenic cell line [3]. This cell line, derived from oxidative muscle and having abundant mitochondria, has the potential to readily utilize fatty acids rather than glucose. However, early insulin signaling in this model appears to be responsive to insulin. Studies in rodent myogenic cell models [4–7] have been complemented by those in which tissue explants have been collected from both human subjects [8–12] and animal models [13–15] and insulin signaling pathways characterized.

In studies of energy partitioning in muscle, it appears that some actions leptin and insulin may be antagonistic as insulin inhibits oxidation of free fatty acids and leptin appears to suppress this insulin effect [16–19]. Evidence for the insertion of the leptin and insulin pathways is also supported as an increase in fatty acid oxidation in muscle associated with insulin resistance appears to be linked to an increase in diacylglycerol synthesis and activation of protein kinase C [20–22], a blunting of insulin-stimulated IRS-1 tyrosine phosphorylation [22] and inhibition of glucose transport and glucose phosphorylation [23]. The effects of leptin treatment on muscle may be partially attenuated by synthetic blockade of PI3-kinase activity [18]. Leptin also directly stimulates fatty-acid oxidation in muscle by activating the 5'-AMP-activated protein kinase (AMPK), an enzyme that phosphorylates and subsequently inactivates acetyl CoA carboxylase [24]. Recent evidence suggests a new paradigm for the role of leptin in the periphery and the interaction of the leptin and insulin signaling axes: Solinas et al. [25] propose that leptin may stimulate thermogenesis in skeletal muscle via a mechanism independent of decoupling of beta oxidation from ATP synthesis (through UCPs). In this scenario, leptin may induce a futile cycling between de novo lipogenesis and lipid oxidation, a process also requiring glucose and thus an interdependence on insulin signaling. It is proposed that AMPK in response to leptin and PI3-kinase in response to insulin play pivotal roles in this process.

The interactions between leptin and insulin signaling pathways are likely to be both tissue-specific and reflect physiological demand [26–30]. Studies in liver or hepatoma cells have demonstrated interaction between Janus Kinases (JAKs) and IRS-1 with apparent downstream effects on signaling via STATs [31,32] also observed in the hypothalamus [33]. Other studies have described effects of leptin on the IRS–PI 3-kinase signaling pathway [27,31], also apparent in hypothalamus [34]. Leptin may also interact with insulin signaling via the MAPK pathway [26]. Thus, it appears that leptin and insulin signaling may intersect at multiple points in each pathway.

In the present study, we have investigated early events in the insulin and leptin signaling cascades in a primary bovine myogenic cell (BMC) model. In terms of the specific actions of insulin on glucose metabolism, bovine muscle provides a model of insulin resistance which has no associated pathology and has evolved due to the unique nutritional

physiology of ruminants. These species are not subjected to large fluxes in blood glucose, fatty acids or amino acids due to the steady release of nutrients from the rumen and insulin may have a less prominent role in glucose regulation in bovine physiology. We report recruitment of early signaling molecules in each of the insulin and leptin signaling pathways in response to time-course treatments of insulin and leptin alone and sequential treatment, 10 min leptin followed by an insulin time-course.

2. Methods

2.1. Myogenic cells and cell culture

Postnatal skeletal muscle stem cells (termed bovine myogenic cells, BMC) were isolated by methods described previously [35,36]. Use of animals in this research was screened by the Animal Care and Use Committee at Washington State University and met the standards imposed by both the United States Department of Agriculture and the Public Health Service. BMC were routinely cultured in DMEM with 10% FCS plus penicillin/streptomycin (CM) at 37 °C, and 5% CO₂-enriched air. Cells were expanded and cultures allowed to grow to 80–90% of confluence, and morphologically remained in an undifferentiated state. For all experiments reported here, cells were used between passages 9 and 11. For insulin, leptin and sequential leptin and insulin treatments, cultures were grown to sub-confluence in CM and the medium changed 18h before the treatments were applied in DMEM plus 0.3 mM BSA, fatty acid free plus 0.3 mM fatty acids, 0.1 mM of each: oleic, linoleic and palmitic acids (Sigma Chemical Co., St Louis, MO).

2.2. Experimental treatments

A time-course response to insulin (100 nM), leptin (60 nM) or leptin (60 nM) 10 min pretreatment and then insulin (100 nM) time-course was determined. Cultures were incubated in the presence of bovine insulin (Gibco Invitrogen, Carlsbad, CA), bovine leptin (A. Gertler) or leptin 10 min and then insulin for either 0,1,10,30 or 120 min.

2.3. Preparation of cell lysates

Following experimental treatments, cultures were washed ($\times 3$) in DMEM, and flooded with 15 ml of liquid nitrogen for 30 s to freeze fracture the cells. Immediately after evaporation of the liquid nitrogen, 10 ml of lysis buffer, 50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, plus antiprotease Complete mini-cocktail (Roche Diagnostics, GmbH, Germany) pH 7.3 was added. Cell lysates were then incubated at 4 °C for 2 h, then aliquoted and stored at

–80 °C. Total protein was determined using the BCA assay (Pierce Chemical Co., Rockford, IL).

