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Interleukin-6 in combination with the interleukin-6 receptor stimulates glucose uptake in resting human skeletal muscle independently of insulin action

RUNNING TITLE: IL-6/IL-6R stimulates glucose uptake in muscle

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ABSTRACT

Aim To examine if physiological concentrations of both IL-6, in combination with IL-6R, are able to stimulate glucose uptake in human skeletal muscle and to identify the associated signalling pathways. **Methods** Skeletal muscle tissue (~60mg) obtained from healthy female volunteers via muscle biopsy was subjected to incubation in the absence or presence of insulin (60 $\mu\text{U}\cdot\text{ml}^{-1}$), rhIL-6 (4 $\text{ng}\cdot\text{ml}^{-1}$), or a combination of rhIL-6 (4 $\text{ng}\cdot\text{ml}^{-1}$) and rhIL-6R (100 $\text{ng}\cdot\text{ml}^{-1}$) for 30 min, with glucose transport measured for each incubation. Western blot analysis was conducted on key signalling proteins, protein kinase B, (PKB/Akt), adenosine monophosphate kinase (AMPK) and mammalian target of rapamycin (mTOR) to gain an early insight into any differing transport mechanisms. **Results** Human skeletal muscle exhibited increased glucose uptake with insulin (1.85 fold; $P<0.05$) and stimulated phosphorylation of PKB/Akt and AMPK (0.98 ± 0.23 and 1.49 ± 0.13 , respectively, phosphorylated: total; $P<0.05$). IL-6/IL-6R increased phosphorylation of mTOR (4-fold, $P<0.05$) compared to insulin, IL-6 alone and basal control. IL-6 did not stimulate glucose uptake but combined with IL-6R, induced 1.5 fold increase in glucose uptake ($P<0.05$) and phosphorylation of AMPK (0.95 ± 0.19 ; phosphorylated: total, $P<0.05$). **Conclusions** IL-6 in combination with IL-6R and not IL-6 alone increased glucose uptake in human skeletal muscle. IL-6/IL-6R mediated glucose uptake occurred independently of PKB/Akt phosphorylation, showing that IL-6/IL-6R induced glucose uptake is dependent on a divergent pathway.

Key Words: Glucose uptake; human skeletal muscle; interleukin-6 and receptor; interleukin; insulin;

INTRODUCTION

Obesity is associated with chronic low-grade inflammation, and cytokines such as interleukin-6 (IL-6) are specifically linked with the development of insulin resistance (1). Both systemic and subcutaneous adipose concentrations of IL-6 have been shown to be associated with the level of insulin resistance (2, 3). Whilst plasma IL-6 is inversely correlated with insulin sensitivity in healthy individuals (3), in type 2 diabetics, IL-6 is correlated with whole body adiposity, rather than tissue specific insulin sensitivity (4), suggesting that IL-6 may be a biomarker of obesity without any direct contribution to the development of insulin resistance. IL-6 and its receptor (IL-6R) are pivotal to the resolution of inflammation, providing the transition between the pro- and anti-inflammatory states, evident in part by its inhibitory effect on the pro-inflammatory cytokine tumour necrosis factor alpha (5).

In contrast to the role of chronically-elevated IL-6 in obesity and diabetes, exercise also results in acute elevations of IL-6 (6) in a duration and intensity dependent manner (7) and is associated with increased glucose uptake in skeletal muscle (1,8). Elevations in plasma IL-6 predominantly occur as a consequence of contraction mediated IL-6 release from skeletal muscle (9) and exerts both a local and systemic effect (6). Given that exercise is an effective intervention to cope with weight gain and insulin resistance, it is reasonable to suggest that exercise induced IL-6 expression may exert a positive effect on signal transduction within skeletal muscle. Indeed, both moderate intensity exercise (10) and high intensity interval training (11, 12) have been shown to improve insulin sensitivity, glucose uptake and glycogen synthesis in skeletal muscle. Moreover, IL-6 knockout mice have been shown to develop late onset obesity and high glucose, which was only alleviated following IL-6 administration (13). Taken together, these reports indicate that IL-6 may have beneficial effects on glucose uptake in skeletal muscle in response to exercise.

