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Effects of 3T3 adipocytes on interleukin-6 expression and insulin signaling in L6 skeletal muscle cells

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ABSTRACT

Objective: Central adiposity and inflammation play key roles in the development of insulin resistance through the effects of pro-inflammatory adipokines such as IL-6, but the effect of infiltrating adipocytes in skeletal muscle tissues is not known. Communications between muscle cells and fat cells may contribute to the inflammatory response associated with insulin resistance.

Methods: In this study we used a co-culture system of skeletal muscle (L6) and adipocyte (3T3-L1) cell lines to study expression of the inflammatory cytokine IL-6 and changes in insulin signaling. This model could mimic the adipocytes infiltrating myocytes that is commonly seen in obese patients.

Results: When plated alone the L6 cells express IL-6 mRNA and secrete IL-6 protein, both of which are increased when the cells are challenged with the bacterial lipopolysaccharide (LPS). In contrast, the 3T3-L1 cells had very little expression of IL-6 mRNA or protein. Co-culture of 3T3-L1 pre-adipocytes with L6 cells, at a density ratio of 1:10, respectively, increased IL-6 expression significantly and decreased insulin-stimulated Akt phosphorylation. To examine the role of IL-6 in insulin sensitivity we incubated the L6 cells with IL-6. A brief challenge of L6 cells with IL-6 enhanced insulin-stimulated Akt phosphorylation. In contrast, incubation of the L6 cells with IL-6 for 96 h markedly decreased insulin-stimulated Akt phosphorylation.

Conclusion: The enhanced IL-6 mRNA expression and IL-6 release in L6 myocytes co-cultured with 3T3-L1 cells indicate an important interaction between adipocytes and myocytes. This observation may shed some light on the long-standing enigma of obesity-induced insulin resistance where infiltration of the skeletal muscle by preadipocytes/adipocytes is evident.

1. Introduction

It is well established that obesity and body fat distribution are associated with increased insulin resistance, diabetes and cardiovascular diseases [1,2]. In obese patients a high level of pro-inflammatory adipokines are secreted. Since adipocytes infiltrate muscle fibers in obese patients; the molecular cross-talk between adipocytes and myocytes may contribute to the development of insulin resistance [3,4]. Two fat compartments are described within the muscles of obese individuals, an intramyocellular lipid depot (IMLD) and an extramyocellular lipid depot (EMLD). Whereas IMLD in obesity has been widely associated with insulin resistance [5–7] the distribution and functional significance of the EMLD is not well known. Paradoxically, IMLD is also seen in trained active athletes who are highly insulin sensitive [8].

Interleukin 6 (IL-6) has been reported to be increased in obese patients and in patients with diabetes [9]. Paradoxically, IL-6 enhances insulin-stimulated glucose disposal during exercise [10]. Insulin-stimulated phosphatidylinositol 3-kinase (PI3 K)-Akt signaling and GLUT-4 translocation are impaired in *fa/fa* rats [11,12] and in patients with Type 2 diabetes mellitus (T2D) [13]. However, exercise-stimulated glucose uptake is not affected by synergistic IL-6 secretion [14–16]. In context of the health benefits of exercise, human studies have showed that rather than causing insulin resistance, IL-6 enhances insulin-stimulated glucose disposal during exercise [10]. It is not clearly known which cell types contribute to the circulating IL-6 production during exercise or in chronic conditions such as obesity. It is reported that co-culture of macrophage and adipocytes result in synergetic effects on IL-6 release with increases up to 100-folds [17].

To investigate the signaling effects of adipocytes on myocytes with the anticipated but controversial involvement of IL-6 in inducible insulin signaling we developed an *in vitro* model using a co-culture system of L6 rat skeletal muscle cell line and 3T3-L1 pre-adipocyte murine cell line. To enhance the inflammatory reaction we challenged the co-culture system with lipopolysaccharide (LPS), a bacterial pro-inflammatory agent. We found that the

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3T3-L1 pre-adipocytes and their conditioned medium enhance the expression of IL-6 mRNA in the L6 muscle cells and the secretion of IL-6 in the medium.