2.4. Immunoprecipitation

For each specific protein studied, 5 µl of specific antibody was preincubated with 20 µl protein A Sepharose (Pierce, Rockford, IL) for 2 h at 4 °C, and 1000 µg of cell lysate protein was then added and incubated with constant agitation overnight at 4 °C. The mixture was then centrifuged at 14000 x g for 2 min, the supernatant aspirated and the pellet resuspended in 1 ml lysis buffer. The beads were washed a further two times and finally suspended in 50 µl non-reducing Laemmli buffer. The specific immunoprecipitating antibodies were each raised in rabbits and were directed against either the IR α -chain, IRS-1, PI3-kinase (p85), or JAK-2 (Upstate Biotechnology, Lake Placid, NY), or GLUT4 (Abcam, Cambridge, UK). For the immunoprecipitation of ObR, a mouse monoclonal anti-extracellular domain of human ObR (clone 10B4), a kind gift of Dr. Christian Strasburger [37] was used.

2.5. Electrophoresis and immunodetection

Proteins in immunoprecipitates were resolved using SDS PAGE under non-reducing conditions (Hoefer Scientific Instruments, San Francisco, CA), transferred to nitrocellulose (Semi-dry Transfer, Hoefer, Model TE-70) and the proteins detected using a rabbit antibody as shown for immunoprecipitation as primary antibody (1:1000); and mouse monoclonal (clone 4G10) anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY) (1:1000) to detect tyrosine phosphorylated proteins. Blots were incubated simultaneously with dual secondary antibodies, (1:5000): Alexa Fluor 680-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and IRDye 800-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA). Total specific proteins and phosphorylated proteins were simultaneously detected using a dual infrared laser fluorescence imager (Odyssey, Li-Cor Biosciences, Lincoln, NE) with resolution set to 169 µm. For each experiment, controls and treatments were applied, resolved and blotted from the same gel and each of these was replicated three to six times. Only within-blot data are reported (paired *t*-test). Data are presented as the mean \pm S.E.M.

2.6. Plasma membrane preparation and GLUT4 analysis

Following treatments, crude plasma membranes were prepared from whole cell lysates as described [3] and 100 µl aliquots were stored at –80 °C. Protein content was determined using the BCA assay with bovine serum albumin as the standard (Pierce Chemical Company, Rockford, IL). GLUT4 was immunoprecipitated using an anti-GLUT4 antibody (Abcam, Cambridge, UK), electrophor-

osed, blotted under reducing conditions and detected as described above.

2.7. Saturation binding analysis

Insulin binding to IR was performed on whole cells using saturation binding analysis, exactly as described previously [3]. Data were modeled using the Ligand Binding Module in SigmaPlot version 8 (SPSS, Chicago, IL), and IR affinity and receptor number determined.

3. Results

3.1. Insulin Receptor Tyrosine kinase (IR)

IR abundance was affected by insulin only after 30-min incubation ($P < 0.1$, Fig. 1a). As expected, there was little change detected in IR abundance, the only other response observed following 10 min of incubation in the presence of leptin ($P < 0.1$). Phosphotyrosyl (pY)-IR was increased in response to insulin after 30 and 120 min incubation ($P < 0.1$, Fig. 1b). pY-IR was also increased by sequential treatment, leptin (plus 1 min insulin, $P < 0.1$). In the leptin alone treatment, pY-IR detection was near the limit of sensitivity of the instrument (data not reported).

3.2. IRS-1 and p85 PI 3-kinase

In response to insulin alone, IRS-1 was only marginally increased ($P < 0.1$) after 10 min and returned to basal levels (Fig. 2a). Leptin alone had no effect on IRS-1. In response to sequential leptin, insulin treatment, IRS-1 rapidly increased (1 min insulin) more than 3.5-fold ($P < 0.05$), suggesting leptin and insulin interact to increase cellular IRS-1 but not necessarily IRS-1 recruitment to either pathway. This response to leptin–insulin was rapidly attenuated and remained unresponsive up to 120 min.

PI 3-kinase (Fig. 2b) showed only a small response to insulin being slightly elevated at 1 min insulin ($P < 0.1$) and returning to basal levels for the remaining experimental period. PI 3-kinase also showed a small response to leptin, being slightly reduced at 1 min ($P < 0.05$), slightly elevated at 10 min ($P < 0.1$), then unresponsive. Sequential leptin, insulin treatment also evoked only a small response, PI 3-kinase being elevated only following 10 and 30 min insulin stimulation ($P < 0.1$).

