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Research Article

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Insulin Receptor Phosphorylation, Insulin Receptor Substrate-1 Phosphorylation, and Phosphatidylinositol 3-Kinase Activity Are Decreased in Intact Skeletal Muscle Strips from Obese Subjects

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Abstract

To determine whether the impaired insulin-stimulated glucose uptake in obese individuals is associated with altered insulin receptor signaling, we measured both glucose uptake and early steps in the insulin action pathway in intact strips of human skeletal muscle. Biopsies of rectus abdominus muscle were taken from eight obese and eight control subjects undergoing elective surgery (body mass index 52.9 ± 3.6 vs 25.7 ± 0.9). Insulin-stimulated 2-deoxyglucose uptake was 53% lower in muscle strips from obese subjects. Additional muscle strips were incubated in the basal state or with 10^{-7} M insulin for 2, 15, or 30 min. In the lean subjects, tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), measured by immunoblotting with anti-phosphotyrosine antibodies, was significantly increased by insulin at all time points. In the skeletal muscle from the obese subjects, insulin was less effective in stimulating tyrosine phosphorylation (maximum receptor and IRS-1 phosphorylation decreased by 35 and 38%, respectively). Insulin stimulation of IRS-1 immunoprecipitable phosphatidylinositol 3-kinase (PI 3-kinase) activity also was markedly lower in obese subjects compared with controls (10- vs 35-fold above basal, respectively). In addition, the obese subjects had a lower abundance of the insulin receptor, IRS-1, and the p85 subunit of PI 3-kinase (55, 54, and 64% of non-obese, respectively). We conclude that impaired insulin-stimulated glucose uptake in skeletal muscle from severely obese subjects is accompanied by a deficiency in insulin receptor signaling, which may contribute to decreased insulin action. (*J. Clin. Invest.* 1995. 95:2195–2204.) Key words: insulin action • insulin resistance • obesity • insulin receptor substrate • tyrosine phosphorylation

Introduction

A significant metabolic abnormality in obesity is the diminished ability of insulin-sensitive tissues to take up and metabolize

glucose (1–3). Skeletal muscle is the primary site of insulin-stimulated glucose disposal and has been suggested to be the major tissue responsible for postprandial hyperglycemia in insulin-resistant subjects (4, 5). Although the defect in insulin-stimulated glucose disposal has been well described in human skeletal muscle, the underlying molecular mechanisms for abnormal insulin responsiveness in this tissue are not clear. It is likely that the impaired insulin action associated with obesity stems from alterations in the expression or function of one or more of the cellular proteins that are components of the insulin receptor signaling pathway, the glucose transport system, or both. In the current investigation we have developed a novel method to study the proximal steps of insulin receptor signaling in intact strips of human skeletal muscle. We have used this method to determine whether human obesity is associated with an alteration in tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1)¹ and in phosphatidylinositol 3-kinase (PI 3-kinase) activity.

Insulin action is initiated through hormone binding to cell surface insulin receptors, which triggers a cascade of intracellular phosphorylation events. Insulin binding to the extracellular α subunit of the receptor results in autophosphorylation of tyrosine residues in the receptor β subunit and activation of a tyrosine kinase intrinsic to the β subunit (6). The stimulation of receptor kinase activity induces the phosphorylation of nonreceptor proteins (7, 8), including IRS-1 (9). Phosphorylation of IRS-1 by the insulin receptor kinase can occur at TyrXXMet (YXXM) and TyrMetXMet (YMXM) motifs (10), which then bind with high affinity to *src*-homology 2 domains of cellular proteins, including the 85-kD regulatory subunit of PI 3-kinase (11). PI 3-kinase phosphorylates cellular phosphoinositides at the D-3 position, resulting in the formation of phosphatidylinositol-3-monophosphate, phosphatidylinositol-3,4-bisphosphate, and phosphatidylinositol-3,4,5-trisphosphate (12). An increase in PI 3-kinase activity occurs after activation of several growth factor receptors (13, 14) and has been implicated in the regulation of multiple cellular functions (15, 16). In insulin-sensitive tissues, such as isolated rat adipocytes (17) and intact rat skeletal muscle (18, 19), insulin dramatically increases PI 3-kinase activity in IRS-1 and tyrosine phosphoprotein immunoprecipitates, resulting in an increase in D-3 phosphoinositides (19, 20). The precise function of PI 3-kinase activation and generation of the D-3 phosphoinositides by insulin is not clear, but recent

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1. *Abbreviations used in this paper:* BMI, body mass index; IRS-1, insulin receptor substrate-1; NIDDM, non-insulin-dependent diabetes mellitus; PI 3-kinase, phosphatidylinositol 3-kinase; PTPase, protein tyrosine phosphatase.

studies in cultured cells suggest that enzyme activity may be necessary for insulin-stimulated translocation of glucose transporters (21–23).