2. Materials and methods

2.1. Cell culture design

L6 cells were seeded in multi-well plates for 24 h in DMEM containing 10% fetal bovine serum (FBS). Confluent L6 cells were induced to differentiate in DMEM containing 2% horse serum for 7–11 days, differentiation is monitored by the appearance of closely aligned and fused myotubes. Samples at different time points, before induction of differentiation (day 1) and after induction (day 2, day 4, day 7 and day 11) were analyzed with quantitative polymerase chain reaction (q-PCR) for skeletal muscle-specific differentiation marker genes (data not shown).

The 3T3-L1 (3T3) pre-adipocyte cell line, originally derived from murine embryo, was from the American Type Culture Collection, and grown in 12-well plates or 6-well plates cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (growth medium). The 3T3-L1 cells were differentiated into adipocytes according to previously described methods [20]. Briefly, 3T3-L1 cells were grown to confluence in a medium supplemented with 250 nmol/l dexamethasone, 25 nmol/l insulin, and 0.5 mmol/l isobutyl methylxanthine (IBMX) for 3–5 days. Both undifferentiated pre-adipocytes and differentiated adipocytes were used in the co-culture system. The 3T3-L1 cells were trypsinized and plated over the differentiated L6 myotubes at a ratio of 1:10, for 3T3-L1 and L6 cells respectively.

2.2. RNA extraction

Total RNA extraction was performed using a kit from Promega. RNA Purification was carried out using the Spin Column Assemblies according to manufacturer's instructions (Promega, USA). Briefly, the cells were trypsinized, washed with PBS and lysed with a cell lysis buffer containing β-mercaptoethanol; the lysate was diluted according to manufacturer's recommendations and the nucleic acids were adsorbed to the column matrix. Adsorbed DNA was hydrolyzed and repeatedly removed using DNase enzymatic digestion. The purified RNA was eluted into nuclease-free water and stored at −80 °C. RNA concentration and purity were determined using absorbance at 260 and 280 nm.

2.3. cDNA synthesis

The mRNA was converted into cDNA using the Promega first strand Reverse Transcription kit (Promega, USA) in a total volume of 20 μl. The reaction mixture was reconstituted with the following components respectively: $MgCl_2$, 10× reverse transcriptase buffer, dNTP mixture, recombinant RNase inhibitor, MMLV reverse transcriptase, and random hexamer primers. The reaction mixture was incubated for 1 h at 42 °C and the reaction was terminated by heating for 5 min at 95 °C followed by cooling at 0–5 °C for 5 min. The cDNA was diluted and stored at −80 °C.

2.4. Primers design

Primer Premier for Windows version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used for searching, aligning, editing and handling primers. The GeneBank National Center for Biotechnology Information Database was used as a source of mRNA sequence. The primers were designed to span one or more exon-exon junctions to avoid interference from genomic DNA as confirmed by single product melt-curves for each primer set. The primer design for optimal secondary structure were set to include: very high stringency, Tm of 47–59, GC% of 40–60 and degeneracy of 1, 3′ end stability of −0.9 to −6.5 kcal/ml, GC clamp dGC <−10 kcal/ml, maximal repeats of 3, maximal dimmer/hairpin of 6 at 3′, and maximal false priming of 8. The designed primers were synthesized at Invitrogen Life Technologies (Invitrogen, USA). For IL-6 we used CACTGCCTTCCCTACTTC as a forward primer and CATCATCGCTGTTCATAC as a reverse primer. For β actin we used CGTTGACATCCGTAAAGAC as a forward primer, and GAAGGTGGACAGTGAGGC as a reverse primer.

2.5. Real-time PCR

Quantitative analysis was conducted using real-time PCR detection system (ABI Step-One Plus System) and the SYBER Green fluorophore (Bio-Rad, USA). The preset PCR cycle condition was 2 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s. Ct values (cycle threshold), the cycle at which the fluorescence reached a preset threshold was used for quantitative analyses after normalization against the house-keeping gene β-actin. Original readings were adjusted by calculating the difference of C_T values of each gene and C_T value of the house keeping gene ($\Delta C_T = C_T$ (target gene) − the C_T (housekeeping gene)). Finally, the relative gene expression was transformed by calculating as $2^{-(S\Delta C_T - C\Delta C_T)}$, where $S\Delta C_T - C\Delta C_T$ is the difference between the sample ($S\Delta C_T$) and the control ($C\Delta C_T$).