3.3. Co-precipitation of IRS-1 and p85 PI 3-kinase with IR

Recruitment of IRS-1 and PI 3-kinase to the early insulin-signaling cascade was determined by their co-immunoprecipitation with IR. IRS-1 recruited specifically to IR showed a persistent, decreased response to insulin treatment (10, 30 and 120 min, $P < 0.1$, $P < 0.05$, and $P < 0.1$, respectively, Fig. 3a), indicating that these cells are apparently insulin

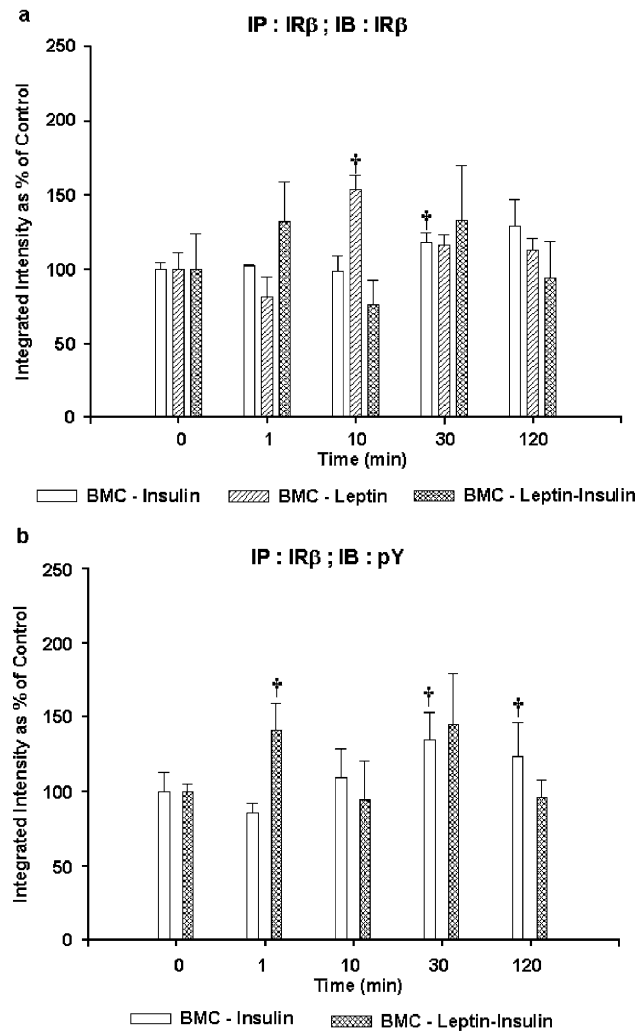


Fig. 1. Bovine myogenic cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course, for the indicated times, lysed and insulin receptor (IR) immunoprecipitated using an excess of specific antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, Western blotted using the same antibody for (a) total IR or (b) anti-phosphotyrosine for pY-IR, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ($^{\dagger}P < 0.1$, versus control, $n=3$).

resistant. Leptin treatment evoked minimal response of IRS-1 recruitment to the IR, showing only a small reduction at 120 min ($P < 0.1$). In the sequential leptin–insulin treatment, leptin apparently blunted the down-regulation of insulin signaling, as there was no significant change in IRS-1 recruitment to the IR throughout the insulin time-course, in contrast to the reduction in IRS-1 recruitment observed in response to insulin alone. Interestingly, PI 3-kinase recruitment to the IR in response to insulin appeared to follow a contrasting pattern to IRS-1, increasing at the 10-min time-point ($P < 0.1$) and remaining numerically, but not statistically higher than basal for the remainder of the experiment (to 120 min, Fig. 3b). Leptin treatment evoked no change in PI 3-kinase recruitment to the IR. Furthermore, following sequential leptin–insulin treatment, PI 3-kinase recruitment to the IR rapidly increased at 1 min insulin ($P < 0.01$), falling to baseline at 10 min insulin and rising again at 30 min insulin ($P < 0.05$). The magnitude and pattern of this response

reflected IR tyrosine phosphorylation (Fig. 1b) and was opposite to that shown for IRS-1 recruitment to the IR.

3.4. GLUT4 translocation to the plasma membrane

To examine the effects of insulin, leptin and the sequential leptin–insulin treatment in the BMC model on an important target of the IR-PI 3-kinase pathway, crude plasma membranes were prepared and the levels of GLUT4 quantified (Fig. 4). Despite the apparent blunted response to insulin in early signaling events, GLUT4 translocation to the plasma membrane in response to insulin was increased at both 10 min ($P < 0.05$) and 120 min ($P < 0.1$). Leptin alone also appeared to slightly increase GLUT4 translocation at 1 min and 120 min ($P < 0.05$). Sequential leptin–insulin treatment appeared to evoke no change in GLUT4 translocation, suggesting that leptin pretreatment had disrupted the insulin-signaling pathway.

