Evidence against a role for insulin-signaling proteins PI 3-kinase and Akt in insulin resistance in human skeletal muscle induced by short-term GH infusion

Niels Jessen,1 Christian B. Djurhuus,1 Jens O. L. Jørgensen,1 Lasse S. Jensen,1 Niels Møller,1 Sten Lund,1 and Ole Schmitz1,2

1Medical Research Laboratory and Medical Department M (Endocrinology and Diabetes), University Hospital of Aarhus, and 2Department of Clinical Pharmacology, University of Aarhus, Aarhus, Denmark

Submitted 31 March 2004; accepted in final form 24 August 2004

GH on insulin-stimulated glucose metabolism and insulin signaling in human skeletal muscle. In a cross-over design, eight healthy male subjects (age 26.0 ± 0.8 yr and body mass index 24.1 ± 0.5 kg/m²) were infused for 360 min with either GH (Norditropin, 45 ng·kg⁻¹·min⁻¹) or saline. During the final 180 min of the infusion, a hyperinsulinemic euglycemic clamp was performed (insulin infusion rate: 1.2 mU·kg⁻¹·min⁻¹). Muscle biopsies from vastus lateralis were taken before GH/saline administration and after 60 min of hyperinsulinemia. GLUT4 content and insulin signaling, as assessed by insulin receptor substrate (IRS)-1-associated phosphatidylinositol 3-kinase activity already at IRS-1 phosphorylation, have shown that high levels of FFAs effectively inhibit the insulin-signaling cascade or reduction in total GLUT4. Yet unknown mechanisms behind this impaired insulin action are still unknown, but more recent studies have shown that binding of insulin to its receptor leads to autophosphorylation of the intracellular domain of the receptor. It binds to the insulin receptor substrate (IRS), which subsequently is tyrosine phosphorylated. Phosphorylated IRS binds and activates the lipid kinase phosphatidylinositol (PI) 3-kinase, and inhibition of this step with the specific inhibitor wortmannin abrogates GLUT4 translocation and insulin-stimulated glucose uptake (10). The steps downstream of the PI 3-kinase are shown in many studies to involve the serine/threonine kinase Akt/PKB (1), but the link that leads to GLUT4 translocation is not identified. Interestingly, after binding to the GH receptor, GH has been shown to phosphorylate IRS-1 through activation of the Janus kinase JAK2 in skeletal muscle from fasted (36), but not fed, rats (9). The physiological relevance of this cross talk between GH and insulin signaling remains unclear.

After 2–4 h of GH stimulation, an anti-insulin-like action of GH is observed, resulting in inhibited glucose utilization (13). The impaired glucose tolerance is mainly due to reduced glucose uptake in skeletal muscle (4, 20, 26), the predominant tissue for insulin-stimulated glucose disposal (14). In contrast, glucose uptake in heart muscle is unchanged (6). The mechanisms behind this impaired insulin action are still unknown, but infusion of GH to achieve physiological circulating concentrations has been shown to increase levels of free fatty acids (FFAs) (28), and this has been proposed as one possible mechanism (27). FFAs were originally proposed to induce insulin resistance by substrate competition (30), but more recent studies have shown that high levels of FFAs effectively inhibit the insulin-signaling cascade activity already at IRS-1 phosphorylation and IRS-1-associated PI 3-kinase activity (16, 18).

Animal studies of long-term GH stimulation using either GH treatment in rats (34, 35) or transgenic animals overexpressing bovine GH (15) have shown inhibition of the insulin-signaling cascade in skeletal muscles that mimics the changes induced by elevated FFAs. Because FFA levels were not reported, it is not clear whether this inhibition is due to a direct effect of GH
stimulation or indirectly through GH-mediated lipolysis and elevated FFAs.

The present study was undertaken to gain further insight into the mechanisms behind the GH-induced insulin resistance by assessing the impact of short-term GH infusion on IRS-1-associated PI 3-kinase and Akt activity in skeletal muscle during concomitant insulin-induced suppression of FFA levels.