2.6. Analysis of IL-6 protein release

Commercially available ELISA was employed using unlabeled and biotinylated polyclonal anti-rat IL-6 antibodies (R&D Systems, Inc., Minneapolis, MN). The primary antibody was diluted to 1 μg/ml in PBS and coated overnight onto Maxisorp ELISA plates (Nunc, Chicago, IL) in 100 μl at room temperature. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked using 200 μl blocking buffer (3% BSA in PBST) for 1 h. The blocking solution was removed, and media samples were added to the plate. After 2 h at room temperature, the plate was washed with PBST, and biotinylated anti-IL-6 antibody (1 μg/ml) was added to each well. After 2 h the plate was washed, and streptavidin horseradish peroxidase (100 ng/ml; Sigma–Aldrich) was added. After 1 h at room temperature, the plate was washed with PBST. Assay substrate, 3,3′, 5,5′-tetramethylbenzidine (0.1 mg/ml) dissolved in citrate buffer 50 mM Na_2HPO_4 and 25 mM citric acid (pH 5.0) was added in 100 μl/well. The reaction was stopped after 10 min by addition of 50 μl 1 M H_3PO_4. Optical density was measured with a Molecular Devices plate reader at 450 nm and well corrected for reference A_{570} ($A_{cor} = A_{450} - A_{570}$). A_{cor} data was then converted to percent of control values calculated using the formula: % of control = $A_s/A_c \times 100$, (where A_s is absorbance of test sample and A_c is absorbance of control sample).

2.7. Western blot

SDS–PAGE was performed in 10–12% polyacrylamide by applying 20–50 mg of total cell lysate protein in each lane. For a given Western analysis, equal protein loading and pre-stained molecular weight marker (Amersham, USA) were included. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Amersham, USA) in a buffer containing 25 mM Tris–HCl, 192 mM glycine, 20% methanol, and 0.01% SDS (pH 8.5) using Bio-Rad transblot semi-dry apparatus at 14 V for 1 h. Residual protein binding sites on the membranes were blocked by incubation for 1 h in TBST buffer (20 mM Tris–HCl, pH 7.6, 250 mM NaCl, 0.5% Tween-20) containing 5% non-fat dry milk. The membranes were incubated

with primary antibody overnight at 4 °C. After washing with TBST, a secondary antibody (anti-IgG conjugated with HRP) was added for 1 h. Finally; the protein bands were visualized by autoradiography using an enhanced chemiluminescence detection system (ECL kit, Amersham, USA).

2.8. Statistical analysis

Statistical analysis was performed using SPSS (version 18). One way analysis of variance (ANOVA) was used to compare means of treatment groups applying Tukey's multiple comparisons, considering a p value of <0.05 as significant. Levels of significance are indicated in the figures and text as appropriate.

3. Results

3.1. IL-6 mRNA expression

Confluent L6 cells induced to differentiate for 7 days and challenged with LPS (1 μg/mL) for 24 h increased IL-6 mRNA levels by 3.6-fold ($p = 0.002$). When L6 myocytes were co-cultured with 3T3-L1 cells at a density of 10:1, IL-6 mRNA expression increased by 2.3-fold if the 3T3 cells were undifferentiated and 2.7-fold if differentiated ($p < 0.05$) when compared to L6 myocytes cultured alone (Fig. 1). Addition of LPS (1 μg/ml) to the co-cultured cells dramatically increased IL-6 mRNA expression by 9.5-fold in presence of undifferentiated 3T3 and 8.2-fold in presence of differentiated 3T3 cells ($p < 0.001$). Similarly, LPS-stimulated IL-6 mRNA expression was significantly increased by 4-folds when L6 myotubes are incubated with conditioned medium from differentiated 3T3-L1 and 5.6-folds when incubated with conditioned medium from differentiated 3T3-L1, respectively ($p < 0.001$) (Fig. 1).

To examine the specificity of the cell-cell interaction between 3T3 and L6 cells we co-cultured the L6 myocytes with a murine osteoblastic cell line, MC4, in the same conditions used for the 3T3/L6 co-cultures. The MC4 cells were selected as a negative control because osteoblasts have a common lineage with adipocytes and myocytes. The presence of MC4 cells with the L6 myocytes did not cause increased IL-6 mRNA expression or IL-6 protein release (Fig. 2A).