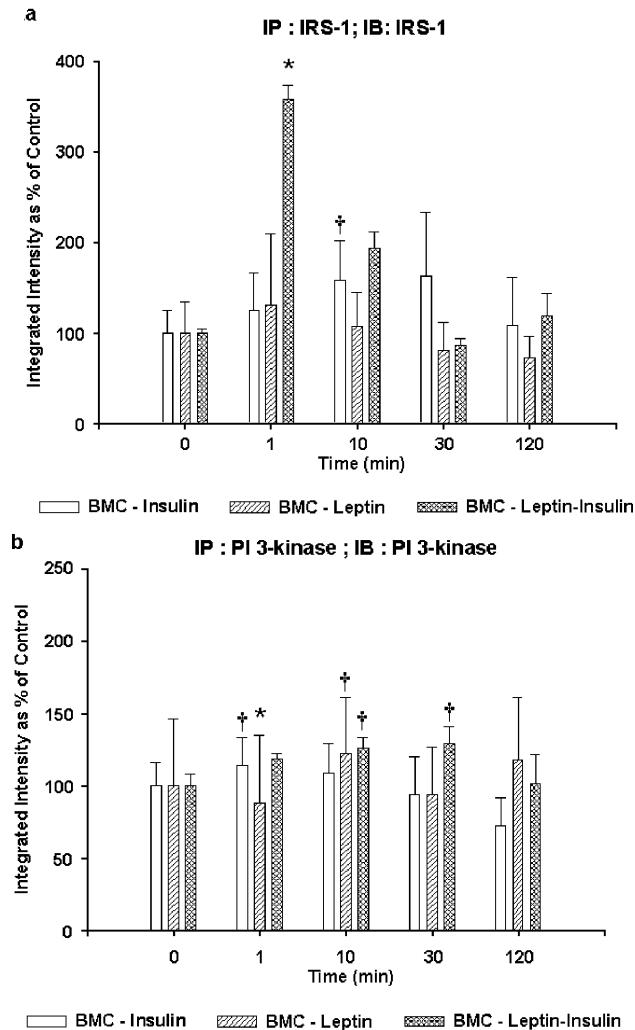


Fig. 2. Bovine Myogenic Cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course, for the indicated times, lysed and (a) IRS-1 or (b) p85 PI 3-kinase immunoprecipitated using an excess of specific antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, Western blotted using the same antibody, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ($^{\dagger}P<0.1$, $*P<0.05$, versus control, $n=3$).

3.5. Leptin receptor (ObR)

ObR abundance appeared to be modulated by insulin treatment, showing erratic changes, being reduced at 1 min insulin ($P<0.1$), fluctuating over time and being significantly increased at 120 min ($P<0.05$, Fig. 5). Leptin alone had little effect on ObR abundance, being increased at only one time-point, 30 min ($P<0.05$). Sequential leptin–insulin treatment evoked no changes in ObR abundance.

3.6. JAK-2 co-precipitation with ObR and JAK-2 phosphorylation

The downstream effects of insulin, leptin and sequential leptin–insulin treatment are most striking in the changes observed in JAK-2 association with ObR. Under basal conditions, JAK-2 was co-precipitated with ObR indicating constitutive association of these molecules (Fig. 6a). Both

insulin and leptin alone induced significant dissociation of the kinase from ObR.

In the insulin treatment, dissociation of these molecules was rapid, being observed at 1 min of insulin stimulation, and persistent, no re-association being observed throughout the experimental period (120 min). Compared to control, JAK-2 was detected from approximately 1/100th of the control value (1, 10 and 120 min) to approximately 1/10th of the control value (30 min, all $P<0.05$). In response to leptin alone, dissociation of JAK-2 from the ObR was of a lesser magnitude, from 1/10th of the control value at 1 min leptin ($P<0.1$), to one quarter of the control value (10 and 30 min, $P<0.1$). In contrast to the insulin response, by 120 min of leptin stimulation, JAK-2 association with ObR was not significantly different from the control. Sequential leptin–insulin treatment appeared to invoke a contrasting response as JAK-2 association with the ObR significantly increased for all time-points from 1 min to 120 min insulin stimulation.

