

IL-6 directly increases glucose metabolism in resting human skeletal muscle

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Stephan Glund¹, Atul Deshmukh¹, Yun Chau Long¹, Theodore Moller¹, Heikki A Koistinen⁴, Kenneth Caidahl³, Juleen R Zierath¹ and Anna Krook^{1,2}

¹Department of Molecular Medicine and Surgery, Section for Integrative Physiology, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

²Department of Physiology and Pharmacology, Section for Integrative Physiology, Karolinska Institutet, Stockholm, Sweden

³Department of Molecular Medicine and Surgery, Section for Clinical Physiology, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

⁴Helsinki University Central Hospital and Biomedicum, Helsinki, Finland

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Address for correspondence:

Anna Krook

Department of Physiology and Pharmacology,

Section for Integrative Physiology,

Karolinska Institutet,

von Eulers väg 4,

SE- 171 77 Stockholm, Sweden

E-mail: anna.krook@ki.se

Abstract

Objective: IL-6 is a pro-inflammatory cytokine shown to modify insulin sensitivity. Elevated plasma levels of IL-6 are observed in insulin resistant states. Interestingly, plasma IL-6 levels also increase during exercise, with skeletal muscle being the predominant source. Thus, IL-6 has also been suggested to promote insulin-mediated glucose utilization. We determined the direct effects of IL-6 on glucose transport and signal transduction in human skeletal muscle.

Research Design and Methods: Skeletal muscle strips were prepared from *vastus lateralis* biopsies obtained from 22 healthy men. Muscle strips were incubated with or without IL-6 (120 ng/ml).

Results: IL-6 increased glucose transport in human skeletal muscle 1.3 fold ($P < 0.05$). A 30 min pre-exposure to IL-6 did not affect insulin-stimulated glucose transport. IL-6 also increased skeletal muscle glucose incorporation into glycogen, and glucose oxidation (1.5 fold and 1.3 fold, respectively, $P < 0.05$). IL-6 increased phosphorylation of STAT3 ($P < 0.05$), AMPK ($P = 0.063$), and p38 MAPK ($P < 0.05$), and reduced phosphorylation of S6 ribosomal protein ($P < 0.05$). In contrast, phosphorylation of PKB/Akt, AS160, GSK3 α as well as IRS1-associated PI 3-kinase activity was unaltered.

Conclusion: Acute IL-6 exposure increases glucose metabolism in resting human skeletal muscle. Insulin-stimulated glucose transport and insulin signaling were unchanged following IL-6 exposure.

Introduction

Obesity is associated with an inflammatory state of adipose tissue. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 have been suggested to contribute to the manifestation of whole body insulin resistance (1). While several lines of evidence implicate TNF- α as a contributing factor in the manifestation of peripheral insulin resistance (2), the role of IL-6 has been challenged (3, 4).

Adipocytes, monocytes/macrophages, fibroblasts, and vascular endothelial cells are important sources of IL-6 production, with adipocytes secreting up to 35% of total circulating levels (5, 6). In type 2 diabetic and non-diabetic obese humans, adipose tissue IL-6 content correlates with impaired insulin-mediated glucose uptake (5, 7). Moreover, plasma IL-6 is inversely related to insulin sensitivity, in healthy subjects (8). However, in people with type 2 diabetes, the circulating IL-6 concentration is correlated with adipose tissue mass, rather than whole body insulin sensitivity (9), suggesting that IL-6 may be marker of obesity without any direct contribution to the development of insulin resistance. IL-6 has also been proposed to enhance glucose uptake and metabolism in skeletal muscle after exercise. During exercise, plasma IL-6 levels increase in an intensity and duration dependent manner (10, 11). The elevation of plasma IL-6 during exercise is contributed mainly by the contracting skeletal muscle, such that during exercise, skeletal muscle may become the predominant source of IL-6 (10). Thus, IL-6 has been proposed to enhance glucose uptake and metabolism in skeletal muscle after exercise (12). Skeletal muscle plays a paramount role in the

regulation of insulin-mediated glucose uptake. Consequently, elevations in plasma IL-6 may directly target skeletal muscle and regulate glucose metabolism.

IL-6 has been reported to have positive and negative actions on metabolic responses in liver, adipose tissue and skeletal muscle. In cultured hepatocytes IL-6 directly impairs insulin action (13-16). IL-6 impairs differentiation of pre-adipocytes and reduces insulin action in mature adipocytes (17, 18). In contrast, in cultured skeletal muscle cells, insulin action following IL-6 exposure is enhanced (19, 20). *In vivo* infusions of IL-6 in rodent models have also yielded divergent results. Some reports provide evidence that IL-6 infusion in mice reduces insulin action on hepatic glucose output and skeletal muscle glucose uptake (21), while others report no effect of IL-6 on whole body glucose disposal (22). Furthermore, IL-6-deficient (IL-6^{-/-}) mice develop obesity at 6–7 months of age, accompanied by disturbances in lipid and carbohydrate metabolism (23).