3.2. IL-6 release by L-6 myocytes

To examine if the increased IL-6 mRNA is reflected in increased IL-6 release, we measured IL-6 concentrations in the medium at different time points (Fig. 2B). LPS significantly increased IL-6 release from L6 myocytes cultured alone at 8 h or longer time of exposure; the increase reached a maximum of 4-fold after 20 h ($p < 0.01$). Co-culturing the L6 myocytes with 3T3-L1 pre-adipocytes dramatically increased LPS-induced IL-6 mRNA ($p < 0.001$) by 3.6-folds at 8 h, 5-folds at 12 h and a 12.7-folds at 20 h. The treatment of L-6 myocytes with pre-adipocyte preconditioned medium and LPS followed a similar increase ($p < 0.01$) from 2.6-folds at 8 h, 3.3-folds at 12 h, 8.2-folds at 20 h and 8.8-folds at 30 h. The 3T3-L1 cells, cultured alone to confluence or to the same cell density as in the co-culture, do not release any detectable amount of IL-6 in the medium (not shown).

3.3. Insulin signaling

We examined insulin-stimulated Akt phosphorylation in the co-culture system of L6 myocytes with undifferentiated or fully differentiated 3T3 cells (Fig. 3). Insulin-induced Akt activation in L6 myocytes cultured alone was increased when the cells were challenged with LPS. The presence of conditioned medium from undifferentiated 3T3 cells had no effect on insulin-induced Akt phosphorylation (Fig. 3, panels B versus A), however, the presence of the 3T3 undifferentiated pre-adipocytes blocked completely insulin-induced Akt phosphorylation (Fig. 3, panel C). In contrast, both the conditioned medium (Fig. 3, panels E versus D) and the differentiated 3T3 adipocytes (Fig. 3, panels F versus D) significantly suppressed insulin-induced Akt phosphorylation (Fig. 3).

Since IL-6 released in the medium may influence insulin signaling we incubated the myocytes with IL-6 for different time periods and examined insulin-stimulated Akt phosphorylation (Fig. 4). The presence of IL-6 in growth medium over an extended period of time did not influence insulin dependent phosphorylation of Akt;

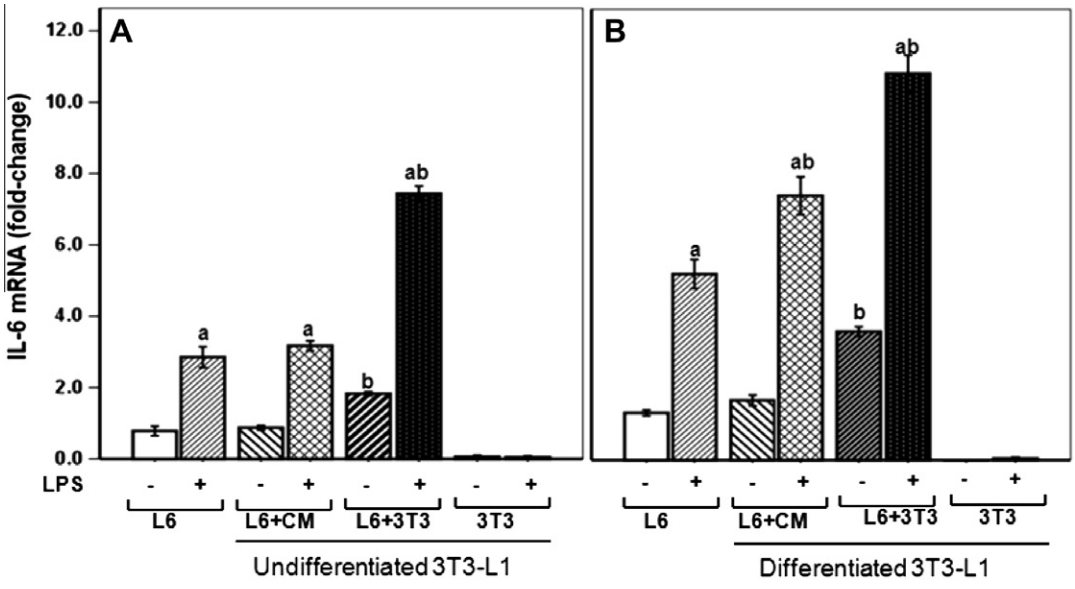

Fig. 1. Expression of IL-6 mRNA in L6 myocytes cultured alone or in combination with undifferentiated or differentiated 3T3-L1 adipocytes. L6 cells were first grown to confluence and induced to differentiate into myotubes in DMEM medium containing 2% horse serum for 7 days. The adipocyte to myocyte ratio was 1–10. Cultures were treated with 1 μg/ml LPS or vehicle. Similar experiments were performed using conditioned media (CM) from differentiated or undifferentiated 3T3-L1 cells. Total RNA was extracted; mRNA was converted to cDNA and analyzed by real-time PCR (rt-PCR). The Ct values were normalized for that of β-actin. (A) $p < 0.01$ + LPS versus-LPS; (B) $p < 0.01$ CM or 3T3 versus corresponding control cultures.