The current understanding of IL-6 regulation in skeletal muscle glucose uptake requires clarification as the exact role of IL-6 currently remains under debate. *In vitro* and *in vivo* experiments in both animal and human derived skeletal muscle suggest that supra-physiological levels of IL-6 are required to stimulate glucose uptake (8,14). However, the addition of IL-6 has been shown to increase glucose uptake in human skeletal muscle (1). Moreover, our previous work demonstrates that physiological levels of IL-6 in combination with the soluble IL-6R (sIL-6R) increases glucose uptake in murine skeletal muscle, partly via AMPK dependent signalling (15).

For IL-6 to initiate cell signalling it must bind with its receptor (IL-6R) and gp130, forming a membrane-bound IL-6/IL-6R/gp130 complex (16, 17). Although gp130 is an ubiquitously distributed membrane-bound receptor (18), some tissues are deficient in membrane bound IL-6R (19), or have low levels, such as in untrained skeletal muscle, with expression increasing after acute exercise and prolonged training (6, 20). IL-6 binding to the soluble IL-6R allows IL-6 signalling to occur in tissues that are deficient in membrane bound IL-6R by 'trans-signalling,' which is responsible for the pro-inflammatory effect of IL-6 (21). Elevated IL-6R has been detected in a number of inflammatory disease states, and thus has the potential to regulate local and systemic responses to IL-6 (16). This suggests that some of the muscle-specific insulin-sensitising effects of IL-6 are mediated via the IL-6R, but the underlying molecular mechanism is unclear.

The mammalian target of rapamycin (mTOR) has been linked to impaired glucose uptake and insulin resistance as a consequence of two mechanisms. Firstly, activation of a downstream effector of mTOR complex 1, ribosomal protein S6 kinase results in phosphorylation and degradation of the insulin receptor substrate 1 (22) and leads to peripheral insulin resistance. Secondly, mTOR complex 2 activates PKB/Akt, a crucial downstream effector of insulin action (23) therefore, its inhibition is likely to impair insulin sensitivity. Indeed, mTOR inhibition has recently been shown to result in increased insulin resistance (24).

The current discrepancy in the literature regarding the effect of IL-6 on glucose uptake may be due to the low sequence homology between human and murine IL-6 (25) and the supra-physiological doses of IL-6 used in many studies. Therefore, it was the aim of the present study to examine if physiological concentrations of both IL-6 and IL-6R affect glucose uptake in human skeletal muscle independently of insulin. We hypothesise that physiological levels of IL-6 in isolation will have no effect on glucose uptake, whereas physiological concentrations of IL-6 in combination with IL-6R will have an impact on glucose uptake.

METHOD

Materials

All reagents were purchased from Sigma-Aldrich Ltd (Poole, UK) unless otherwise stated.

Participants

Eleven young, healthy, untrained, female participants were recruited for the glucose uptake experiments ($n=11$, aged 22 ± 2.5 years, height 169.5 ± 8.4 cm, weight 61.7 ± 6.0 kg, BMI 21.5 ± 1.3 kg.m⁻²). Owing to the limitations of the sample size of the muscle biopsy a subsequent cohort of 7 females were recruited for the signalling experiments ($n=7$, aged 24 ± 0.7 years, height 167 ± 0 cm, weight 62.7 ± 2.6 kg, BMI 22.65 ± 0.9 kg.m⁻²). Experimental procedures were carried out in accordance with the Declaration of Helsinki and ethics approval was granted by the local Ethics Advisory Committee. Participants all signed an approved informed consent form and health questionnaire. None of the participants smoked, had any underlying health problems nor reported taking any medication. Participants were asked to refrain from exercise, caffeine and alcohol for 48 hours prior to the muscle biopsy which was performed within 7-21 days of the start of their menstrual cycle, with all biopsies taken at the same time of day.

Muscle biopsy

Muscle biopsies were obtained from the *vastus lateralis*. Two passes per participant were obtained using an 11G ACECUT micro-biopsy needle (TSK Laboratory, Europe B.V.). The procedure was completed under local anaesthetic (2 ml 1% (w/v) lidocaine solution; Taro Pharmaceutical Ltd, County Tipperary, Ireland). After dissection of connective tissue, the *vastus lateralis* samples were allowed to recover at 35 °C for 60 min in a stoppered flask containing Krebs-Henseleit Bicarbonate (KHB) Buffer supplemented with 8 mM glucose, 32 mM mannitol, 0.1 % bovine serum albumin (BSA) and bubbled with a gas phase of 95 % O₂– 5 % CO₂.

