Direct Cross-talk of Interleukin-6 and Insulin Signal Transduction via Insulin Receptor Substrate-1 in Skeletal Muscle Cells*

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The exercise-induced interleukin (IL)-6 production and secretion within skeletal muscle fibers has raised the question of a putative tissue-specific function of IL-6 in the energy metabolism of the muscle during and after the exercise. In the present study, we followed the hypothesis that IL-6 signaling may directly interact with insulin receptor substrate (IRS)-1, a keystone in the insulin signaling cascade. We showed that IL-6 induces a rapid recruitment of IRS-1 to the IL-6 receptor complex in cultured skeletal muscle cells. Moreover, IL-6 induced a rapid and transient phosphorylation of Ser-318 of IRS-1 in muscle cells and in muscle tissue, but not in the liver of IL-6-treated mice, probably via the IL-6-induced co-recruitment of protein kinase C-β. This Ser-318 phosphorylation improved insulin-stimulated Akt phosphorylation and glucose uptake in myotubes since transfection with an IRS-1/Glu-318 mutant simulating a permanent phospho-Ser-318 modification increased Akt phosphorylation and glucose uptake. Noteworthily, two inhibitory mechanisms of IL-6 on insulin action, phosphorylation of the inhibitory Ser-307 residue of IRS-1 and induction of SOCS-3 expression, were only found in liver but not in muscle of IL-6-treated mice. Thus, the data provided evidence for a possible molecular mechanism of the physiological metabolic effects of IL-6 in skeletal muscle, thereby exerting short term beneficial effects on insulin action.

Over the last two decades, the pleiotropic cytokine interleukin-6 (IL-6) has been looked upon as a major component in the inflammatory network and the acute immune response (1). Since 1997, when the adipose tissue was recognized as a relevant IL-6 producing organ, accounting for 10–35% of circulating IL-6 plasma levels in humans (2), this view was renewed and broadened. It has been established in numerous clinical trials and association studies that IL-6 plasma concentrations increased with weight gain and are associated with the development of insulin resistance (3–6). Moreover, some reports provide evidence for elevated IL-6 levels as a risk factor for the manifestation of type 2 diabetes (3, 4). Well in accordance with these clinical data, chronic IL-6 treatment of mice reduces insulin signal transduction in the liver and produces a mild state of insulin resistance in these animals (7, 8). Thus, it is widely accepted that IL-6, similar to tumor necrosis factor-α, induces insulin resistance in the liver and adipose tissue (9).

However, there is overwhelming evidence that besides immune competent cells and adipose tissue, the skeletal muscle is a further important source of IL-6 (10, 11). Noteworthily, the IL-6 production in the muscle fibers is markedly increased by exercise, reaching interstitial IL-6 concentrations >1 ng/ml (i.e. 100-fold above physiological plasma concentrations) and, depending on the duration and intensity of the exercise, leading to elevated IL-6 plasma concentrations (12, 13). Since exercise is one of the most important therapeutic interventions to cope with weight gain and insulin resistance (14), it is obvious to suggest that exercise-induced IL-6 expression may exert positive effects on insulin signal transduction in the skeletal muscle. In fact, moderate exercise has been shown to improve insulin sensitivity, glucose uptake, and glycogen synthesis in muscle (15–17). An increasing number of reports suggest that IL-6 could act as a myocyte-derived “exercise factor” that improves the energy supply of the muscle by increasing the output of glucose and fatty acids from the energy-storing organs liver and fat and by enhancing glucose uptake, fatty acid oxidation, and glycogen synthesis in muscle (18–21). Apparently, this argues for some muscle-specific insulin-sensing effects of IL-6, but the underlying molecular mechanism is unclear.

IRS-1 is a key player in insulin signaling in muscle, and its cross-talk with metabolic and mitogenic pathways is modulated in a very subtle and complex manner using the phosphorylation pattern of the Ser/Thr-phosphorylation sites of IRS-1 as a regulatory mechanism for the interaction with its important downstream mediators, e.g. phosphatidylinositol-3 kinase (PI3K) (22–28). Since IL-6 regulates via the signal transducer and activator of transcription (STAT)-3-Janus kinase (JAK) pathway and the mitogen-activated protein kinase (MAPK) pathway several Ser/Thr kinases shown to phosphorylate IRS-1 (29), we hypothesize that IL-6 could cross-talk with the insulin signaling cascade by phosphorylation of IRS-1. We showed in the present study that IL-6 induces the Ser-318 phosphorylation of IRS-1 in various muscle cell lines and in muscle tissue of mice but not in the liver, that IRS-1 co-immunoprecipitates with the IL-6 receptor (IL-6R) and protein kinase C (PKC)-β in an IL-6-dependent manner, and that Ser-318 phosphorylation may improve insulin action.