Insulin receptor preparations from the skeletal muscle of human subjects have been used to study receptor kinase activity *in vitro* (24, 25). In patients with non-insulin-dependent diabetes mellitus (NIDDM) and obesity, these previous studies have shown variable changes in insulin receptor autophosphorylation, which has been alternatively described as impaired (24) or unchanged (25). In addition, assays of kinase activity toward added (exogenous) substrates reveal consistently decreased activity in these subjects (24, 25). Although these studies have described receptor function using isolated receptor preparations, the effects of insulin on receptor phosphorylation, IRS-1 phosphorylation, and PI 3-kinase activity in intact human skeletal muscle preparations are not known. In rat skeletal muscle recent studies have assessed insulin-induced tyrosine phosphorylation of muscle proteins *in vivo* (26–28). These methods are advantageous in that the manipulation of intact tissue rather than partially purified receptor preparations minimizes procedurally induced changes in receptor function and allows for the measurement of endogenous rather than artificial substrate phosphorylation. In animal models of diabetes and insulin resistance, significant differences between experiments performed *in vivo* and *in vitro* have become evident. For example, isolated receptors from streptozotocin-diabetic skeletal muscle demonstrate decreased exogenous substrate kinase activity with histone H₂b and angiotensin II as phosphoacceptors (29), whereas streptozotocin-diabetes results in increased tyrosine phosphorylation of IRS-1 in intact muscle tissue (26, 27). In addition, whereas insulin receptors from the skeletal muscle of hypercortisolemic rats show normal receptor autophosphorylation and kinase activity *in vitro* (30), this condition results in decreased receptor tyrosine phosphorylation *in vivo* (28). Thus, it is crucial to our understanding of insulin action in human tissues to study the insulin signaling pathway in intact skeletal muscle preparations.

In the current study, we have used a new method to measure both glucose uptake and proximal steps in insulin signaling in the same intact human skeletal muscle preparations. This method has been used to measure tyrosine phosphorylation of the insulin receptor and IRS-1, PI 3-kinase activity, and expression of the insulin receptor, IRS-1, and the p85 subunit of PI 3-kinase in intact skeletal muscle of obese individuals. Our results demonstrate that impaired insulin-stimulated glucose uptake in the skeletal muscle of obese patients is associated with decreased activation of the molecules involved in the initial steps of the insulin signaling pathway.

Methods

Materials. Protein A-Sepharose was from Pierce Chemical Co. (Rockford, IL). Reagents for SDS-PAGE and protein assay kits were from Bio Rad Laboratories (Richmond, CA). ¹²⁵I-labeled protein A was purchased from ICN Biomedical (Costa Mesa, CA). 2-[1,2-³H(*n*)]-deoxy-D-glucose and [γ -³²P]ATP were from New England Nuclear (Boston, MA). Phosphatidylinositol was from Avanti Polar Lipids Inc. (Alabaster, AL). Anti-PI 3-kinase was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Human subjects. The surgical protocol was approved by the East Carolina University Policy and Review Committee on Human Research. Informed consent was obtained from all subjects after a thorough expla-

nation of the experimental protocol. Control muscle was obtained from non-obese subjects undergoing hysterectomy. The obese group consisted of severely obese subjects undergoing gastric bypass surgery for treatment of obesity. All subjects were female.

Muscle strip preparation. The method for obtaining the muscle specimens has been described in detail (2). After an overnight fast, the subjects had general anesthesia induced with a short acting barbiturate and maintained with phentanyl and a nitrous oxide-oxygen mixture. Only saline was given intravenously before the muscle biopsy. The abdomen was opened, and ~3 × 2 × 2-cm biopsy from the rectus abdominus muscle was clamped, excised, and placed in oxygenated Krebs-Henseleit buffer. The specimen was rapidly transported to the laboratory, where 18 muscle strips weighing 30–40 mg each were teased from the mounted sample. The remaining muscle tissue was frozen in liquid nitrogen.

Glucose transport measurements and muscle incubation and processing. To measure basal and insulin-stimulated glucose transport, muscle strips were incubated in triplicate in Krebs-Henseleit buffer in the absence or presence of 10⁻⁷ M insulin as previously described (2). Briefly, the muscle strips were preincubated for 30 min in Krebs-Henseleit buffer containing 1% BSA and 1.0 mM pyruvate. The strips were then transferred to medium containing 5 mM 2-deoxyglucose, 20 mM sorbitol, 0.4 μ Ci/mmol [³H]2-deoxyglucose, and 0.005 μ Ci/mmol [¹⁴C]sorbitol for 10 min. Both incubations were done at 37°C with the media continuously gassed with 95% O₂, 5% CO₂. After washing twice by incubating for 5 min in ice-cold Krebs-Henseleit buffer, the samples were blotted, weighed, digested, and counted by liquid scintillation for ³H and ¹⁴C radioactivity.