Muscle Treatments

Glucose uptake experiments

Following the recovery period, where possible ($n=8$) muscle biopsy samples were each divided by wet weight into 3, and then incubated for 30 min at 35 °C in KHB buffer and subjected to no addition (control); 60 μ U.ml⁻¹ of purified human insulin; or rhIL-6 (4 ng.ml⁻¹) and rhIL-6R α (IL-6R; 100 ng.ml⁻¹). On 4 occasions, due to limited tissue sample from the biopsy, it was only possible to obtain 2 samples which were then subjected, in duplicate, to the following treatments: no addition (control); or rhIL-6 (4 ng.ml⁻¹) to act as controls. These recombinant proteins were purchased from R & D systems (Minneapolis, MN, USA;

products 206-IL and 227-SR respectively). The IL-6R protein used encodes the extracellular domain of the IL-6R; residues Leu20-Asp358. The concentrations of IL-6 and IL-6R employed represent levels previously reported after strenuous exercise (26, 27).

Signalling experiments

Biopsy samples were incubated for 30 min at 35 °C in KHB buffer in the presence of no addition (control), insulin (60 $\mu\text{U}\cdot\text{ml}^{-1}$), rhIL-6 (4 $\text{ng}\cdot\text{ml}^{-1}$) alone or rhIL-6 (4 $\text{ng}\cdot\text{ml}^{-1}$) in combination with rhIL-6R α (IL-6R; 100 $\text{ng}\cdot\text{ml}^{-1}$). Following 30 min incubation, tissue was snap frozen.

Glucose transport

Muscles were subsequently rinsed for 10 min at 29 °C in KHB containing 40 mM mannitol, 0.1 % bovine serum albumin and appropriate treatments if present in the previous medium. Following this, muscles were incubated for 20 min at 29 °C in KHB with 4 mM 2-Deoxy-D-glucose spiked with tritiated 2-Deoxy-D-glucose (2-[1,2- $^3\text{H}(\text{N})$],2-DG; Perkin Elmer, Bucks, UK), 36 mM mannitol and a gas phase of 95 % O_2 -5 % CO_2 [24]. Muscles were rinsed in ice-cold KHB buffer containing 40 mM mannitol, 0.1% bovine serum albumin, then placed in 1 ml Goldisol (Meridian Biotech Ltd, Surrey, UK) and solubilised for 2 hours at 50 °C. Following solubilisation, 10 ml of Prosafe TS scintillation cocktail (Meridian Biotech Ltd, Surrey, UK) was added and the determination of 2-DG accumulation measured from counting tritium in a Packard Liquid Scintillation counter (Tri-Carb 2500TR, Cranberra Packard). All tritium counting measurements were performed in duplicate from which an average was calculated. Internal tritium standards were also processed in duplicate (from which an average was calculated) for every sample measured to correct for any alteration in tritium counting efficiency between samples.

Western blot analysis

Samples were homogenised on ice in 10:1 (w/v) of Protein Extraction Lysis buffer containing 1 mM protease inhibitor cocktail (Sigma). Homogenised samples were centrifuged for 10 min at 12,000 x g at 4 °C, and the supernatant removed for protein analysis. Supernatant protein concentrations were determined using the Bradford protein assay (Biorad Laboratories, Hemel Hemstead, UK), using BSA as a protein standard. Ten micrograms of each experimental sample were heated and denatured in Laemmli sample buffer and then