EXPERIMENTAL PROCEDURES

Materials—C2C12 cells were from ATCC (Manassas, VA). Parental L6 cells and L6 GLUT4Myc cells were kindly provided from A. Klip.
Hospital for Sick Children, Toronto, Canada. Cell culture media and supplements were from Invitrogen (Eggenstein, Germany). Human, mouse, and rat recombinant IL-6 were from Sigma (Munich, Germany) and R&D Systems (Wiesbaden, Germany). Oligonucleotides were synthesized by Invitrogen (Karlsruhe, Germany); reagents for cDNA synthesis and the LightCycler system were from Roche Applied Science (Mannheim, Germany). Antibodies against phospho-STAT-3 Tyr-705, STAT-3, and phospho-Akt Ser-473 were from Cell Signaling (Frankfurt, Germany); antibodies against IRS-1 (C terminus) and phosphotyrosine (4G10) were from Upstate Biotechnology (Lake Placid, NY), antibodies against PKC-δ were from BD Biosciences, antibodies against IL-6R used for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibody against IL-6R used for immunoprecipitation was from Biologend (San Diego, CA). The phospho-IRS-1 Ser-307 antibody was a kind gift from M. F. White, Harvard Medical School, Boston, MA. Polyclonal anti-phospho-Ser-318 antiseraum was raised against a synthetic peptide (SMYGGPKPSGRFRASSSDK) flanking Ser-318 in IRS-1, conserved among mouse, rat, and humans, and characterized as described in Ref. 24. 2-[3H(G)]Deoxy-D-glucose (185–370 GBq/mmol) was from PerkinElmer Life Sciences. The cytokemalovirus promoter-based expression vector for rat IRS-1 was described in Ref. 30, and the cytokemalovirus promoter-based expression vectors for murine PKC-δ and dominant kinase-negative PKC-δ were described in Ref. 31. Mutation of Ser-318 of IRS-1 to alanine or glutamate was made by oligonucleotide-mediated mutagenesis with the mutagenic upstream primers IRS-1/Ala-318 5′-ccagtatggtgggtgggaaaccaggtcaggctccagc-3′ and IRS-1/Glu-318 5′-ccagtatggtgggtgggaaaccaggtcaggctccagc-3′ (32).

In Vivo IL-6 Treatment of Mice—Ten-week-old male C57Bl/6 mice were obtained from The Jackson Laboratory and studied after 2 weeks of acclimatization. They were maintained on a normal light/dark cycle and kept on a regular diet. For short term IL-6 effects, mice were anesthesitized with an intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight), and a total of 2.5 μl of 0.9% NaCl and lysed with 250 μl of 2-deoxy-glucose buffer containing 0.25 μl/ml 2-[3H]deoxy-glucose and 10 mM 2-deoxy-glucose was added. After incubation at 37 °C, 5% CO2 for 7 min, cells were washed three times with 0.9% NaCl and lysed with 250 μl of lysis buffer. Total amounts of cell lysates were counted by liquid scintillation counting. Protein content of lysates generally varied <10%.

Reverse Transcription-PCR and Real-time Quantitative PCR Analysis—Total RNA of muscle and liver was isolated with PEGOLD TriFast (Peglab, Erlangen, Germany). Reverse transcription of total RNA (1 μg) was performed using avian myeloblastosis virus reverse transcriptase with the first-strand cDNA synthesis kit for reverse transcription-PCR. Aliquots (2 μl) of the reverse transcription reactions were then submitted to online quantitative PCR with the LightCycler system with SYBR® green using the FastStart DNA-MasterSYBR Green I as described (36). The following SOCS-3 primer pair was used: sense, 5′-gctggccaaagaaataacca-3′; antisense, 5′-agtcaccagcccttca-3′, product of 224 bp. The PCR was performed in a volume of 20 μl: 2 μl of FastStart DNA-MasterSYBR Green I, 3 mM MgCl2, and primers corresponding to a primer concentration of 1 μM. The instrument settings were: Denaturing at 95 °C for 10 min, 45× denaturing at 95 °C for 15 s, annealing at 66 °C for 10 s, elongation for 9 s.