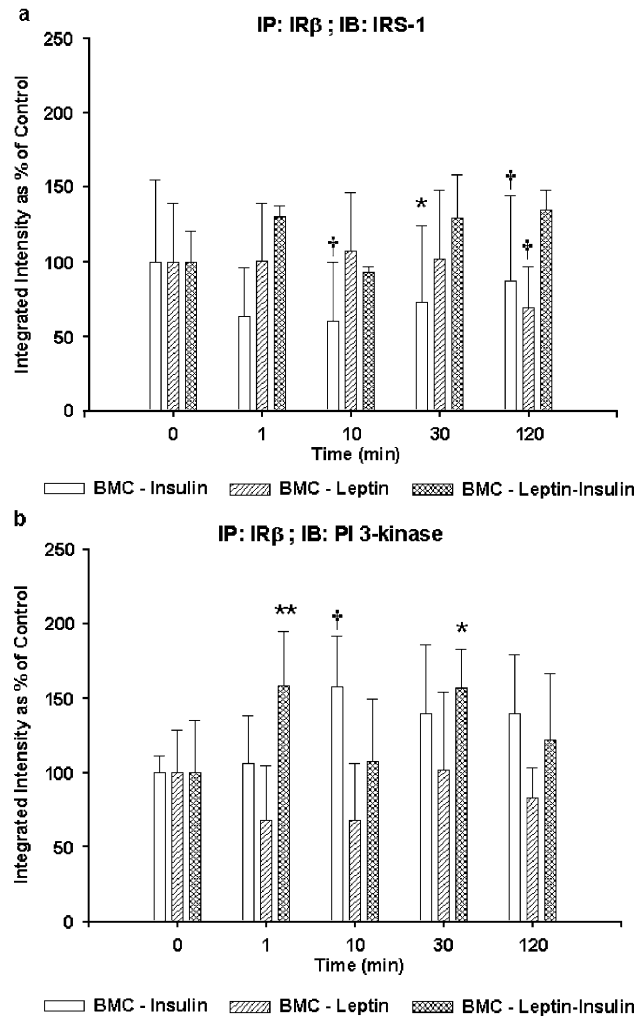


Fig. 3. Bovine myogenic cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course for the indicated times, lysed and immunoprecipitated using an excess of anti-IR antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed and Western-blotted using (a) anti-IRS-1 antibody or (b) anti-p85-PI 3-kinase antibody, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ($^{\dagger}P<0.1$, $*P<0.05$, $**P<0.01$ versus control, $n=3$).

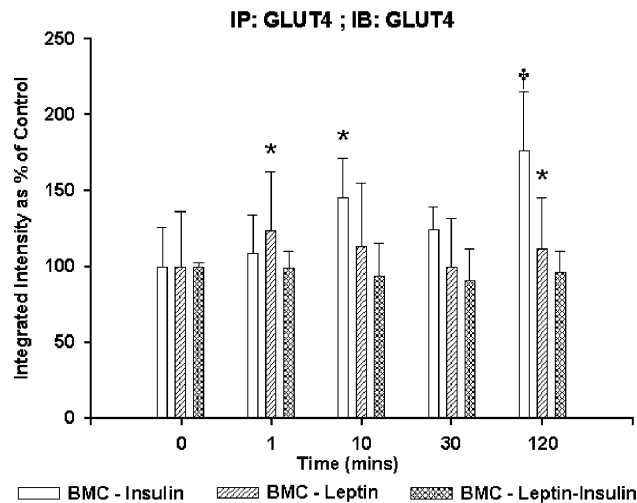


Fig. 4. Bovine myogenic cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course, for the indicated times, and lysed. Plasma membranes were prepared and proteins immunoprecipitated using an excess of anti-GLUT4 antibody bound to Protein A agarose. Immunoprecipitates were electrophoresed, Western blotted using the same antibody and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ($^{\dagger}P<0.1$, $*P<0.05$ versus control, $n=3$).

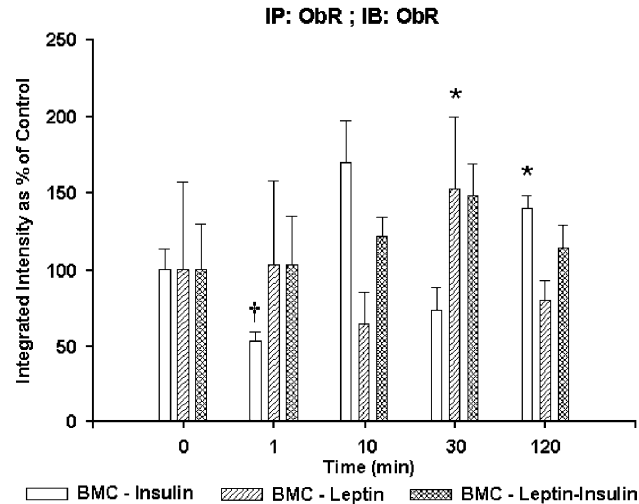


Fig. 5. Bovine myogenic cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course, for the indicated times, lysed and immunoprecipitated using an excess of anti-ObR antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed and Western blotted using the same antibody, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ([†] $P < 0.1$, * $P < 0.05$, versus control, $n = 3$).

Following pre-incubation with leptin and 1 min insulin, JAK-2 association with the ObR was greater than 15-fold basal level ($P < 0.01$) and remained elevated (greater than 7-fold basal) for the latter time-points ($P < 0.1$, $P < 0.01$ and $P < 0.001$, respectively). Thus, these data may suggest that although both insulin treatment alone and leptin treatment alone result in dissociation of JAK-2 from the ObR, when leptin is followed by insulin, these signaling pathways are completely reprogrammed, increasing the association of JAK-2 with ObR and maintaining this association at an elevated level. The mechanisms at work underlying this great contrast in the responses to insulin and leptin alone and the sequential leptin–insulin treatment may be clarified by observations of the tyrosine phosphorylation status of JAK-2 (Fig. 6b).

Insulin treatment alone resulted in rapid dephosphorylation of JAK-2, being approximately 1/10th basal level at 1 min insulin ($P < 0.05$). Although JAK-2 phosphorylation remained decreased, it appeared to slowly return toward basal levels (10 min $P < 0.1$, 30 min, $P < 0.05$), indicating that the insulin signaling pathway to JAK-2 was being modulated. Thus, insulin treatment caused rapid JAK-2 dissociation from ObR (Fig. 6a) in addition to rapid dephosphorylation of JAK-2 (Fig. 6b), suggesting that insulin acutely deactivates the leptin signaling pathway in these cells.