SUBJECTS AND METHODS

Subjects. Eight healthy male volunteers participated in the study. Their age was 26.0 ± 0.8 yr (means ± SE), and body mass index was 24.1 ± 0.5 kg/m². None of them had a family history of diabetes, and none were receiving any form of medication. The Scientific Ethics Committee of Aarhus County approved the study protocol, and all study participants gave informed consent according to the second declaration of Helsinki.

Design. Each participant underwent two studies in random order with and without GH infusion ~4 wk apart. Intense physical exertion was avoided for 24 h before the examinations. The studies commenced at 8:00 AM after a 10-h overnight fast and were conducted in the supine position. A Venflon catheter was inserted in an antecubital vein for administration of the infusates. For blood sampling, a wrist vein of the contralateral hand was cannulated and kept in a heating box to provide arterialized blood. Before infusions, a “baseline” muscle biopsy was taken from vastus lateralis muscle with a Bergström biopsy needle under local anesthesia (1% lidocaine), a small incision having been made through the skin and muscle sheath 15–20 cm above the knee. A total amount of ~200 mg of muscle was aspirated; biopsies were cleaned for blood (within 15 s) and snap-frozen in liquid nitrogen. Muscle biopsies were stored at −80°C until analyzed. After baseline blood sampling at time 0, either saline or GH (Norditropin; Novo Nordisk, Gentofte, Denmark) was infused. GH was infused at a rate of 45 ng.kg⁻¹.min⁻¹ for 330 min. At 180 min, a hyperinsulinaemic (insulin infusion rate: 1.2 mU.kg⁻¹.min⁻¹) euglycaemic (plasma glucose ~5 mmol/l) clamp was commenced and continued for the next 150 min. At 240 min, a second muscle biopsy was obtained from the thigh at a distance of ~5 cm from the first incision. The glucose infusion rate (GIR) during this procedure was considered an estimate of insulin-stimulated glucose uptake, as the endogenous glucose release at this degree of hyperinsulinaemia is presumed to be very close to zero.

Analytic methods. All samples were analyzed in duplicate. Plasma glucose was measured immediately by a glucose analyzer (Beckman Instruments, Palo Alto, CA). Serum insulin concentrations were measured in duplicate by a two-site immunospecific insulin enzyme-linked immunosorbent assay (2). Serum FFA concentrations were determined by radioimmunoassays (DELFIA; Wallac Oy, Turku, Finland). Plasma glucagon concentrations were determined by radioimmunoassays as described by Ørskov et al. (29) with modifications.

Muscle preparations for insulin-signaling assays. Muscles were homogenized as described by Wojtaszewski et al. (38). In brief, frozen muscles biopsies (~80 mg) were homogenized in ice-cold solubilization buffer (50 mM HEPEs, 137 mM NaCl, 10 mM NaPO₄, 10 mM NaF, 1 mM MgCl₂, 1 mM CaCl₂, 1% NP-40, 10% glycerol, 2 mM Na₂VO₃, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 10 μg/ml anti-pain, 1.5 mg/ml benzamidine, and 100 μmol/l AEBSF [4-[(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride], pH 7.4] and rotated for 1 h at 4°C. Insoluble materials were removed by centrifugation at 16,000 g for 60 min at 4°C, and protein content on the supernatant was determined with a bichinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL).

PI 3-kinase assay. PI 3-kinase activity was assessed as previously described (38), with minor modifications. Briefly, aliquots of protein were immunoprecipitated overnight with protein A-agarose-coupled anti-IRS-1 antibody (Upstate Biotechnology, Lake Placid, NY). The immune complexes were washed thoroughly, and IRS-1-associated PI 3-kinase activity was assessed directly on the protein A-agarose complex in a buffer containing 10 mM Tris·HCl, 1 mM EDTA, 1 mM MgCl₂, 75 μM ATP, 50 mM NaCl, and 6 μCi [γ-³²P]ATP (NEN, Boston, MA). Reaction products were resolved by thin-layer chromatography and were quantified using a phosphorimage (Packard BioScience, Meriden, CT).