Small Interfering RNA (siRNA)—siRNA oligonucleotides targeting PKC-δ were designed and synthesized and annealed at Dharmacon Research, Lafayette, CO. An unrelated siRNA targeting firefly luciferase was used as control in all experiments. Transfection was performed with CellPhect (Amersham Biosciences, Buckinghamshire, UK) with 100 pmol of siRNA according to the instructions of the manufacturer. Briefly, 1 × 105 of C2C12 cells/well were seeded in 6-well plates and transfected in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% FCS without antibiotics. 24 h after the glycerol shock, cells were stimulated as indicated.

Statistical Analysis—Results presented are derived from at least three independent experiments. Means ± S.E. were calculated, and groups of data were compared using Student’s t test. Statistical significance was set at p < 0.05.

RESULTS

IL-6 Induces Ser-318 Phosphorylation of IRS-1 in Muscle and in Muscle Cell Culture Models—Since previous studies showed that serine phosphorylation of IRS-1 modulates insulin signal transduction, we
studies whether IL-6 treatment of mice (2.5 μg/kg intravenously for 5 min) induces Ser phosphorylation of IRS-1 using phospho-site-specific antibodies against inhibitory serine residue 307 (23) and the Ser-318 phosphorylation previously reported from our group (24, 32). We detected a rapid and significant increase in the phosphorylation of Ser-307 in liver but not in muscle of IL-6-treated mice (Fig. 1, A and C). Phosphorylation of Tyr-705 of STAT-3 demonstrated the activation of IL-6-dependent pathways. Insulin treatment resulted in a clear phosphorylation of Ser-307 in muscle (Fig. 1A) as reported previously (23). When we studied the phosphorylation of Ser-318 of IRS-1 in these animals, we found just the opposite; IL-6 induced a rapid (5 min) and significant phosphorylation of this serine residue in muscle but not in liver (Fig. 1, B and C). Noteworthily, we observed a clear although less pronounced phosphorylation of STAT-3 after IL-6 treatment in muscle when compared with liver tissue, indicating that the muscle responds to IL-6.

To investigate the rapid and unexpected IL-6-induced phosphorylation of Ser-318 in skeletal muscle, we studied this effect in cultured skeletal muscle cells. Using three different cell models, we observed a rapid (2 min) and transient IL-6-induced phosphorylation of Ser-318 in human myotubes, murine C2C12 myoblasts, and rat L6 myotubes, which reached a maximum after 5–10 min of IL-6 stimulation, whereas the phosphorylation of STAT-3 proceeded (Fig. 2, A–C). Together the data show that IL-6 induces a very rapid phosphorylation of a distinct serine residue (i.e. Ser-318 in muscle) on IRS-1, an adaptor molecule previously described as an essential mediator in the signal transduction of insulin and IGF-1 (38). The following questions arise from this novel finding. First, how are the IL-6 and the insulin signaling pathways linked to each other; second, which kinase is responsible for the IL-6-stimulated Ser-318 phosphorylation in human myotubes. The values of unstimulated cells were set as 1 (n = 4; mean ± S.E.; *p < 0.05 versus untreated mice), relative.

Cross-talk of IL-6 and Insulin Signal Transduction via IRS-1

![Image](https://example.com/image1.png)

**Figure 1. IL-6 treatment induces different Ser phosphorylation of IRS-1 in muscle and liver.** Ten-week-old male C57Bl/6 mice were treated with recombinant IL-6 (2.5 μg/kg intravenously) or 5 IU of insulin (Ins) for 5 min, muscle and liver tissue was obtained, and protein extracts were prepared as described under "Experimental Procedures." Proteins (150 μg) were separated by 5% SDS-PAGE, and Ser phosphorylation of IRS-1 was studied using anti-phospho-Ser-307 (A, p-Ser-307) and anti-phospho-Ser-318 (B, p-Ser-318) antibodies. Phosphorylation of STAT-3 was monitored to show activation of the IL-6 signaling pathway. Membranes were reprobed for protein amount. A: control, B: p-Tyr-705, C: densitometric quantification of the relative IL-6-induced phosphorylation of Ser-307 and Ser-318 in muscle and liver extracts related to IRS-1 protein. The values of untreated mice were set as 1 (n = 5; mean ± S.E.; *p < 0.05 versus untreated mice), relative.