We have recently reported that IL-6 directly enhances glucose uptake and metabolism and enhances insulin sensitivity in cultured myotubes prepared from human skeletal muscle biopsies (20). Signaling pathways via 5'-AMP-activated protein kinase (AMPK) (19) and phosphatidylinositol (PI) 3-kinase (20) have been implicated in the effects of IL-6 on glucose metabolism in cultured cells. However, the direct effect of IL-6 on glucose uptake and metabolism in intact human skeletal muscle is unknown. This is a relevant question to resolve, given the clinical and experimental evidence linking IL-6 with the metabolic regulation of glucose homeostasis. Thus, we determined the direct effect of IL-6 on signal transduction and glucose

metabolism in human skeletal muscle *in vitro*. In addition we assessed whether IL-6 exposure modified insulin action on signal transduction and glucose metabolism.

Materials and Methods

Subjects

The study protocol was approved by the ethical committee of Karolinska Institutet. Informed consent was received from all subjects before participation. The clinical characteristics of the healthy male volunteers (n=22) are presented in Table 1. Glucose, insulin and HbA1c values were within normal range, and serum aminotransferases were not elevated. None of the subjects were smokers or reported taking any medication. The subjects were asked to refrain from strenuous exercise for 48 h prior to the study and to report to the laboratory after an overnight fast.

Muscle biopsy procedure

Skeletal muscle (~1 g) was obtained by means of an open biopsy. Biopsies were taken under local anesthesia (mepivakain chloride 5 mg/ml) from the *vastus lateralis* portion of the *quadriceps femoris* (24). Muscle specimens (10 mg) were dissected from the biopsy material, mounted on Plexiglas clips (9 mm in width), and incubated for 30 min in individual flasks containing oxygenated (95 % O₂, 5 % CO₂) Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mmol/l glucose, 15 mmol/l mannitol and 0.1 % bovine serum albumin (BSA).

Glucose transport

Skeletal muscle was incubated in KHB supplemented with 5 mmol/l glucose, 15 mmol/l mannitol and 0.1 % BSA. Muscles were pre-incubated for 30 min in the absence or presence of 120 ng/ml IL-6

(human recombinant, Roche). The IL-6 concentration was maintained throughout all subsequent incubation steps. Thereafter, muscles were incubated in KHB supplemented with 18 mmol/l mannitol, 2 mmol/l pyruvate, 0.1 % BSA and incubated for 30 min in the absence or presence of 0.36 nM or 60 nM insulin (Insulin Actrapid, Novo Nordisk). The concentration of insulin was maintained throughout all subsequent incubation steps. Subsequently, skeletal muscle strips were incubated for 20 min in KHB containing 5 mmol/l 3-O-methyl-[³H]glucose (800 μCi/mmol) and 15 mmol/l [¹⁴C]mannitol (53 μCi/mmol). At the end of the incubation protocol, skeletal muscle specimens were blotted of excess fluid, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. Glucose transport was determined by the accumulation of intracellular 3-O-methyl-[³H]glucose. Muscle lysate was stored at -80 °C for subsequent signal transduction analysis

Glucose incorporation into glycogen and oxidation

Muscles were incubated in sealed vials containing 2 ml of oxygenated (95 % O₂, 5 % CO₂) KHB supplemented with 15 mmol/l mannitol, 0.1 % BSA, 5 mM [U-¹⁴C]glucose (0.3 μCi/ml). Muscles were incubated in the absence (basal) or presence of either 120 ng/ml IL-6 or 120 nM insulin. Oxygen supply was stopped after the first 15 min of the incubation period. After 1 h, vials were placed on ice for 3 min. Muscles were quickly removed and frozen between tongs cooled to the temperature of liquid nitrogen. Glucose oxidation measurements were performed as described (25). Vials were quickly resealed and a center well containing 200 μl Protosol (PerkinElmer Life Sciences) was introduced. The media was acidified

with 0.5 ml of 15 % perchloric acid and released CO₂ was collected for 1 h. Thereafter, center wells were removed and [¹⁴C] was determined. Glucose incorporation into glycogen was determined as described (26). Muscles were weighed and dissolved in 0.5 ml 1 N sodium hydroxide (NaOH) for 30 min. Subsequently, 0.5 ml of 20 % trichloric acid was added, and samples were mixed and subjected to centrifugation (3500 g at 10 °C for 15 min). The supernatant was transferred to a new vial. Thereafter, 200 µl of a glycogen solution (20 mg/ml) and 2 ml of 95 % ethanol were sequentially added and the mixture was incubated at -20 °C for 1 h. After centrifugation (15 min at 2000 g), the resulting pellet was dissolved in water.

Muscle incubation protocol for signal transduction analysis

To establish a time-course for the effects of IL-6 on signal transduction, skeletal muscle was incubated in KHB supplemented with 5 mmol/l glucose, 15 mmol/l mannitol and 0.1 % BSA. Muscle specimens were incubated for 15 or 30 min in the absence or presence of IL-6. At the end of the incubation period, muscle specimens were blotted of excess fluid, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. Furthermore, skeletal muscle samples from the glucose transport incubation were also analyzed, and thus a time course of 15, 30 and 80 minutes IL-6 exposure was determined.