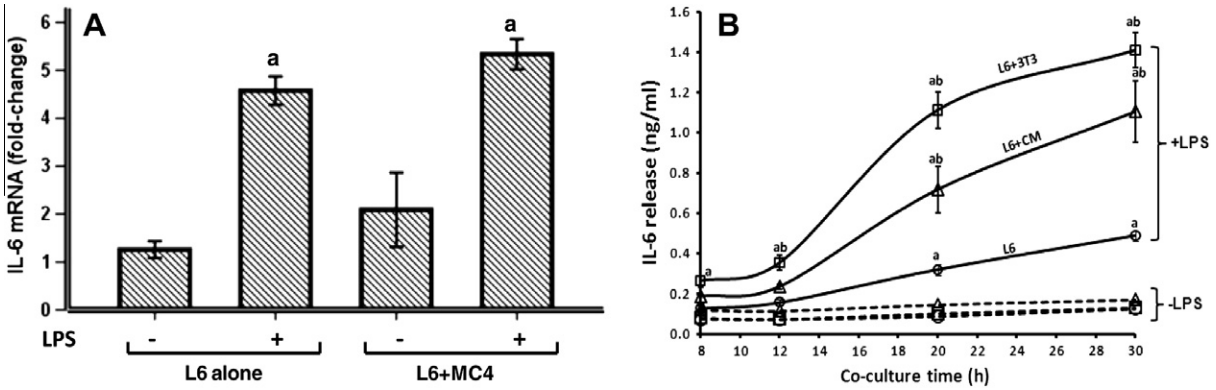

Fig. 2. (A) IL-6 mRNA expression by L6 myocytes co-cultured with murine MC4 cells. The co-cultures were performed similar to Fig. 1 except that MC4 cells were used instead of 3T3-L1 cells at a ratio 1:10. (A) $p < 0.01$; +LPS versus −LPS. (B) Effects of 3T3-L1 preadipocyte on IL-6 release in the co-cultures. L6 myocytes co-cultured with 3T3-L1 preadipocytes or their conditioned medium. IL-6 release was measured ELISA. (A) $p < 0.01$ and (B) $p < 0.01$.