For studies of insulin signaling, additional strips from the same biopsied muscle specimen were preincubated for 30 min in Krebs-Henseleit buffer containing 1% BSA and 1.0 mM pyruvate. For the insulin-treated muscle, the strips were then transferred to medium containing insulin (10⁻⁷ M) and incubated for 2, 4, 15, or 30 min. For basal treatment, the muscle strips were incubated in the absence of insulin for the corresponding times (2–30 min). Three strips were incubated for each condition/time point. After incubation, the muscle strips were rapidly frozen in liquid nitrogen. The three strips from each treatment were pooled and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in ice-cold buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM NaPP, 2 mM Na₃VO₄, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 2 mM EDTA, 2 mM PMSF, 5 μ g/ml leupeptin, 1% NP-40, 10% glycerol). The homogenate was incubated for 45 min at 4°C with gentle mixing and then centrifuged at 35,000 *g* for 60 min. The supernatant was collected and assayed for protein concentration using the Bradford dye binding assay kit (31).

Tyrosine phosphorylation of the insulin receptor and IRS-1. For the study of tyrosine phosphoproteins, equal amounts of solubilized proteins (300 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose membranes using a semidry transfer apparatus (Owl Scientific, Cambridge, MA). To reduce nonspecific antibody binding, the nitrocellulose membranes were blocked with 5% albumin for 2 h at 37°C in a Tris-NaCl buffer (pH 7.8) containing 0.01% sodium azide (TNA). The membranes were then incubated with affinity-purified anti-phosphotyrosine antibody (α PY) (26) in TNA plus 5% BSA for 12–16 h at 4°C. The membranes were subjected to two 5-min washes in TNA plus 0.05% NP-40 and one 5-min wash in TNA plus 0.1% Tween 20. Bound antibodies were detected by incubation with ¹²⁵I-labeled protein A (1 μ Ci/ml) for 1 h at 25°C. The membranes were then washed twice, for 10 min each, in TNA plus 0.05% NP-40 and twice, for 10 min each, in TNA plus 0.1% Tween 20. Quantitation of specific protein bands was determined on a phosphorimager.

PI 3-kinase activity measurements. To determine the effects of obesity on basal and insulin-stimulated PI 3-kinase, enzyme activity was measured in immunoprecipitates obtained with antibodies to IRS-1 (α IRS-1, affinity-purified polyclonal antibody prepared by injecting rabbits with a synthetic peptide corresponding to the last 14 amino acids in the COOH-terminal region of rat IRS-1). PI 3-kinase activity in

Table I. Descriptive Data

	Lean	Obese
n	8	8
Age (yr)	39.3±2.3	40.0±2.0
Race	4AA, 4W	4AA, 4W
BMI (kg/m ²)	25.7±0.9	52.9±3.6*
Waist/hip ratio	ND	0.89±0.2
Glucose (mg/dl)	92.4±4.7	105.4±12.4
Insulin (μU/ml)	4.0±1.0	7.5±2.1

Data are mean±SE. AA, African-American; W, white; ND, not determined. * $P < 0.05$ vs lean group.

reconstituted immunoprecipitates was assayed as previously described (18, 32) with some modifications. A 2-mg aliquot of extracted muscle proteins (see previously described procedure) was immunoprecipitated with α IRS-1. After incubation with protein A-Sepharose, immunocomplexes were collected by centrifugation, washed three times with PBS containing 1% NP-40 and 100 μ M Na₃VO₄, three times with 100 mM Tris-HCl (pH 7.5) containing 500 mM LiCl₂, and two times with 10 mM Tris (pH 7.5) containing 0.1 M NaCl, 1 mM EDTA, and 100 mM Na₃VO₄. The pellet was resuspended in 50 μ l of Tris-NaCl buffer, and 12 mM MgCl₂ and phosphatidylinositol (10 μ g) were added. The PI 3-kinase reaction was performed at room temperature and was started by adding 10 μ l of 440 μ M ATP containing 30 μ Ci of [³²P]ATP. After 10 min of vigorous vortexing, the reaction was stopped by the addition of 20 μ l of 8 N HCl and 160 μ l of CHCl₃/CH₂OH (1:1). The phases were separated by centrifugation, and 50 μ l of the lower organic phase containing the reaction products was spotted on an aluminum-backed silicon TLC plate pretreated with oxalate. The lipids were resolved by chromatography in CH₃OH/CHCl₃/H₂O/NH₄OH (60:47:11.3:2) and visualized by autoradiography. The radioactivity in the spots that comigrated with a PI 3-phosphate standard was quantitated by densitometry.