loaded onto 10% SDS-PAGE gels and run for 2 h at 200 V. Proteins were then transferred onto a nitrocellulose membrane. Membranes were washed in Tris Buffered Saline with 0.05% Tween-20 (TBS-T), and then blocked with 5% BSA in TBS-T for 1 hour at room temperature. Blots were probed overnight at 4 °C with total anti-PKB/Akt or anti-phospho-PKB/Akt (serine-473) primary antibodies (1:1000 dilution; Cell Signalling Tech, Danvers, MA, USA), total anti-AMPK α or anti-phospho-AMPK (threonine-172) primary antibodies (1:1000 dilution; Cell Signalling Tech, Danvers, MA, USA), total anti-p38 MAPK or anti-phospho-p38 MAPK (threonine-180/tyrosine-182) primary antibodies (1:1000 dilution; Cell Signalling Tech, Danvers, MA, USA), total anti-mTOR or anti-phospho-mTOR (serine-2448) primary antibodies (1:1000 dilution; Cell Signalling Tech, Danvers, MA, USA), followed by incubation for 1hr at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000 dilution; Cell Signalling Tech, Danvers, MA, USA). Blots were developed using Supersignal West Pico enhanced chemiluminescence (Pierce Biotechnology, UK), with light captured on a ChemiDoc XRS+ (Biorad Laboratories, Hemel Hemstead, UK). Relative fold difference levels of total PKB/Akt, AMPK α , p38 MAPK, mTOR, to their corresponding phosphorylated (and activated) counterparts were quantified with Quantity One software using the ChemiDoc XRS+ and Licor Image Studio Lite Version 3.1.

Statistical analysis

Data were analysed using Microsoft Excel (version 7.0), SPSS (version 16.0) and GraphPad (version 5.0) software. Results are presented as the mean \pm standard error of the mean (S.E.M). For non-multiple comparisons, paired *t*-tests were used. Multiple comparisons were analysed using one-way repeated measures ANOVA and Tukey's *post-hoc* test. Results were considered statistically significant when $P < 0.05$. All experiments were performed in quadruplicate, unless otherwise stated.

RESULTS

Insulin, IL-6 and IL-6/IL-6R, mediated glucose uptake

The addition of IL-6 alone at physiological levels (4 ng.ml⁻¹) had no significant effect ($P > 0.05$) on glucose transport compared to basal levels in female untrained *vastus lateralis* muscle (Figure 1a). Incubation of physiological concentrations of IL-6 (4 ng.ml⁻¹) in combination with the IL-6R (100 ng.ml⁻¹) resulted in a significant increase (1.5 fold) in

glucose uptake ($P<0.05$) relative to basal (Figure 1b). The incubation of *vastus lateralis* muscle with insulin ($60 \mu\text{U}\cdot\text{ml}^{-1}$) for 30 min stimulated an approximately 1.85-fold increase ($P<0.05$) in glucose transport (Figure 1b).

FIGURE 1

IL-6 and IL-6R mediated intracellular signalling

AMPK. Incubation of *vastus lateralis* muscle with insulin alone, and the combination of IL-6 with the IL-6R, induced a significant ($P<0.05$) increase in the relative levels of phospho-AMPK to total AMPK when compared with those at basal levels and IL-6 alone (Figure 2a). AMPK phosphorylation peaked with insulin administration 1.49 ± 0.13 phospho/total. With co-incubation of IL-6 and IL-6R the relative fold increase was 0.95 ± 0.19 phospho/total and was significantly ($P<0.05$) higher than in the presence of IL-6 alone (0.35 ± 0.02 phospho/total).

FIGURE 2

PKB/Akt. In primary human skeletal muscle cells, IL-6-mediated glucose metabolism is dependent on signal transduction via PI 3-kinase (27). Here we show in human skeletal muscle, incubation with IL-6 alone or in combination with the IL-6R does not increase the proportion of phosphorylated PKB/Akt ($P>0.05$), 0.19 ± 0.14 phospho/total and 0.32 ± 0.08 phospho/total, respectively (Figure 2b). Incubation of *vastus lateralis* muscle with insulin did induce significantly ($P<0.05$) the highest increase in phosphorylated PKB/Akt and was approximately 3-fold greater than all other incubation conditions (0.98 ± 0.23 phospho/total; Figure 2b).

p38 MAPK. Although there was a trend for increased p38 MAPK phosphorylation following 30 min for insulin, IL-6 and co-incubation of IL-6 and IL-6R being approximately 2-fold greater than basal conditions (0.51 ± 0.18 , basal; 1.20 ± 0.25 , insulin; 0.87 ± 0.11 , IL-6; 1.063 ± 0.24 , IL-6 and IL-6R, phospho/total) this effect did not reach statistical significance ($P = 0.21$; Figure 2c).