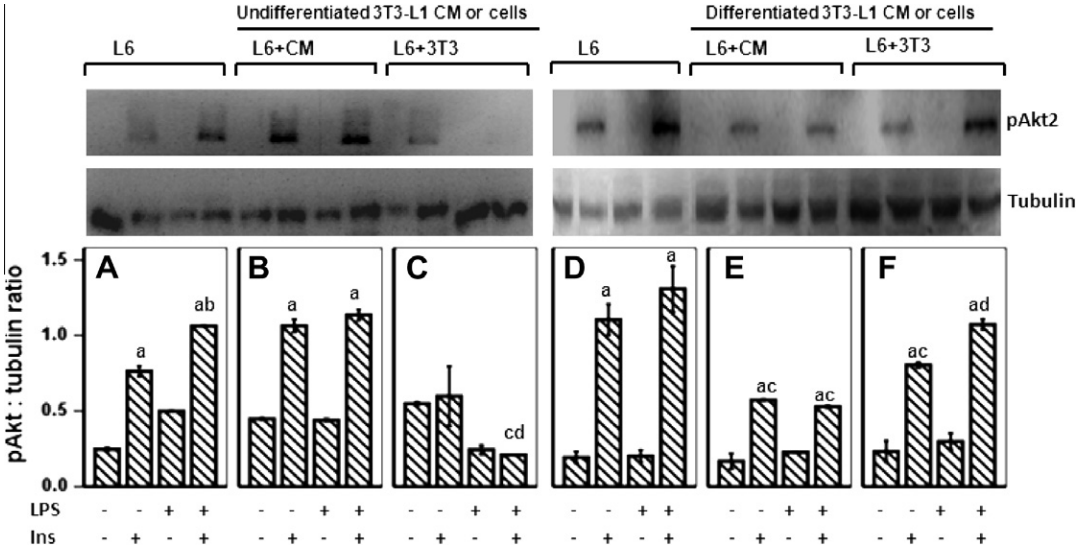

Fig. 3. Effects of insulin and LPS on Akt activation in L6 myocytes cultured alone or in presence of 3T3-L1 cells or its conditioned medium. The culture conditions are similar to Fig. 1. Western blot were done using pAkt antiserum against Ser473. Densitometry values of pAkt corrected for tubulin (means ± SEM) are calculated from at least three independent experiments. (A) $p < 0.01$ for the effects of insulin versus corresponding control values, (B) $p < 0.01$ for the effects of LPS versus corresponding values without LPS, (C) $p < 0.05$ for the effect of cell-to-cell interaction or conditioned medium compared to comparable L6 alone cells, and (D) $p < 0.05$ for the effect of cell-to-cell compared to corresponding conditioned medium.

although we observed a tendency toward enhanced insulin signaling at 24–72 h and decreased signaling at 96 h.

4. Discussion

The co-culture system was developed to study the paracrine interactions between myocytes and adipocytes. The presence of 3T3-L1 cells or their conditioned medium in direct contact with L6 myocytes increased IL-6 mRNA expression and protein production by the L6 myotubes. The IL-6 production is markedly enhanced when the co-culture was challenged by LPS.

Adipocytes secrete numerous cytokines, but only few are known to modulate insulin action including the pro-inflammatory TNF-α and IL-6; which cause insulin resistance in adipose, hepatic and muscle cells [18,19]. Adipocytes also secrete anti-inflammatory cytokines, especially IL-10, reported to be beneficial in insulin-regulated glucose homeostasis [19]. In our attempts to determine the effects of humeral factors, we used conditioned media generated from pre-adipocytes and adipocytes, which was found to be effective in producing the targeted changes, especially IL-6 secretion. It was reported that 3T3-L1 cells could be stimulated by LPS to secrete IL-6 [20]. However, in our study the preadipocytes and adipocytes cultured alone did not release detectable levels of IL-6; perhaps because these cells only represent 10% of the total cell number of the co-culture. Yet, we consistently found that the presence of the 3T3-L1 cells in the co-cultures enhances IL-6 secretion by L-6 cells both at mRNA and protein levels.

The skeletal muscles of obese individuals are invaded by EMLD, which may contribute to development of insulin resistance. In fact, circulating IL-6 levels are significantly elevated in obese patients and in patients with diabetes or insulin resistance [9,21]. In line with these observations, weight loss induced by calorie restriction in patients with diabetes has been shown to considerably lower circulating levels of IL-6 and its mRNA expression in adipose tissues [22,23]. From these reports one can surmise that IL-6 is associated with insulin resistance and could be a major player in metabolic syndrome. The detailed mechanism why IL-6 is chronically elevated in patients with obesity and diabetes is not well known.

Contrary to the above reports that describe the association of IL-6 and insulin resistance, there are other reports that show the