Western Blot Analysis

Total proteins extracted from the muscle strips that were incubated for signal transduction analysis, as well as for the determination of glucose transport, were subjected to Western blot analysis. Muscle specimens were homogenized in

0.3 ml of ice-cold lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 10 mM NaF, 1 mM MgCl₂, 1 mM Na₃VO₄, 0.2 mM phenylmethylsulfonyl fluoride, 10 % glycerol, 1 % Triton X 100, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 nM microcystin using a glass-on-glass system with motor pestle. Homogenates were solubilized by end-over-end mixing at 4 °C for 60 min and subjected to centrifugation (4 °C at 12000 g for 10 min). The supernatant was stored at -80 °C until use. Total protein was determined using a commercially available kit (Pierce, Rockford, IL). Equal amounts of protein (40 µg) were diluted in Laemmli sample buffer. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed using the following phospho-specific antibodies against STAT3 (Y705) (Upstate), AMPK (Thr172) (Cell Signaling) GSK3 alpha/beta (Cell Signaling), PKB/Akt (Ser473) (New England Biolabs), PKB/Akt (Thr308) (Cell Signaling), p38 MAPK (Thr180/Tyr182) (Cell Signaling), PAS (Cell Signaling), S6 ribosomal protein (Ser235/236) (Cell Signaling). Equal loading was ensured using a NADH ubiquinol oxidoreductase (NUO) protein specific antibody (Molecular Probes, Eugene, OR). Proteins were visualized by chemiluminescence and quantified by densitometry.

PI 3-kinase activity

An aliquot of the supernatant fraction (800 µg of protein) was immunoprecipitated overnight (4 °C) with anti-IRS1 antibody coupled to protein-A-sepharose. IRS-1 associated PI 3-kinase activity was determined as described previously (27). The reaction products

were separated by thin layer chromatography (Silica Gel 60; Merck, Darmstadt, Germany). The radioactivity incorporated into phosphatidylinositol was quantified by a phospho imager (Image Reader BAS-1800 II; Fujifilm, Düsseldorf, Germany) and the results were normalized to a standard consisting of insulin-stimulated mouse liver.

Restriction fragment-length polymorphism IL-6 gene analysis.

Determination of IL-6 polymorphism at -174 was determined as described (28). The promoter region surrounding -174 was amplified by polymerase chain reaction using primers as described (28). The reaction was carried out in a final volume of 50 μ l containing 1.5 mmol/l MgCl₂, 0.2 mmol/l of each dNTP, 0.2 mmol/l of sense and anti-sense primer, and 2.5 U Taq polymerase. DNA was amplified by an initial denaturation of 10 min at 94 °C, 35 cycles consisting of 1 min denaturation at 94 °C, 1 min and 35 s annealing at 55 °C, and 1 min extension at 72 °C, and a final extension of 10 min at 72 °C. Resultant PCR products were digested with SfaNI restriction enzyme at 37 °C overnight and separated on a 2 % agarose gel. SfaNI restriction fragment-length polymorphism (RFLP) was detected by ethidium bromide staining. Genotypes were classified according to the presence or absence of the enzyme restriction sites; SfaNI. G/G, G/C, and C/C are homozygous for the presence of the site (140/58 bp), heterozygotes for the presence and absence of the site (198/140/58 bp), and homozygous for the absence of the site (198 bp), respectively.

Statistics

Data are presented as mean \pm SE. Differences between groups to determine the effect of IL-6 on insulin-stimulated

signaling and metabolism were detected by ANOVA, followed by Fisher's least significant post hoc analysis. A paired t-test was applied when testing the direct effect of IL-6 on signaling and metabolism. Statistical significance was accepted for P<0.05. As expected, the effect of insulin alone was significant for glucose transport, glucose incorporation into glycogen, glucose oxidation and PI 3-kinase activity. Insulin alone also increased the phosphorylation of components downstream of the insulin receptor (PKB/Akt, GSK3 alpha/beta, AS160, S6 ribosomal protein). As the effect of insulin was not the scope of this study, only the effects of IL-6 on subsequent signaling are reported.

Results

Subject characteristics

The metabolic characteristics of the subjects are presented in Table 1. All subjects were healthy and had no known first degree relative with type 2 diabetes. Genotyping was performed on 10 subjects for *IL-6* gene promoter polymorphism at position -174. One third of subjects were heterozygous for GC and the remainder homozygous for GG. None of the subjects were homozygous for the rarer CC allele (the CC allele has been linked to lower *IL-6* transcription) (29).

Glucose transport

IL-6 has a direct effect on glucose metabolism in primary cultures established from human skeletal muscle satellite cells (20; 30). We determined the effects of *IL-6* on glucose uptake and metabolism in intact human skeletal muscle. Muscle strips were incubated in the absence or presence of 120 ng/ml *IL-6* and 3-O-methyl-glucose transport was assessed. *IL-6* increased skeletal muscle glucose transport 1.3 fold (Fig. 1, P<0.05).