![Image](https://example.com/image2.png)

**Figure 2. IL-6 induces rapid and transient phosphorylation of Ser-318 in skeletal muscle cells.** Human myotubes, C2C12 cells, and L6 myotubes were stimulated with 20 ng/ml recombinant human IL-6, 20 ng/ml recombinant mouse IL-6, or 50 ng/ml recombinant rat IL-6, respectively, for different time points as indicated in the figure. Cell extracts were separated by 7.5% SDS-PAGE, and phosphorylation of Ser-318 was studied using the site-specific phospho-antibody against Ser-318 of IRS-1. Membranes were reprobed for IRS-1 protein. A: control, B: densitometric quantification of the relative IL-6-induced phosphorylation of Ser-318 in human myotubes. The values of unstimulated cells were set as 1 (n = 4; mean ± S.E.; *p < 0.05 versus untreated mice); two representative immunoblots of myotubes obtained from two different subjects are shown. p-Ser-318: anti-phospho-Ser-318, relative. B: control, C: relative. 8, time-dependent IL-6-induced Ser-318 phosphorylation in C2C12 cells, and C: in L6 myotubes with the concomitantly induction of phosphorylation of STAT-3 shown in the lower part of each figure. p-Tyr-705, phospho-Tyr-705.
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was similar in wild-type and IRS-1-transfected cells (Fig. 3A). In non-transfected cells, we detected a faint signal for endogenous IRS-1 in the gp130 immunoprecipitate that increased after short term IL-6 stimulation (Fig. 3B). In IRS-1-transfected cells, the basal and IL-6-stimulated IRS-1 association was much more pronounced (Fig. 3B).

Further studies using this in vitro approach revealed that all three classes of PKC isoforms, classical, novel and atypical PKCs, can phosphorylate Ser-318 (40). Since an important role for PKC-isoforms, classical, novel and atypical PKCs, can phosphorylate Ser-318 as a PKC-dependent phosphorylation site (24, 39). Further GST fusion proteins and subsequent mass spectrometry-based complex stimulation rapidly recruits IRS-1.

**FIGURE 3.** IRS-1 co-immunoprecipitates with gp130 and IL-6R. C2C12 cells were transfected with IRS-1 as indicated and stimulated with 20 ng/ml mouse recombinant IL-6 for 10 min. Control cells were transfected with empty expression vector pCDNA3 (con). A, cell extracts were monitored for IRS-1 expression and phosphorylation of STAT-3. p-Tyr-705, phospho-Tyr-705. Cell extracts shown in A were immunoprecipitated (IP) with anti-gp130 (B) or anti-IL-6R antibodies (C), and IRS-1 was detected in the immunocomplexes by immunoblotting (IB). Representative immunoblots of four independent experiments are shown.

Co-recruited PKC-δ Phosphorylates Ser-318—The serine 318 residue of IRS-1 was previously identified by in vitro phosphorylation of IRS-1-GST fusion proteins and subsequent mass spectrometry-based sequencing as a PKC-dependent phosphorylation site (24, 39). Further studies using this in vitro approach revealed that all three classes of PKC isoforms, classical, novel and atypical PKCs, can phosphorylate Ser-318 (40). Since an important role for PKC-δ in IL-6 signaling has been demonstrated (41), we hypothesized that this PKC isoform could be responsible for IL-6-induced Ser-318 phosphorylation of IRS-1. To elucidate the possible participation of PKC-δ, we co-transfected active or kinase-negative PKC-δ and IRS-1 in C2C12 cells. Overexpression of active PKC-δ caused a very strong increase in Ser-318 phosphorylation in unstimulated cells, with no further obvious increase after IL-6 stimulation (Fig. 4A). In control cells, IL-6 treatment enhances Ser-318 phosphorylation as expected, whereas overexpression of the kinase-negative PKC-δ caused no change in Ser-318 phosphorylation (Fig. 4A). The data suggest that this serine residue can be phosphorylated by PKC-δ in skeletal muscle cells. To strengthen our hypothesis, we investigated the effect of IL-6 on Ser-318 phosphorylation in C2C12 cells after knockdown of PKC-δ using a siRNA silencing approach (Fig. 4B). The IL-6-induced phosphorylation of STAT-3 remained unaffected in siRNA oligonucleotides targeting PKC-δ (si-PKC-δ)-transfected cells, but phosphorylation of Ser-318 was blocked completely (Fig. 4B). Thus, we conclude that PKC-δ mediates the effect of IL-6 on Ser-318 phosphorylation.