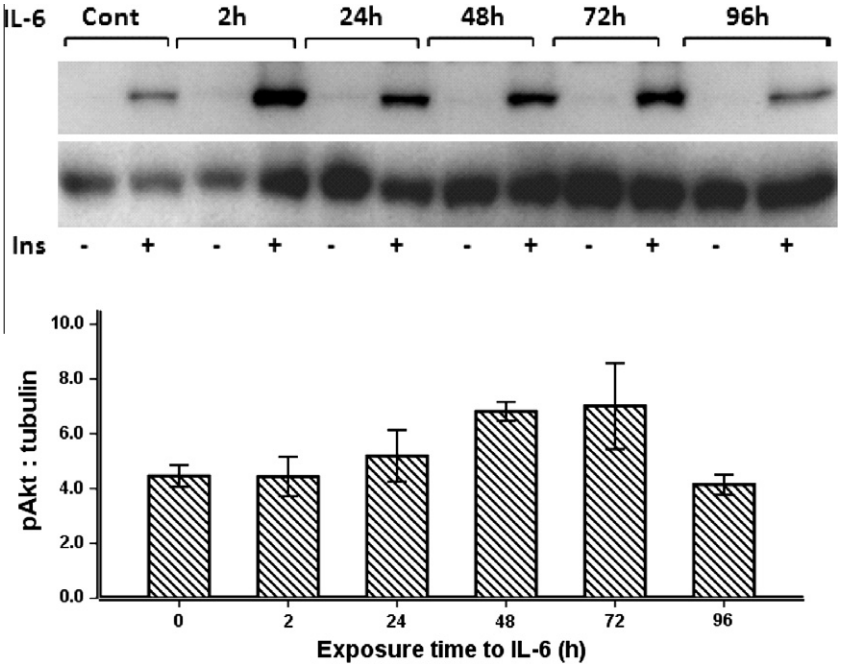

Fig. 4. Effects of interleukin-6 on insulin-activation of Akt in L6 myocytes. Cells were treated with 10 nM of recombinant IL-6 for 96 h. pAkt antiserum was as described in Fig. 3.

opposite effects of IL-6. One of the conditions where the action of IL-6 is dramatically displayed is at time of exercise. During exercise plasma levels of IL-6 increase considerably up to 100-fold [10,24,25]. Such high levels of IL-6 during exercise can by no means be marker of insulin resistance. The harmful and detrimental effects of IL-6 might occur after continuous exposure of tissues to high levels of IL-6 for a prolonged period of time such as in obesity and T2DM. IL-6 levels attained during exercise are much higher than those seen in individuals with diabetes and obesity although only for a period of brief exposure [26]. The beneficial/detrimental effects of IL-6 secretion in humans may be dependent on the level and contexts in which they are secreted.

In addition to the exercise induced surge in IL-6 levels, that possibly have beneficial effects on insulin signaling, it was shown that skeletal muscle strips prepared from vastus lateralis increase their glucose uptake when incubated by IL-6 [27]. Similarly, IL-6 infusion in humans during hyperinsulinemic-euglycemic clamp increased glucose disposal, fatty acid oxidation, basal and insulin stimulated glucose uptake, translocation of GLUT 4 to the plasma membrane and AMPK-mediated cellular responses [28]. In contrast, a human hepatocarcinoma cell line, primary mouse hepatocytes and 3T3-L1 cells showed inhibition of insulin receptor signal transduction and 75% reduction in glycogen synthesis when incubated with IL-6 [29,30].

The mechanism through which IL-6 modulates insulin sensitivity is not clearly known. In our study we found that L-6 cells produce high levels of IL-6 when co-cultured with 3T3-L1 cells and when challenged by LPS. The combination of 3T3-L1 and LPS produced more than the additive effects of either agent. The enhanced production of IL-6 when L6 myocytes are cultured in 3T3-L1 cell conditioned medium suggests the presence of soluble cellular factors produced by the adipocytes that promote IL-6 expression and secretion by the myocytes. These findings provide an understanding of the possible pathologic role of infiltrating preadipocytes and adipocytes within the muscle fibers of patients with diabetes and obesity.

The fact that the osteoblastic cell line, MC4, did not increase IL-6 expression by the L6 myocytes suggests that the effects of the preadipocytes and adipocytes on the myocytes are cell specific. This indicates an important role for the interaction between muscle and fat cells in the development of muscle dependent insulin resistance. These observations corroborate the presence of a cell-specific inflammatory response due to the interaction of myocytes and adipocytes that involves their humeral products.

An important observation emerged from this study that may explain the differential effects of IL-6 as beneficial or detrimental agent in cross-talk between skeletal muscle and fat cells. Pre-adipocytes rather than fully differentiated adipocytes showed inhibition of Akt phosphorylation, an important insulin signaling pathway. Previous studies on C57BL/6J and *ob*/*ob* mice [20] and humans [31] reported that IL-6 expression is more abundant in preadipocytes than in adipocytes which may be partly responsible for insulin sensitivity observed in obese patients. The result of this co-culture system suggests autocrine and paracrine interactions between myocytes and adipocytes depending on the presence of inflammatory conditions and whether infiltrating adipocytes are differentiated or not. The differentiated adipocytes may secrete a number of beneficial adipocytokines such as adiponectin that enhance insulin signaling and thus neutralizes the detrimental effects. On the other hand the fact that conditioned medium from undifferentiated adipocytes did not affect insulin signaling might signify that the undifferentiated adipocytes are not mature enough to produce the factor that suppresses insulin signaling. The results of this *in vitro* system indicate the importance of future studies using animal models to understand the cross-talks between muscle and preadipocytes and adipocytes in terms of the role played by IL-6 and related cytokines.

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