Insulin receptor, IRS-1, and p85 protein content. To determine the content of the insulin receptor, IRS-1, and p85 subunit of PI 3-kinase, muscle extracts were prepared from muscle samples that were frozen in liquid nitrogen at the time of surgery without incubation. For determination of IRS-1 and p85, ~1 g of the frozen muscle was powdered with a mortar and pestle, homogenized in ice-cold buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM NaPP, 2 mM Na₃VO₄, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 2 mM EDTA, 2 mM PMSF, 5 μ g/ml leupeptin, 1% NP-40, 10% glycerol), and processed as previously described for the α PY studies. To detect insulin receptor protein by immunoblotting, crude membranes were prepared from the untreated frozen muscle specimens. Approximately 0.5 g of muscle was homogenized in ice-cold buffer (250 mM sucrose, 10 mM Tris, pH 8.0, containing 10 mM DTT). After centrifugation at 1,000 g for 15 min to remove large debris and nuclei, the supernatant was centrifuged at 200,000 g for 45 min at 4°C. The pellet was resuspended in the sucrose-Tris buffer, and protein concentration was measured using the Bradford dye binding assay kit (31).

Immunoblotting procedures for the detection of the insulin receptor and p85 proteins were as previously described for immunoblotting with α PY. For insulin receptor immunoblotting, an affinity-purified COOH-terminal anti-peptide antibody was used (α IRct) (26). For detection of the p85 protein, a polyclonal antibody directed against the 85-kD subunit of PI 3-kinase was used (α p85). Immunoblotting of the IRS-1 protein was done using an affinity-purified anti-peptide antibody (α IRS-1C) (33). For IRS-1 immunoblotting, nonspecific antibody binding was reduced by incubating in 5% milk (TNA containing 5% Carnation nonfat powdered milk and 0.2% Tween 20) for 2 h at 25°C. Specific antibody binding was done with α IRS-1C in TNA plus 1% milk and 0.2% Tween



Figure 1. Basal and insulin-stimulated glucose uptake in human skeletal muscle. Rectus abdominus muscle was removed from eight lean and eight obese subjects undergoing abdominal surgery. Multiple muscle strips were teased from the muscle specimen and incubated under basal conditions or in the presence of insulin (10^{-7} M) as described in Methods. Data are mean±SE. * Significantly different from the corresponding basal value ($P < 0.01$); # significantly different from the corresponding lean value ($P < 0.05$).

20. Bound antibodies were detected with ¹²⁵I-labeled protein A as previously described.

Statistical analyses. All data are expressed as mean±SE. Differences between the lean and obese groups were determined using an unpaired Student's t test.

Results

Subject characteristics and glucose uptake measurements. The clinical characteristics of the subjects are shown in Table I. Age and racial makeup were similar in the two groups. The body mass index (BMI) for the control, non-obese group was 25.7 kg/m², whereas that for obese group indicates severe obesity, with BMIs ranging from 41 to 67 kg/m². Glucose and insulin concentrations measured in the fasted state were not statistically different between the lean and obese subjects (Table I).

Glucose uptake was measured in strips of rectus abdominus muscle obtained during surgery. Muscle strips from each subject were incubated in the absence or presence of 10^{-7} M insulin, and glucose uptake was measured using [³H]2-deoxyglucose. Basal rates of glucose uptake in the muscle strips were not different in the lean and obese groups (Fig. 1). In contrast, insulin-stimulated glucose uptake was significantly lower in muscles from the obese subjects as compared with the lean subjects ($P < 0.05$).

Insulin receptor and IRS-1 tyrosine phosphorylation and protein content. Preliminary experiments were done on rectus abdominus muscle strips obtained from three lean subjects. To determine whether insulin stimulates tyrosine phosphorylation of the insulin receptor β subunit and IRS-1 in intact human skeletal muscle, strips were incubated in buffer containing insulin (10^{-7} M) for 2, 4, or 15 min. Figure 2 is a representative autoradiograph from one subject showing that insulin incubation increased tyrosine phosphorylation of two major proteins, one with a molecular mass of ~95 kD and one with a molecular mass of ~170 kD. Subsequent experiments using specific antibodies to the insulin receptor β subunit (α IRct) and IRS-1 (α IRS-1C) demonstrated that the two major insulin-stimulated tyrosine phosphoproteins, pp95 and pp170, are the β subunit of