We then studied whether IL-6 stimulates the association of both IRS-1 and PKC-δ with the IL-6R. As shown in Fig. 4C, the minor co-precipitation of endogenous PKC-δ with IL-6R in wild-type cells is increased by IL-6 treatment, and this increase is similar in IRS-1-transfected cells. Transfection of C2C12 cells with PKC-δ or both PKC-δ and IRS-1 led to a comparable stimulation; the amount of endogenous as well as co-transfected PKC-δ was increased in the IL-6R immunopre-
FIGURE 5. Effect of IL-6 on IRS-1 tyrosine phosphorylation in C2C12 cells. A, C2C12 cells were transfected with IRS-1 and stimulated with 20 ng/ml recombinant IL-6 for 10 min or 10 nM insulin for 5 min. Co-immunoprecipitation was performed with anti-IL-6R antibodies or anti-IRS-1 antibodies (IP), and tyrosine phosphorylation was detected in the immunocomplexes by immunoblotting. Membranes were reprobed for IRS-1 protein. A representative immunoblot is shown in the upper part of the figure. Densitometric data of three independent experiments were summarized as histogram, and the values of insulin-stimulated IRS-1 WT-transfected cells were set as 1 (mean ± S.E.; *, p < 0.05 versus unstimulated cells; n.s., no significant difference versus IRS-1 WT-transfected cells).

FIGURE 6. Effect of IL-6 on IRS-1 tyrosine phosphorylation in L6 myotubes. L6 myotubes were stimulated with 20 ng/ml rat recombinant IL-6 for 10 min or 10 nM insulin for 5 min. A, cell extracts were separated by 7.5% SDS-PAGE and immunoblotted for IRS-1, phospho-STAT-3, and phospho-Akt. p-Tyr-705, phospho-Tyr-705; p-Ser-473, phospho-Ser-473. B, co-immunoprecipitation was performed with anti-IL-6R antibodies or anti-IRS-1 antibodies (IP), and tyrosine phosphorylation was detected in the immunocomplexes by immunoblotting (IB). Membranes were reprobed for IRS-1.

IL-6 Treatment Does Not Influence Tyrosine Phosphorylation of IRS-1—To study the biological function of the IL-6-stimulated recruitment of IRS-1 to the IL-6R complex and the subsequent Ser-318 phosphorylation of IRS-1, we investigated the tyrosine phosphorylation of IRS-1 in IRS-1-transfected C2C12 cells after stimulation with IL-6 for 10 min or insulin for 5 min. Although IL-6 treatment of the IRS-1-transfected cells did not induce the tyrosine phosphorylation of total IRS-1 protein, stimulation with insulin led to the expected increase of this phosphorylation (Fig. 5A). Although a huge amount of IRS-1 was co-immunoprecipitated with the IL-6R, no tyrosine phosphorylation of this IRS-1 fraction was detectable in both IL-6- and insulin-stimulated cells (Fig. 5A). Similarly, we observed no tyrosine phosphorylation of co-immunoprecipitated IRS-1 in control cells (Fig. 5A). To further investigate the role of Ser-318, we transfected C2C12 cells with IRS-1/Ala-318 to exclude phosphorylation at this site and IRS-1/Glu-318 to mimic permanent phosphorylation. We observed no effect of Ala-318 or Glu-318 on the tyrosine phosphorylation of total IRS-1 after stimulation with IL-6, insulin, or both (Fig. 5B). Preincubation with IL-6 neither reduced nor increased the amount of tyrosine-phosphorylated IRS-1 (Fig. 5B). These data indicate first that treatment of C2C12 cells with IL-6 did not inhibit insulin signal transduction at the level of IRS-1 tyrosine phosphorylation. Secondly, a potential regulation of insulin action by IL-6-induced phosphorylation of Ser-318 takes place downstream of IRS-1.