Akt/PKB protein expression. Aliquots of protein were resolved by SDS-PAGE using the Bio-Rad Mini Protein II system (10% polyacrylamide gels), transferred to nitrocellulose, blocked with 5% nonfat milk in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) and incubated with anti-PKB/Akt antibody (New England BioLabs, Beverly, MA). The membranes were then washed and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Pierce Chemical) as secondary antibody, and proteins were visualized by BioWest enhanced chemiluminescence (UVU, Upland, CA) and quantified by UVP BioImaging System.

Akt/PKB activity assay. Aliquots of protein were immunoprecipitated overnight with protein G-agarose-coupled anti-Akt/PKB antibody, which reacts with both the α- and β-subunits (Upstate Biotechnology, Lake Placid, NY). The activity was assessed as described by Sherwood et al. (33), with minor modifications. The complex was washed, and Akt/PKB activity was assessed in buffer containing 50 mM Tris·HCl (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 0.01% PKA inhibitor peptide (Upstate Biotechnology), 0.1 mM PKB-serum- and glucocorticoid-induced kinase (SGK)-specific peptide (Upstate Biotechnology), 3 μCi [γ-³²P]ATP (NEN), and 5 mM ATP at a final volume of 30 μl at 30°C for 30 min. At the end of the reaction, a 20-ml aliquot was removed and spotted on Whatman P81 paper. The papers were washed six times for 20 min each in 1% phosphoric acid and once with acetone, and radioactivity was quantified by scintillation counting (Wallac 1409; Wallac).

Total muscle GLUT4 content. Total crude membranes were prepared from ~20 mg of skeletal muscles, and aliquots of protein were resolved as previously described (7) by use of a polyclonal anti-COOH-terminal peptide GLUT4 antibody (23). Proteins were visualized by chemiluminescence (Pierce Super Signal) and quantified with the UVP BioImaging System.

Statistics. All data are presented as means ± SE. Statistical analysis was performed using SPSS for Windows (v. 11.0; SPSS, Chicago, IL). Normality of the data was tested with the Kolmogorov-Smirnov test of normal distribution. Where P > 0.20 the data was considered to be normally distributed. Statistical evaluation of differences between normally distributed data was done with a paired t-test. Differences between groups were considered statistically significant if P < 0.05.

RESULTS

Circulating hormones, substrates, and GIRs. Serum GH increased gradually during the GH infusion, eventually reaching a plateau of 20.2 ± 2.3 μg/l during the final 30 min of the exposure compared with 0.8 ± 0.4 μg/l during saline infusion (P < 0.01; Fig. 1). Plasma glucose levels were stable before and during the clamp (5.2 ± 0.07 and 5.1 ± 0.04 mM from 0–180 min and 5.2 ± 0.2 and 5.1 ± 0.2 mM from 180–330 min, GH vs. saline infusion). Average serum insulin was identical in the two conditions before the hyperinsulinemic clamp (43.7 ± 9.3 and 44.7 ± 2.0 μg/l, GH vs. saline). During insulin infusion, circulating insulin rose sharply, ~10-fold, to levels that were comparable in the GH and saline condition. Average serum insulin during the last 30 min of the clamp was 485.9 ± 16.8 and 509.4 ± 16.5 μg/l (GH vs. saline), and at 240 min, i.e., when the second biopsy was taken, serum insulin was...
GH administration, serum FFA increased from 0.53 ± 0.08 mM at 0 min to a peak concentration of 0.98 ± 0.10 mM just before initiation of hyperinsulinemia compared with a peak concentration of 0.51 ± 0.10 mM during saline infusion (P < 0.01). During insulin infusion, serum FFA was markedly suppressed to 0.15 ± 0.01 and 0.05 ± 0.01 mM (GH vs. saline infusion, P < 0.01) at 240 min and was further reduced to 0.10 ± 0.02 and 0.03 ± 0.00 mM (GH vs. saline) during the final 30 min of the clamp period. The latter reduction amounted to 81 and 92%, respectively, of the basal FFA concentrations.