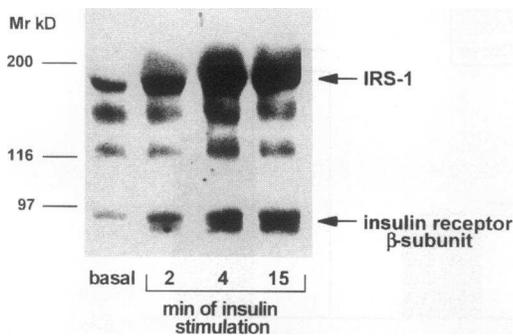


Figure 2. Effect of insulin on insulin receptor and IRS-1 tyrosine phosphorylation in human skeletal muscle. Rectus abdominus muscle was removed from one lean patient undergoing abdominal surgery. Multiple muscle strips were teased from the muscle specimen and incubated under basal conditions or in the presence of insulin (10^{-7} M) for 2, 4, or 15 min. Equal amounts ($300 \mu\text{g}$) of solubilized muscle protein prepared from the muscle strips were resolved by SDS-PAGE and immunoblotted with αPY .

the insulin receptor and IRS-1, respectively (data not shown). Similar to our previous studies in rat skeletal muscle, there was a rapid increase in phosphorylation of the insulin receptor and IRS-1 in the human skeletal muscle (26). However, in contrast to studies in the intact rat, in which IRS-1 phosphorylation was transient (returned to basal levels by 4 min) (26), there was no decrease in tyrosine phosphorylation of IRS-1 in the human muscle strips after 15 min of insulin incubation (Fig. 2). In subsequent experiments, muscle strips were incubated for 2, 15, or 30 min.

To determine whether there was an effect of obesity on tyrosine phosphorylation of the insulin receptor or IRS-1, muscle strips from eight lean and eight obese subjects were studied in pairs. Incubated muscle strips from one lean subject and one obese subject were homogenized and processed in the same experiment, and equal amounts of muscle proteins were loaded and separated on the same gel. Figure 3 is a representative experiment showing a decreased level of tyrosine phosphoproteins in the obese subject and decreased insulin-stimulated tyrosine phosphorylation of both the insulin receptor and IRS-1. Quantitative data for all eight lean and eight obese subjects

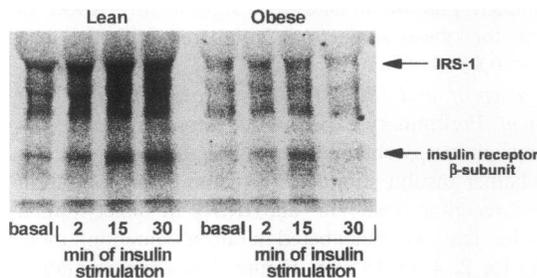


Figure 3. Insulin receptor and IRS-1 tyrosine phosphorylation in skeletal muscle from lean and obese subjects. This image shows tyrosine phosphoproteins from one lean subject and one obese subject. Multiple muscle strips were incubated in the absence or presence of insulin (10^{-7} M) for 2, 15, or 30 min. Equal amounts ($300 \mu\text{g}$) of solubilized muscle protein prepared from the muscle strips were resolved by SDS-PAGE and immunoblotted with αPY .

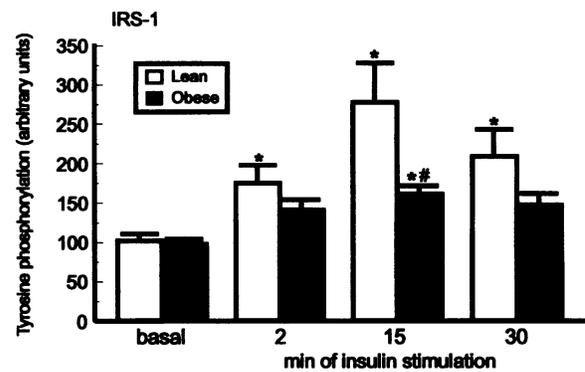
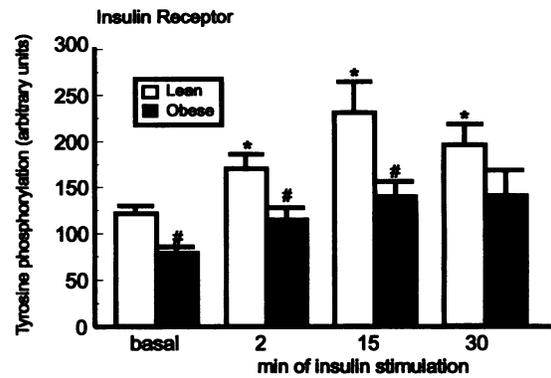