Effect of IL-6 on IRS-1 Recruitment and Tyrosine Phosphorylation in L6 Myotubes—As demonstrated in Fig. 2C, IL-6 induced phosphorylation of Ser-318 in L6 myotubes with a similar time course when compared with C2C12 cells (Fig. 2B). To confirm the results on IRS-1/IL-6R complex formation and tyrosine phosphorylation of IRS-1 obtained in C2C12 cells, we studied the effects of IL-6 in L6 myotubes after stimulation with IL-6 or insulin (Fig. 6A). Again, we observed no effect of IL-6 on tyrosine phosphorylation of total IRS-1, whereas insulin induced this tyrosine phosphorylation (Fig. 6B). IL-6 increased the amount of co-immunoprecipitated IRS-1 in the IL-6R complex, but again, no tyrosine phosphorylation of this IRS-1 fraction was detected (Fig. 6B).

Effect of IL-6-induced Ser-318 Phosphorylation on Insulin Signal Transduction—Several reports on the pathophysiological function of serine phosphorylation of IRS-1 pointed to a desensitizing action of specific serine residues on insulin signal transduction, e.g. among others, the intensively studied Ser-307 phosphorylation (22). However, there is increasing evidence for a more subtle regulation of insulin action by serine phosphorylation of IRS-1 with some serine sites also positively modulating insulin signal transduction (28, 42). Very recently, we could demonstrate that the phosphorylation of Ser-318 is not per se inhibitory but could even improve insulin signal transduction in the early phase of insulin action (32). Here, we support our data using L6GLUT4 myotubes, which in contrast to C2C12 cells exhibit insulin-sensitive glucose uptake (35), stably transfected with IRS-1 WT, IRS-1/Ala-318, or IRS-1/Glu-318. Mimicking a permanent Ser-318 phosphorylation in the Glu-318-transfected cells led to an enhanced increase in phosphorylation of Akt after 5 min of insulin stimulation when compared with IRS-1 WT and Ala-318-transfected cells (Fig. 7A). This improvement of insulin action is further reflected by the increase in insulin-stimulated glucose uptake in the Glu-318 myotubes, determined as incorporated 2-[3H]deoxyglucose. The introduction of Glu-318 markedly enhanced insulin-stimulated glucose uptake up to 1.97 ± 0.07 after 5 min of insulin stimulation and reached a 2.62 ± 0.11 increase after 20 min of insulin stimulation, both significantly above values obtained in IRS-1 WT and IRS-1/Ala-318 myotubes (1.53 ± 0.07 and 2.0 ± 0.11 in IRS-1 WT; 1.47 ± 0.08 and 1.63 ± 0.08 in Ala-318 cells, respectively; Fig. 7B). Similar data have been reported very recently by...
our group (32). These results indicate that IL-6-induced phosphorylation of Ser-318 could enhance insulin-stimulated glucose uptake in muscle cells. Of note, treatment of IRS-1 WT myotubes with IL-6 caused a slight but significant increase in glucose uptake in the absence of insulin (1.14 ± 0.05-fold; p < 0.007), which was not observed in IRS-1/Ala-318-transfected cells (0.98 ± 0.07-fold; data not shown).

IL-6 Treatment of Mice Induces a Rapid and Marked Induction of SOCS-3 Expression in Liver but Not in Skeletal Muscle—Our data did not support any inhibitory action of IL-6 on insulin signal transduction in skeletal muscle, as has been reported, especially for liver and hepatocytes. Since the rapid induction of SOCS-3 expression by IL-6 has been demonstrated as one important mechanism for the IL-6-mediated reduction of insulin action in hepatocytes (43), we hypothesized a different regulation of SOCS-3 expression in muscle and liver. Therefore, we measured SOCS-3 mRNA expression in vivo in muscle and liver tissue of mice treated for 30 and 60 min with 2.5 μg/kg of IL-6. IL-6 plasma levels in these animals increased up to 63.3 ± 6.3 and 60.1 ± 9.4 pg/ml after 30 and 60 min of IL-6 intraperitoneal application when compared with 7.2 ± 0.3 pg/ml in control mice. In the liver, we observed a strong, ~10-fold induction of SOCS-3 mRNA expression, both after 30 min and after 60 min of IL-6 treatment, whereas the increase in SOCS-3 levels in muscle was not significant (Fig. 8).