Although leptin alone rapidly decreased JAK-2 association with ObR (Fig. 6a), phosphorylated JAK-2 appeared to increase numerically, approximately 1.5- to 2-fold at 1 and 10 min leptin, reaching significance at the 30-min and 120-min time-points (for both, $P < 0.1$). Thus, the leptin signaling pathway appeared to be down-regulated by 60 nM leptin (dissociation of JAK-2 from ObR), but phosphorylated JAK-2 increased, suggesting that the down-regulated leptin pathway was stimulated to signal via JAK-2.

The great contrast in the response of JAK-2 association with ObR following sequential treatment with leptin–

insulin (Fig. 6a), compared to the response to both insulin and leptin alone, was accompanied by an increase in JAK-2 phosphorylation (Fig. 6b) ranging in magnitude from approximately 4-fold basal at 1 min insulin to 15-fold basal at 10 min insulin, 7-fold basal at 30 min and 4-fold basal at 120 min (for all, $P < 0.05$). The leptin signaling pathway was not down-regulated as observed for leptin treatment alone, but rather was up-regulated (JAK-2 association with ObR) following 10 min leptin and only 1 min of insulin stimulation. Up-regulation of this early leptin signaling accompanied by the large increase in JAK-2 phosphorylation suggests that this response to leptin–insulin involves enhancement and active signaling of the leptin–JAK-2 pathway. It appears that the influence of leptin was retained in the post-leptin treatment (insulin time-course) phase having a profound effect in terms of both JAK-2 association with ObR and on JAK-2 phosphorylation. Although insulin treatment effects persisted through to the end of the experimental period, both JAK-2 association with ObR and phosphorylated JAK-2 became numerically diminished at the latter time-points indicating that the response to insulin had become attenuated.

3.7. Saturation binding analysis of IR

In order to determine whether the insulin resistance observed in BMC was associated with either IR abundance or perhaps diminished affinity for insulin, we used radioligand saturation binding analysis to study intact cells under basal conditions, and following 10 min insulin stimulation to determine effects on receptor number (B_{max}) and affinity (K_D). B_{max} was not changed by insulin stimulation (29.7 ± 6.8 pM, and 40.4 ± 5.9 pM, respectively (n.s.)). Under basal conditions and insulin stimulation, the K_D fell within the expected range 3.1 ± 1.0 nM and 5.3 ± 1.0 nM, respectively

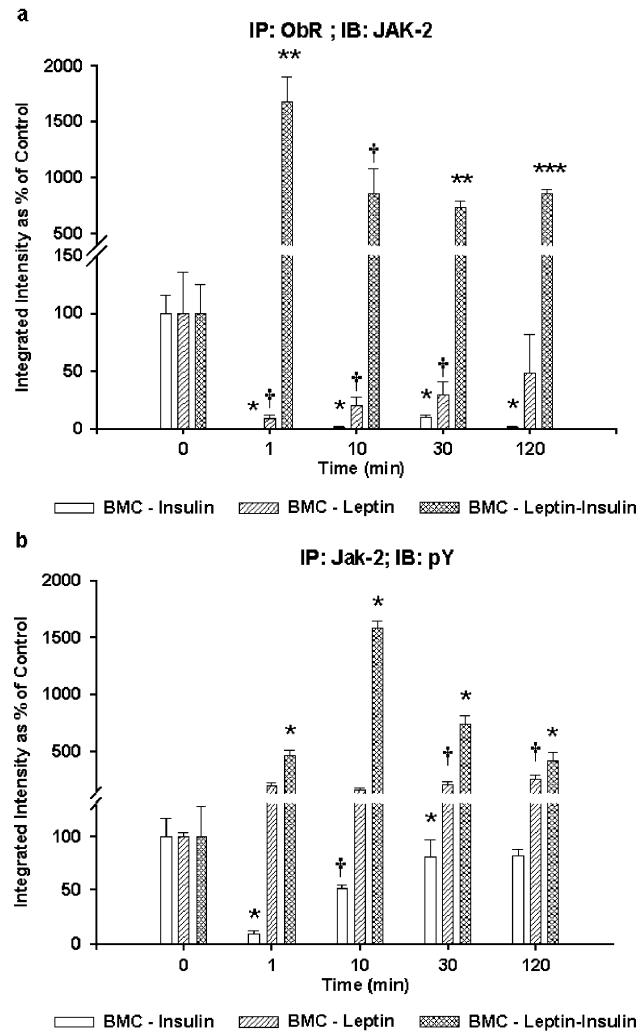


Fig. 6. Bovine myogenic cells (BMC) were stimulated with 100 nM insulin, 60 nM leptin or pre-incubated with 60 nM leptin prior to insulin (100 nM) time-course, for the indicated times, lysed and immunoprecipitated using an excess of (a) anti-ObR antibody or (b) anti-JAK-2 antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed and Western blotted using (a) anti-JAK-2 antibody or (b) anti-phosphotyrosine antibody, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each experiment ($^{\dagger}P<0.1$, $*P<0.05$, $**P<0.01$, $***P<0.001$, versus control, $n=3$).