Figure 4. Quantitation of insulin receptor phosphorylation (*top*) and IRS-1 tyrosine phosphorylation (*bottom*) in skeletal muscle from lean and obese subjects. Muscle strips were incubated and processed as described in Fig. 2 and in Methods. Muscle strips from one lean subject and one obese subject were processed in the same experiment and separated on the same gel. For each paired experiment, the densitometric units of each basal sample from the lean and obese subjects were averaged and set to 100%. Values for each sample from that gel were then calculated relative to the 100%. Data are mean \pm SE, $n = 8$ per group. * Significantly different from the corresponding basal value ($P < 0.05$); # significantly different from the corresponding lean value ($P < 0.05$).

are shown in Fig. 4. In the lean subjects, insulin significantly increased tyrosine phosphorylation of the insulin receptor β subunit at all incubation time points studied (Fig. 4, *top*). In the obese subjects, insulin-stimulated receptor phosphorylation was lower, with only the 15-min incubation time point close to a statistically significant elevation ($P = 0.06$). Likewise, insulin increased IRS-1 tyrosine phosphorylation at all incubation times in the lean subjects, whereas phosphorylation was significantly increased only with 15 min of insulin incubation in the obese subjects (Fig. 4, *bottom*).

To determine whether the decrease in receptor and IRS-1 phosphorylation in the obese subjects was associated with a decreased expression of these proteins, we measured the relative abundance of the insulin receptor and IRS-1 in the skeletal muscle samples. As illustrated in the phosphoimages in Figs. 5 and 6, there was considerable variability in insulin receptor and IRS-1 protein levels among the lean subjects. When insulin receptor and IRS-1 protein content of the eight lean and eight obese subjects was calculated by densitometry, the mean levels of both the insulin receptor and IRS-1 proteins were slightly

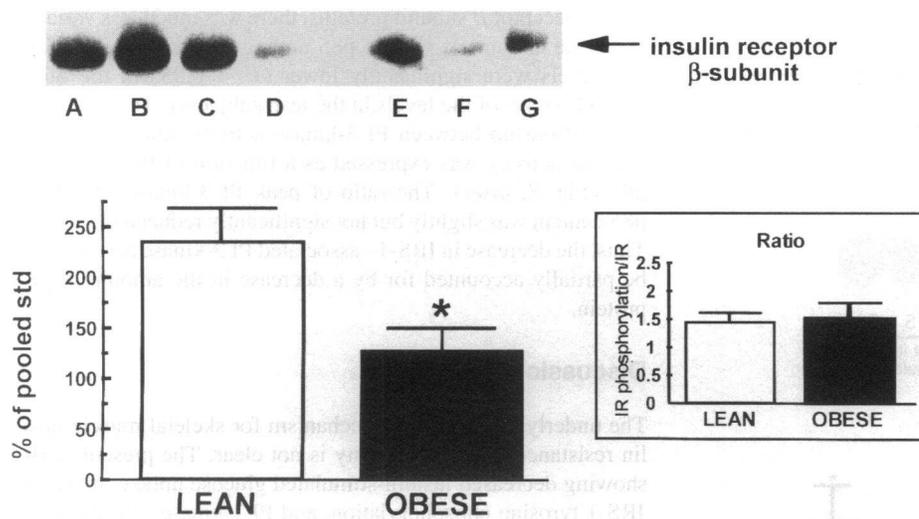


Figure 5. Insulin receptor protein content in human skeletal muscle. The image shows immunoreactive IRS-1 from four individual lean subjects (left, A-D) and three individual obese subjects (right, E-G). The bar graph on the left is quantitation from 14 lean and 11 obese subjects. The inset is insulin receptor tyrosine phosphorylation per insulin receptor protein, which was calculated from the peak tyrosine phosphorylation from the eight lean and eight obese subjects and expressed as a ratio. Muscle extracts were prepared from frozen muscle samples as described in Methods. Immunoblotting was done as described in Methods using α IR β . Data are mean \pm SE. * Significantly different from the lean value ($P < 0.05$).

higher in the lean compared with the obese subjects (data not shown), but these differences did not reach statistical significance ($P = 0.08$). To determine whether there was a clear difference in receptor and IRS-1 levels between lean and obese subjects, we measured insulin receptor protein in six additional lean and three additional obese subjects. These subjects were similar to the 16 subjects characterized in Table I, but had only frozen, untreated muscle samples obtained at the time of surgery.

Figure 5 (top) is a representative phosphoimage showing the insulin receptor protein from four individual lean and three obese subjects. The bar graph on the left summarizes the data from all 14 lean and 11 obese subjects. There was a significant decrease in insulin receptor levels in the obese subjects (55% of lean; $P < 0.05$). To examine the relationship between insulin receptor phosphorylation and insulin receptor protein, we expressed receptor phosphorylation as a function of the amount of receptor protein in each subject (Fig. 5, inset). This ratio was calculated by dividing the peak tyrosine phosphorylation from each of the eight subjects (2-, 15-, or 30-min time point) by the receptor protein from each of these subjects. The similar ratio in the lean and obese subjects suggests that the decrease

in tyrosine-phosphorylated receptor may largely be explained by the decrease in the abundance of the insulin receptor protein.