DISCUSSION

The exercise-induced IL-6 production and secretion within skeletal muscle fibers have raised the question of a putative role of IL-6 in the energy supply and energy storage of the muscle during and after exercise. This function has to be considered as a short term, physiological, and probably local effect of IL-6 within minutes or probably a few hours. In the present study, we followed the hypothesis that IL-6 may influence muscle energy metabolism by direct interaction of the IL-6 receptor complex with important molecules of the insulin signaling cascade in muscle.

We have provided here for the first time evidence that IL-6 induces a rapid, occurring within 10 min, recruitment of IRS-1, one key element of the insulin receptor complex. We detected endogenous IRS-1 as well as co-expressed IRS-1 by immunoprecipitation using antibodies against both IL-6 receptor subunits, gp130 and the IL-6R, in IL-6-stimulated C2C12 cells. The sequential regulation of this rapid complex formation is yet unknown. Theoretically, IRS-1 could directly associate with the phosphorylated tyrosine residues of the receptor subunit gp130 via the phosphotyrosine binding or Src homology (SH) 2 domains. Since the exact peptide sequence that is required for the binding of IRS-1 to the tyrosine-phosphorylated insulin receptor (NPXY motif) is not present in gp130, it could be speculated that SH2 domains-containing adaptor molecules that are tyrosine-phosphorylated by IL-6 mediate the ligand-induced binding of IRS-1 to the IL-6 receptor complex.

Another major finding of the present study was that IL-6 modifies IRS-1 by the rapid and transient phosphorylation of Ser-318, which was demonstrated in the muscle of IL-6-treated mice and in various skeletal muscle cell lines. Noteworthy, this modification was not observed in the liver of these mice, although the activation of IL-6 signaling pathways by the IL-6 treatment, as demonstrated by the phosphorylation of STAT-3, was much more pronounced in liver when compared with muscle.

We have provided several lines of evidence that PKC-δ could be the kinase responsible for the IL-6-induced Ser-318 phosphorylation. First, PKC-δ was recruited to the IRS-1-containing IL-6 receptor complex by IL-6 stimulation, which is well in line with a previous report demonstrating the association of this PKC with gp130 via STAT-3, thereby regulating IL-6 signaling (41). Second, overexpression of PKC-δ resulted in a strong phosphorylation of Ser-318 also in unstimulated cells, whereas overexpression of a kinase-negative PKC-δ was uneffective, and third, knock-down of PKC-δ prevented the IL-6-induced Ser-318 phosphorylation. Moreover, PKC-δ was able to phosphorylate this serine residue using an in vitro phosphorylation assay (data not shown).
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Of note, another PKC isoform, namely atypical PKC-ζ, has been demonstrated to mediate the insulin-dependent Ser-318 phosphorylation in baby hamster kidney cells (24) and in skeletal muscle cells (32). Thus, one serine residue could be phosphorylated by different PKC isoforms depending on the stimulus.

The functional relevance of the Ser-318 phosphorylation was demonstrated in L6 GLUT4 myotubes expressing IRS-1 WT, IRS-1/Ala-318, the non-phosphorylated form, or IRS-1/Glu-318, which simulates a permanent phospho-Ser-318 modification. The permanent Ser-318 "phosphorylation" of IRS-1 enhances insulin-stimulated Akt phosphorylation and glucose uptake after short term insulin stimulation of the myotubes, as shown in the present study and in a very recent study (32), whereas mutation of Ser-318 to alanine reduced Akt phosphorylation in the early phase of insulin stimulation. Thus, the phosphorylation of this serine residue could initially improve early insulin action. Of note, the IL-6-induced Ser-318 phosphorylation is a very rapid and transient modulation and was no longer visible after 30 min of IL-6 stimulation.