(n.s.). Thus, the data of Fig. 1a indicating that 10-min insulin had no effect on IR abundance were recapitulated by this experiment.

4. Discussion

BMC appear to be insulin resistant. Increased phosphorylation of IR in response to insulin was delayed (first detected after 30 min insulin stimulation), of small magnitude and was associated with an apparent decrease in IR-associated IRS-1 (to 60–70% of basal) and transient increase in IR-associated p85 PI 3-kinase (140–150% of basal). These contrasting changes suggest that PI 3-kinase may associate directly with the phosphorylated IR. The complex interactions among the three early signaling molecules, IR, IRS-1 and PI 3-kinase, appear to be finely

regulated in response to insulin. It is possible that tri-molecular complexes were co-precipitated in IR immunoprecipitates accounting for some PI 3-kinase apparently associated with the IR. However, the increase in IR-associated PI 3-kinase paralleled a decrease in IR-associated IRS-1, suggesting direct association of PI 3-kinase with IR. Bovine IR and IRS-1 may have markedly different SH2-binding motifs from other more insulin-sensitive species. The high affinity binding site, pYMXM recognized by the SH2 domains of PI 3-kinase [38] occurs at four sites in human IRS-1, while the human IR (and the type 1 IGF receptor) do not possess such a high affinity site but have a lower affinity, C-terminal binding site, pYTHM, which is also recognized by PI 3-kinase SH2 domains. Unfortunately, bovine IRS-1 has not been sequenced and only short segments are known. We hypothesize that the relative affinities of SH2-binding motifs on IRS-1 versus IR is a

mechanism which affects the distribution of PI 3-kinase between the IR and IRS-1 and species differences in these affinities and basal concentrations of each messenger may explain subtle differences in recruitment patterns in similar sub-plasma membrane compartments. More complete gene sequence information is required to aid in resolving these issues. This mechanism believed to down-regulate insulin signaling [14,39,40], has been demonstrated in the present study to be invoked in BMC. Shao et al. [14] have demonstrated the insulin signaling inhibition of this mechanism in a mouse model of gestational diabetes (GDM). We have also recently reported parallel increases in both IRS-1 and PI 3-kinase association with the IR following insulin stimulation in an insulin-sensitive mouse myogenic cell (Sol8) model [3]. In contrast, the present study in BMC showed a decrease in IR-associated IRS-1 following insulin stimulation. The time-course of the BMC response to insulin was similar to our previous report in the Sol8 model [3], and other insulin sensitive models reported in the literature [14,41–43].

Leptin treatment alone was almost without effect on the early insulin signaling cascade in the present study. However, 10 min leptin followed by insulin time-course led to a rapid but transient increase in phosphorylated IR, increased PI 3-kinase associated with the IR with no change in IRS-1 associated with the IR. These data suggest that leptin signaling may redirect the IRS-1–PI 3-kinase pathway into a futile signaling loop, via direct interaction of PI 3-kinase with the IR. This notion is corroborated by the data of Fig. 4, showing that although GLUT4 translocation to the plasma membrane resulted from insulin stimulation, pretreatment with leptin followed by insulin did not change plasma membrane GLUT4 from control values at any time-point. In studies of cross-talk of these pathways in other cell or tissue types, differing responses of IRS-1 have been described. In hepatoma cells, Cohen et al. [44] found that a similar sequential leptin–insulin treatment slightly up-regulated phosphorylation of IR (1 min insulin), in agreement with the present study, although IRS-1 phosphorylation was down-regulated (IRS-1 abundance not reported). In the present study, IRS-1 was acutely, but only transiently increased, showing no change in IR-associated IRS-1 levels (representative of tyrosine phosphorylated IRS-1). Szanto and Kahn [27], also studying an hepatoma model, found that tyrosine phosphorylated IRS-1 was increased following 1 to 10 min of leptin, then 1 min insulin stimulation. Thus, the present study in BMC and the two cited studies in hepatoma cells all found varying results in response to the same treatment protocol. Many factors may contribute to these differing results. As well as genetic variation amongst these three examples, in all studies, cells were ‘serum starved’ prior to hormone treatments, and the biological effects of this commonly used *in vitro* procedure are not clear. The present study utilized a medium with addition of physiological concentrations of fatty acids plus BSA, one which we have shown maintains viability of both myogenic [45] and

adipocyte primary cells [46] and cell lines for up to 8 days without evidence of increased apoptosis (data not shown).