**IRS-1-associated PI 3-kinase activity.** Insulin stimulation caused an approximately threefold rise in IRS-1-associated PI 3-kinase activity (Fig. 2). There was no difference in the insulin-stimulated activities during GH and saline infusion (269 ± 105 and 311 ± 71% compared with baseline, GH vs. saline).

**Akt/PKB activity.** To further investigate any potential involvement of the insulin-signaling cascade, the activity of the IRS-1-associated phosphatidylinositol (PI) 3-kinase activity.

**Akt/PKB activity protein expression.** Expression of Akt/PKB did not change from baseline during either GH or saline infusion (data not shown).

**Akt/PKB activity.** To further investigate any potential involvement of the insulin-signaling cascade, the activity of the IRS-1-associated PI 3-kinase activity.

**Fig. 2.** Insulin receptor substrate (IRS)-1-associated phosphatidylinositol (PI) 3-kinase (top) and Akt/PKB activity (bottom) at baseline (open bars) and under insulin stimulation during GH (filled bars) and saline infusions (gray bars). Values are means ± SE. IRS-1-associated PI 3-kinase activity is expressed in arbitrary units and Akt/PKB activity as pmol incorporated ATP·mg protein$^{-1}$·min$^{-1}$.
downstream kinase Akt/PKB was assessed. Insulin caused a substantial increase in activity (~13-fold) under both GH and saline infusions compared with basal, but no difference was observed in the two conditions (1,309 ± 327 and 1,287 ± 173% compared with baseline, GH vs. saline; Fig. 2).

Muscle GLUT4 protein content. Infusion of GH did not alter total GLUT4 protein content (114.7 ± 173% compared with baseline, GH vs. saline; Fig. 3).

DISCUSSION

The present study shows that hypersomatotropinemia induces insulin resistance even though FFA levels are substantially suppressed, indicating that GH also perturbs insulin-stimulated glucose uptake in a non-FFA-dependent manner. Apparently, the GH-induced insulin resistance was not associated with detectable changes in PI 3-kinase or Akt activity or the total expression of GLUT4.

In this study, the second muscle biopsy was performed after 60 min of hyperinsulinemia because it has been established that insulin-signaling activity at this time point has reached maximal levels (37). It should be emphasized that, already at this time point, insulin sensitivity was considerably reduced during GH exposure. Furthermore, we wanted to minimize the role of GH-induced lipolysis in the signaling process. To that end, we used an insulin infusion rate leading to supraphysiologival levels of circulating insulin. Although FFA levels were slightly higher under GH administration than during saline, serum FFA was suppressed to very low levels during both conditions (~15% of baseline levels).

In an earlier study, utilizing the isotope dilution technique in combination with the hyperinsulinemic clamp in an experimental design in many respects comparable to the present one, we demonstrated that GIRs were almost identical to the isotopically determined insulin-stimulated glucose disposal in healthy people after short-term GH exposure (4). This indicates that the endogeneous glucose release in the present study is restrained to insignificant values and, consequently, that the reduced rates of glucose infusion reflect insulin resistance in peripheral tissues. Furthermore, of note, insulin infusion rates were almost twofold higher in the present study, further minimizing the endogenous glucose release.

Recent studies have demonstrated that FFAs severely blunt insulin signaling already at the levels of IRS-1 phosphorylation and IRS-1-associated PI 3-kinase activity (16, 18). This impairment cannot be overcome by high levels of insulin, but insulin signaling returns to normal when the FFA level is normalized (40). Moreover, studies of skeletal muscle from obese subjects (17) and type 2 diabetic patients (5, 22) have shown reduced activity in the insulin-signaling cascade, indicating that impairment of the insulin-signaling cascade can be at least partly responsible for the reduced glucose uptake in skeletal muscle observed with elevated FFAs and/or type 2 diabetes.

Studies of the effects of short-term GH exposure in humans (4) and rodents (21) have shown insulin resistance combined with a reduced activity in the downstream target of insulin action glycogen synthase. These findings have been supported by animal studies of long-term GH exposure where a reduced activity in the proximal insulin-signaling cascade in skeletal muscle was found. The impairment was observed at the IRS-1 level (34) and on the downstream target PI 3-kinase (15, 35). These alterations are very similar to what has been observed after FFA exposure, and, as none of the animal studies focusing on the upstream insulin-signaling cascade include measurements of FFAs, the isolated role of GH on proximal insulin signaling in skeletal muscles cannot be evaluated.