The first published participation of Ser-318 in the reduction of insulin signal transduction (24) is only visible after long term insulin treatment with high insulin doses, representing an insulin-resistant state but not the situation during physical activity when insulin concentrations are low. Further studies indicate that besides a positive modulation of insulin action in the early phase, the insulin-induced phosphorylation of Ser-318 is more likely to be a signal for the physiological attenuation of insulin action. Following this line, in the IRS-1/Glu-318 myotubes treated for a long term with insulin, the glucose uptake is attenuated similar to the wild-type cells (32).

The exact molecular mechanism for the insulin-sensitizing effect of the phosphorylation of Ser-318 on glucose uptake in skeletal muscle cells remains open. We detected no effect of IL-6 on the tyrosine phosphorylation of the IRS-1 fraction recruited to the IL-6R complex or of IL-6 or Ser-318 on insulin-stimulated tyrosine phosphorylation of total IRS-1. These data suggest that under some circumstances, serine/threonine phosphorylation could regulate the function of IRS-1 without direct effects on its tyrosine phosphorylation. It could be speculated that IRS-1 might serve as a multidocking site protein in receptor complexes beside the insulin receptor.

The next downstream effector of the insulin signaling cascade is P13K. An IL-6-dependent P13K activation was described in other cell culture models (44). We also observed an IL-6-induced association of the p85 subunit to the IL-6 receptor complex in C2C12 cells.3 This could provide evidence for the hypothesis that the delicate molecular balance of the regulatory p85 and the catalytic subunit p110 of P13K is regulated by IL-6, leading to a sensitization of the P13K-Akt pathway for insulin since the p85 subunits exhibits a negative effect on insulin signaling, probably due to their greater abundance when compared with the p110 subunit (45). However, we found no evidence for a regulation of the p85 recruitment to both IL-6R complex and IRS-1 by phosphorylation of Ser-318.3 Thus, the role of P13K in IL-6-enhanced insulin action in skeletal muscle cells remains open.

Our data may help explain the discrepancy of how IL-6 may act in an apparently opposite way on insulin signal transduction in different tissues and tissue-specific cell types. From previous reports, one could assume different molecular mechanisms by which IL-6 could reduce insulin signal transduction; one could include the mammalian target of rapamycin (mTOR) or Jun-activated kinase-mediated phosphorylation of Ser-307 of IRS-1, which in turn reduces insulin signaling (46). Another mechanism includes IL-6-induced expression of SOCS-3, which in turn attenuates insulin signaling in hepatocytes (43). In the present study, we have shown that none of these mechanisms is operating in skeletal muscle, supporting the observation that IL-6 is not reducing insulin action in skeletal muscle cells (36). Moreover, our data have provided evidence for an IL-6-induced stimulatory effect on insulin action via muscle-specific phosphorylation of Ser-318 of IRS-1. Together with the recently reported muscle-specific IL-6-activated Ser-473 phosphorylation of Akt, which in turn enhances insulin-stimulated glycogen synthesis in human myotubes (47), our data suggest a molecular mechanism concerning how insulin signaling is modulated differently in different tissues.

The putative metabolic function of the contraction-induced IL-6 production described here could be a local function within skeletal muscle during or after exercise. Such a role for IL-6 in skeletal muscle energy balance has been hypothesized by several groups, including the improvement of fatty acid oxidation (48, 49), enhancement of glucose disposal rate (19), activation of AMP-activated kinase (50), and more in general, involvement in exercise endurance (51). Noteworthily, this physiological role of the exercise-induced IL-6 in muscle is not in contrast to several reports demonstrating a role for pathophysiological elevated IL-6 plasma levels in the development of insulin resistance (3–6). First, systemic levels of IL-6 are chronically increased by obesity or inflammation, whereas exercise-induced IL-6 production is transient; second, exercise predominantly increases local concentrations of IL-6 in muscle; and third, IL-6 appears to exert tissue-specific effects.

In conclusion, IL-6 is able to induce a direct interaction of the IL-6 receptor complex with IRS-1 in skeletal muscle cells, thereby improving insulin action. This possibly physiological and local function of exercise-induced IL-6 has to be discriminated from the pathophysiological function of chronically elevated circulating levels of IL-6 that are able to induce insulin resistance.

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