Figure 6 (top) is a representative phosphoimage showing immunoreactive IRS-1 from four individual lean subjects (left) and four individual obese subjects (right). As with the insulin receptor, there was considerable variability in IRS-1 among the lean subjects. The bar graph on the left summarizes the data from the 14 lean and 11 obese subjects for each group. IRS-1 levels were 46% lower in the obese subjects as compared with the lean ($P < 0.05$). The inset shows IRS-1 tyrosine phosphorylation per IRS-1 protein, calculated from the peak tyrosine phosphorylation from the eight subjects for each group and expressed as a ratio (Fig. 6, inset). As with the insulin receptor protein, the similar ratio in the lean and obese subjects suggests that the decrease in IRS-1 is largely responsible for the decrease in insulin-stimulated tyrosine phosphorylation of IRS-1.

PI 3-kinase activity and p85 content. Basal and insulin-stimulated PI 3-kinase activity was measured in IRS-1 immunoprecipitates. The same muscle extracts that were used for the detection of tyrosine phosphoproteins were used to measure enzyme activity in basal muscles and in muscles that were incubated with 10^{-7} M insulin for 2, 15, or 30. A representative

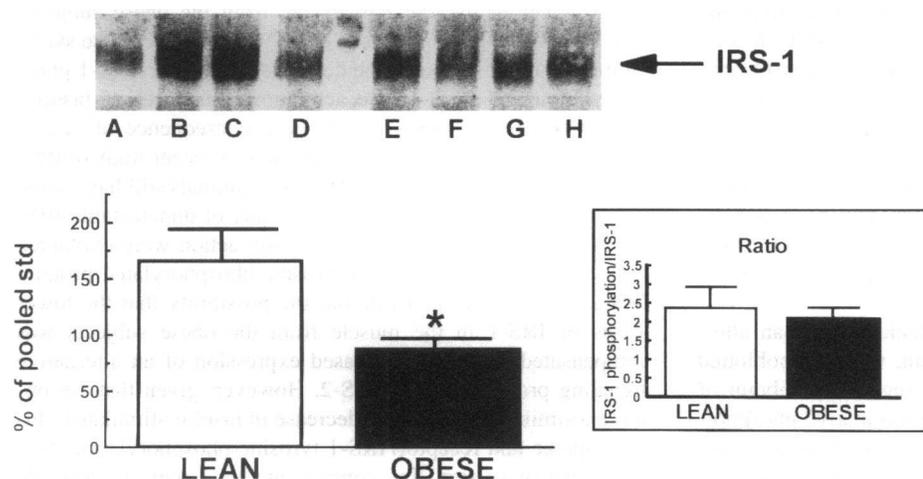


Figure 6. IRS-1 protein content in human skeletal muscle. The image shows immunoreactive IRS-1 from four individual lean subjects (left, A-D) and four individual obese subjects (right, E-H). The bar graph on the left is quantitation from 14 lean and 11 obese subjects. The inset is IRS-1 tyrosine phosphorylation per IRS-1 protein, which was calculated from the peak tyrosine phosphorylation from the eight lean and eight obese subjects and expressed as a ratio. Muscle extracts were prepared from frozen muscle samples as described in Methods. Immunoblotting was done as described in Methods using α IRS-1C. Data are mean \pm SE. * Significantly different from the lean value ($P < 0.05$).

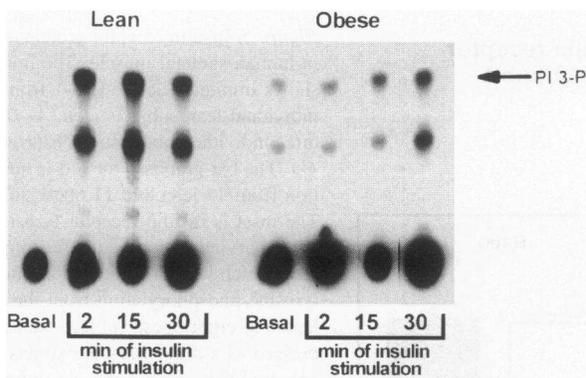


Figure 7. PI 3-kinase activity (IRS-1 immunoprecipitable) in skeletal muscle from lean and obese subjects. Equal amounts (2 mg) of solubilized protein were immunoprecipitated with α IRS-1. The kinase reaction was performed in the presence of phosphatidylinositol and [32 P]ATP, and the lipid products were separated by TLC (*top*). The spots that comigrated with a PI 3-phosphate (PI 3-P) standard were quantitated by densitometry (*bottom*). For each paired experiment, the densitometric units of the basal samples from the lean and obese subjects were averaged and set to 100%, as described in Fig. 4. Data are mean \pm SE; $n = 8$ per group. * Significantly different from the corresponding basal value ($P < 0.05$); # significantly different from the corresponding lean value ($P < 0.05$).