The direct effect of GH on IRS-1 phosphorylation in skeletal muscle has been observed in fasted (36) but not fed rats (9), whereas insulin induces phosphorylation in both situations. This indicates that the phosphorylation of IRS-1 induced by the relatively low levels of insulin in the fed rat obliterates the effects of GH stimulation. Similarly, GH has been shown to induce Akt/PKB phosphorylation in vitro in 3T3-L1 adipocytes (39) when incubated with GH at concentrations ~25 times higher than the plasma concentrations reached in this study. Given these observations, it cannot be completely excluded that the insulin-signaling phosphorylation was slightly elevated at the initiation of the clamp. However, it has been established that the muscle biopsy procedure per se induces insulin resistance (19); consequently, to include an extra muscle biopsy immediately before the hyperinsulinemic clamp may have a destructive effect on interpretation of the data.

In the present study, insulin stimulation resulted in a three-fold rise in IRS-1-associated PI 3-kinase activity during both GH and saline infusions; thus no effect of GH infusion was noted. To further investigate the insulin-signaling cascade, insulin-stimulated Akt/PKB activity and protein expression were assessed. Insulin promotes glycogen synthase by inhibiting glycogen synthase kinase-3 through activation of Akt/PKB (12). We have previously shown that GH infusion inhibits glycogen synthase activity (4). In the present study, we failed to demonstrate any difference in insulin-stimulated Akt/PKB expression or activity or that the reduced glycogen synthase activity may be due to reduced glucose availability in the cell. The finding of normal activity in the insulin-signaling cascade, despite significantly reduced insulin sensitivity, emphasizes that GH and FFAs might inhibit insulin-stimulated glucose transport in distinct manners, and this finding is in line with a previous study that shows that GH infusion imposes insulin resistance before any changes in lipolysis are observed (24). Clearly, we cannot rule out that chronic elevation of GH, as

![Figure 3](http://ajpendo.physiology.org/)

Fig. 3. Total crude membrane GLUT4 content at baseline (open bars) and under insulin stimulation during GH (filled bar) and saline infusions (gray bar). Values are means ± SE and are expressed in arbitrary units.
present in acromegaly, may affect the insulin-signaling cascade in a different manner.

During physiological conditions, translocation of the insulin-sensitive GLUT4 to the cell surface has been shown to be the limiting factor for insulin-stimulated glucose transport (11). Total GLUT4 content is not affected in insulin-resistant states like type 2 diabetes mellitus (3), and animals receiving long-term GH treatment show normal GLUT4 expression (8). This is consistent with the present data that demonstrate that short-term GH infusion does not affect expression of GLUT4 in human skeletal muscles.

In conclusion, the present study shows that GH infusion is able to induce profound insulin resistance in the presence of suppressed circulating FFA levels. The underlying molecular mechanisms do not appear to involve impairment of IRS-1-associated PI 3-kinase or PKB/Akt activity or changes in total insulin-sensitive GLUT4 to the cell surface has been shown to be able to induce profound insulin resistance in the presence of suppressed circulating FFA levels. The underlying molecular mechanisms do not appear to involve impairment of IRS-1-associated phosphatidylinositol 3-kinase activity.

**ACKNOWLEDGMENTS**

E. Carstensen, E. Hornemann, A. Mengel, and H. Petersen are thanked for excellent technical assistance. L. Goodyear, Joslin Diabetes Center, Harvard Medical School, Boston, MA, is thanked for stimulating discussions.

N. Jensen is currently a fellow at the Joslin Diabetes Center, Boston, MA.

**GRANTS**

This study was supported by the Novo Nordisk Foundation and the Institute of Experimental Clinical Research, University of Aarhus, Denmark.

**REFERENCES**


E198 EFFECTS OF GH ON HUMAN SKELETAL MUSCLE