FIGURE 3

mTOR. IL-6 and IL-6R co-incubation and not insulin alone induced the greatest fold increase in mTOR phosphorylation (Figure 3). mTOR phosphorylation was similar across basal levels, insulin incubation (0.19 ± 0.03 phospho/total) and IL-6 incubation alone (0.18 ± 0.04 phospho/total; $P > 0.05$). With addition of IL-6R to IL-6, an increase in mTOR phosphorylation was observed (0.83 ± 0.26 phospho/total; $P < 0.05$).

DISCUSSION

Data from the present study show that IL-6 in combination with IL-6R is required to stimulate an increase in human skeletal muscle glucose uptake, independently of the insulin-signalling pathway. Furthermore, unlike much of the current literature, this response was demonstrated at physiologically relevant concentrations of IL-6 and IL-6R, and replicates our previous findings in murine skeletal muscle (15). This indicates that the low sequence homology between human and murine IL-6 does not limit similar biological function despite human IL-6 sharing only 42% of the amino acid sequence identity of its murine equivalent (25). Our current findings showing improved glucose uptake in the presence of IL-6 combined with IL-6R and independent of insulin action may have therapeutic potential for diabetic patients. In addition to the general benefits to health and well-being, exercise and regular training enhances IL-6R expression (20) and may therefore provide a complimentary insulin-independent mechanism to facilitate skeletal muscle glucose uptake and improve glucose control.

From the available literature, it appears that IL-6 can have both positive and negative effects on glycaemic control. It has been shown previously that IL-6 is positively correlated to insulin resistance and obesity (3). Furthermore, the importance of IL-6 in regulating glycaemic control has been shown in IL-6 knockout mice, which develop late onset obesity and high glucose and triglyceride levels, and alleviated upon IL-6 administration (13). These studies and others, point to elevations in IL-6 contributing to the development of insulin resistance. Conversely, following acute exercise, glucose uptake is significantly elevated and IL-6 is released from skeletal muscle (9) in concentrations above those associated with chronic inflammatory diseases (7), thus suggesting that elevations in IL-6 may be beneficial for GLUT4 translocation and glucose control (8).

The impact of IL-6 in isolation on glucose transport has produced equivocal data in rat, murine and human skeletal muscle. For example, Geiger *et al.* report no effect of

physiological or supra-physiological concentrations of IL-6 ($120 \text{ ng}\cdot\text{mL}^{-1}$) on glucose transport in rat soleus muscle (14), whereas others report positive effects of supra-physiological IL-6 on elevating glucose transport in cell cultures, rat and human muscle (1,8). However, none of these studies have previously considered the role of IL-6R as a potential mediator of IL-6 in the regulation of glucose uptake in skeletal muscle. It is possible that the divergent results may result from differing expression of the membrane bound IL-6R, with low IL-6R expression acting as a limiting factor of signal transduction (17). In these cases, an alternative mechanism is to utilise the sIL-6R to initiate trans-signalling. In instances where muscle has been studied following a training intervention, there is an elevation in IL-6R expression on the cell membrane (20), thus resulting in a reduced dependency on sIL-6R on IL-6 function. Where IL-6R has been omitted from the incubation medium this latter option is not available, meaning that the role of soluble IL-6R may have been largely overlooked. These data, combined with those reported here from untrained individuals, suggest that both the soluble and membrane bound IL-6R have essential roles in IL-6 mediated glucose uptake. However, this remains to be clarified in trained individuals with elevated mIL-6R expression.

Membrane bound IL-6R expression has been shown to be limited in resting tissue (18), which in turn may restrict IL-6 binding to its receptor. As exercise training is well known to improve both glucose regulation and IL-6R expression (20), it follows that an increase in IL-6R may be important for improving glucose control. In the present study, IL-6 alone had no impact on glucose uptake, which we speculate is due to the low membrane bound IL-6R in the present cohort of untrained females. Moreover, the IL-6:IL-6R:gp130 ratio on the cell membrane has been suggested to be of importance in determining the outcome of IL-6 mediated cell signalling (17), supporting our proposal that IL-6R is a key regulator in the mediation of IL-6 stimulated glucose uptake. However, given the difficulty in quantifying the total membrane bound IL-6R without the relevant standards, measuring the total membrane bound IL-6R remains difficult regardless of training status, and therefore can only be reported as fold change overtime. The increase in sIL-6R following acute exercise had been shown to be as a result of both proteolytic cleavage and differential IL-6R mRNA splicing (6), with sIL-6R derived from proteolytic cleavage of IL-6R occurring rapidly in response to its activators, including C-reactive protein (28).