In the present study, the effects of greatest magnitude were observed in the early leptin signal cascade. The profound down-regulation of both ObR-associated JAK-2 and dephosphorylation of JAK-2 in response to insulin alone indicates that insulin is likely to silence leptin signaling in BMC. Kim et al. [26] studied effects on leptin and insulin *in vivo* in rats and found that insulin tended to increase STAT-3 phosphorylation in muscle suggesting increased leptin signaling in response to insulin. However, induction of endogenous leptin or other cytokine signaling cannot be ruled out in this study. Carvalheira et al. [33] found increased JAK-2 phosphorylation *in vivo* in rat hypothalamus following 1- to 3-min insulin stimulation. Similarly, actions of insulin *in vivo*, including induction of endogenous leptin or signaling of other cytokines, cannot be ruled out. However, it is possible that in insulin sensitive models, the insulin effect on leptin signaling is different from that observed in the present insulin-resistant model. To further support our contention that insulin does, in fact, silence leptin signaling in BMC, additional study in which brief pretreatment with insulin followed by leptin time-course is required.

The effect of leptin alone in the present study appears to indicate that the concentration of leptin used, 60 nM down-regulated the leptin signal cascade as leptin caused JAK-2 to dissociate from the ObR. However, signaling of the cascade appeared to remain intact and functional, JAK-2 phosphorylation increasing in response to leptin. It would be of interest to determine whether a lower concentration of leptin might elicit similar results. Use of this and higher concentrations of leptin in previous signaling studies [24,27,47], and knowledge that leptin is hydrophobic, with a propensity for binding to plastic-ware *in vitro* [48] (and reviewed in [30]), suggests that the effective concentration of leptin at the plasma membrane may be considerably lower than the concentration added to the system.

The large effect of leptin–insulin sequential treatment, up-regulating ObR-associated JAK-2 and JAK-2 phosphorylation indicates a profound reprogramming of the leptin signal cascade in comparison to either hormone treatment alone. Carvalheira et al. [31] found in study of rat liver that combined insulin and leptin treatment led to a marked increase in JAK-2 phosphorylation when compared to administration of insulin or leptin alone a result in close agreement with the present study. Thus, leptin signaling in the BMC model appears to be strongly down-regulated by insulin alone, but in contrast, is reprogrammed by 10 min pre-incubation with leptin followed by insulin. In terms of energy substrate utilization, insulin signals the abundance of extracellular glucose, while leptin signals the abundance of extracellular triglyceride (and fatty acids). The previously demonstrated antagonism between leptin and insulin in partitioning of energy substrate utilization [16–19] by muscle supports a mechanism in which insulin down-

regulates leptin signaling as observed in the present study. The contrasting data showing that pre-incubation with leptin followed by insulin showing enhanced leptin pathway signaling is consistent with the notion that BMC provides a model of muscle which has evolved in a species in which high extracellular glucose flux does not normally occur. These data are also consistent with the data of Dulloo et al. [25,49,50] and the notion that leptin induces a futile cycle between de novo lipogenesis and lipid oxidation, also requiring glucose. In addition, this response to leptin–insulin was also reported in another insulin-sensitive model (rat liver) [31].

Several studies have reported a central role of the protein tyrosine phosphatase, PTP1B in negative regulation of insulin signaling. PTP1B also recognizes a specific consensus substrate motif on JAK-2 (reviewed in [51]), and is a negative regulator of leptin signaling [52]. PTP1B is implicated in the development of insulin resistance and Type 2 diabetes. Thus, the leptin signaling pathway inhibition observed in response to insulin in BMC implicates PTP1B as a key intermediate in pathway cross-talk in this model. This also suggests that BMC may prove to be a useful model in understanding the mechanisms underlying the development of insulin resistance and Type 2 diabetes.

The lack of response of the insulin signaling pathway in BMC is not due to compromised insulin binding or low abundance of IR on the plasma membrane. Saturation binding analysis indicated that there was no change in IR abundance (B_{\max}) or receptor affinity (K_D), due to insulin treatment. Furthermore, the values for B_{\max} in this model were similar to those we have previously reported [3] for an insulin sensitive, mouse myogenic cell model (Sol8), 3 to 6 fmol per 10^6 cells, in comparison to the present study, 30–40 pM, equivalent to 9 to 12 fmol per 10^6 cells. The K_D values determined for BMC in the present study were also similar to those we have reported previously (Sol8, 2.6 to 4.8 nM, versus BMC, 3.1 to 5.3 nM), and in close agreement with others [53].

In conclusion, BMC appear to be insulin resistant, indicated by modulation of the IR-PI 3-kinase pathway and the slow and intermittent response of translocation of GLUT4 to the plasma membrane. However, the BMC leptin signaling cascade is highly responsive to insulin, showing marked down-regulation. This effect is completely reprogrammed by pre-incubation with leptin, followed by insulin which results in marked up-regulation of the leptin signaling pathway. These contrasting data may point to the ability of these cells to respond to energy substrate availability, as bovine muscle has evolved to utilize lipids and fatty acids in response to a metabolism which provides only limited glucose, which rarely rises above basal levels as it does in monogastric species. This cross-talk between insulin and leptin signaling pathways points to a better understanding of the mechanisms driving energy substrate utilization in ruminant muscle and may provide a useful model for greater understanding of the molecular mechanisms under-

lying the development of insulin resistance and Type 2 diabetes in man.

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