Data suggest that IL-6 related changes in glucose uptake occur as a consequence of AMPK phosphorylation (15, 30). Increases in circulating IL-6 correlate with activation of AMPK (30) and phosphorylated AMPK stimulates glucose transport (1). However, our present data

highlight that IL-6 alone does not phosphorylate AMPK and increase glucose uptake, unless accompanied by sIL-6R, replicating our previous findings in murine skeletal muscle (15). The present data show that IL-6/sIL-6R activation of AMPK was less than with insulin administration even though the combination of IL-6/sIL-6R induced comparable levels of glucose uptake observed with administration of insulin alone. This suggests that there may be an additional IL-6 mediated pathway triggering GLUT4 translocation and increased skeletal muscle glucose uptake (8). The differences observed in our studies regarding the role of IL-6R being essential for mediating IL-6-induced AMPK activation, and those of others reporting that IL-6 alone can trigger the AMPK pathway may be due the use of supra-physiological levels of IL-6 inducing signal saturation. Thus, the present study demonstrates the essential role of IL-6R in AMPK signal transduction at physiological levels of IL-6 in human skeletal muscle.

The present data show that when skeletal muscle glucose uptake is increased in response to incubation with IL-6/IL-6R, mTOR activation was also maximal but occurred independently of insulin/PKB/Akt pathway. This indicates there is a divergent pathway by which IL-6/IL-6R phosphorylates mTOR. Inhibition of mTOR has been implicated in the aetiology of diabetes, as its inhibition by rapamycin leads to insulin resistance despite concurrent weight loss (24). Although not the focus of the present study, the mechanism underlying the apparent differing signalling pathways involved in the phosphorylation of mTOR and glucose uptake in response to either insulin or IL-6/IL-6R is one of interest and justifies further investigation.

In summary, the present data demonstrate that within a normal physiological range, IL-6 in combination with the soluble IL-6R is necessary to enhance glucose uptake in human skeletal muscle independently of insulin and the subsequent well defined pathway. Furthermore, we demonstrate that the IL-6/sIL-6R combination also results in increased phosphorylation AMPK, whereas IL-6 alone does not. In conclusion, we believe that we are the first to demonstrate that it is the presence of IL-6 in combination with the sIL-6 receptor that is of critical importance in IL-6 intracellular signal transduction and glucose transport in human skeletal muscle.

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Conflict of interest

The authors report no potential conflict of interest

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Figure 1. IL-6 is dependent on IL-6R to stimulate glucose uptake in human skeletal muscle. A) Effect of IL-6 ($4 \text{ ng}\cdot\text{ml}^{-1}$) on glucose transport in human *vastus lateralis* muscle ($n = 4$) $P > 0.05$; not significant. B) Effect of Insulin ($60 \text{ }\mu\text{U}\cdot\text{ml}^{-1}$) and a combination of IL-6 with the IL-6R ($4 \text{ ng}\cdot\text{ml}^{-1}$ and $100 \text{ ng}\cdot\text{ml}^{-1}$ respectively) on glucose transport in human *vastus lateralis* muscle ($n = 8$). Glucose transport is presented as $\mu\text{mol}\cdot\text{mg}^{-1}$ tissue. Asterisk denotes a significant difference from basal ($P < 0.05$). Data presented as mean \pm SEM

Figure 2. The effect of IL-6 and IL-6R on phosphorylation of AMPK, PKB/Akt and p38 MAPK. Bands are representative of blots for A) total and phosphorylated AMPK (p-AMPK), B) total and phosphorylated PKB (p-PKB/Akt) and C) total and phosphorylated p38 MAPK (p-38). Asterisk denotes a significant difference ($P < 0.05$) from all other conditions, (+) denotes a significant difference ($P < 0.05$) to basal and IL-6 only. Data are presented as mean + S.E.M. ($n = 6$)

Figure 3. IL-6 and IL-6R co-incubation and insulin alone on induction of mTOR phosphorylation in human skeletal muscle *in vitro*. Asterisk denotes a significant difference ($P < 0.05$) from all other conditions. Data are presented as mean + S.E.M.