TLC phosphoimage of IRS-1-immunoprecipitable PI 3-kinase activity from the skeletal muscle of one lean subject and one obese subject is shown in Fig. 7 (*top*). In the lean subject, insulin markedly increased IRS-1-associated PI 3-kinase activity at all time points. Following the same pattern as receptor and IRS-1 tyrosine phosphorylation, in the obese subject, insulin-stimulated PI 3-kinase activity was significantly lower. Quantitation of the IRS-1-associated PI 3-kinase activity from all of the subjects shows that insulin increased enzyme activity by ~ 10 - to 35-fold in the lean subjects (Fig. 6, *bottom*). In the obese subjects, insulin significantly increased PI 3-kinase activity at all time points studied. However, the increase in IRS-1-associated enzyme activity was significantly lower in the obese as compared with the lean subjects, increasing to only four- to tenfold above basal activity.

To determine whether obesity is associated with an alteration in the amount of PI 3-kinase protein, we immunoblotted the skeletal muscle samples for the p85 regulatory subunit of the PI 3-kinase enzyme (Fig. 8). The representative phosphoimage in Fig. 8 shows the p85 protein from four lean (*left*) and three obese (*right*) subjects. In comparison with the IRS-1 and

insulin receptor β subunit proteins, there was much less variability in the amount of muscle p85 among the lean subjects. The p85 levels were significantly lower ($P < 0.05$) in the obese subjects (64% of the levels in the lean subjects). To understand the relationship between PI 3-kinase activity and p85 protein, enzyme activity was expressed as a function of the amount of p85 (Fig. 8, *inset*). The ratio of peak PI 3-kinase activity to p85 content was slightly but not significantly reduced ($P = 0.1$). Thus, the decrease in IRS-1-associated PI 3-kinase activity may be partially accounted for by a decrease in the amount of p85 protein.

Discussion

The underlying molecular mechanism for skeletal muscle insulin resistance in human obesity is not clear. The present study, showing decreased insulin-stimulated glucose uptake, receptor/IRS-1 tyrosine phosphorylation, and PI 3-kinase activity in the same intact muscle, strongly suggests that impairment of initial steps in insulin signaling contributes to the defect in insulin-stimulated skeletal muscle glucose uptake. To our knowledge, these are the first results obtained in intact human skeletal muscle tissue showing that multiple steps of the insulin receptor signaling pathway are altered in obese, insulin-resistant subjects. These results support the concept that fully functional insulin receptor phosphorylation, IRS-1 phosphorylation, and PI 3-kinase activation are required for normal insulin stimulation of glucose uptake. Evidence for this hypothesis was originally provided by studies in Chinese hamster ovary cells, in which impairment of insulin-stimulated receptor kinase activity by mutation of the tyrosine kinase domain (34) or ATP-binding region (35, 36) resulted in decreased insulin-stimulated glucose uptake. More recent studies have shown that inhibition of PI 3-kinase activation blocks insulin-stimulated glucose uptake and GLUT-4 translocation in 3T3-L1 adipocytes (21, 37) and CHO-GLUT4myc cells (23). Although none of these studies demonstrated a direct link between insulin receptor signaling and glucose transport, taken together they provide a strong case for a role of these signaling steps in insulin regulation of glucose uptake. Our data demonstrating that the defect in insulin-stimulated glucose uptake is associated with reduced activation of the initial steps in insulin signaling in skeletal muscle suggest a coupling of these mechanisms in this human tissue.

Although insulin-stimulated glucose uptake was significantly lower in the skeletal muscle from the obese subjects, insulin still increased rates of glucose uptake in the obese skeletal muscle by 65%. Given the decrease in receptor/IRS-1 phosphorylation and PI 3-kinase activation, this level of insulin-stimulated glucose uptake could be a consequence of insulin activating alternative signaling pathways. A recent study of IRS-1-deficient mice has revealed that these animals still have some insulin-stimulated glucose uptake in spite of undetectable IRS-1 (38). These residual levels of insulin action were explained by the appearance of a novel tyrosine-phosphorylated protein, IRS-2. Thus, we cannot rule out the possibility that the lower levels of IRS-1 in the muscle from the obese subjects was compensated for by an increased expression of an alternative signaling protein, such as IRS-2. However, given that we observe a similar magnitude of decrease in insulin-stimulated glucose uptake and receptor/IRS-1 tyrosine phosphorylation, it is likely that these signaling components are largely responsible

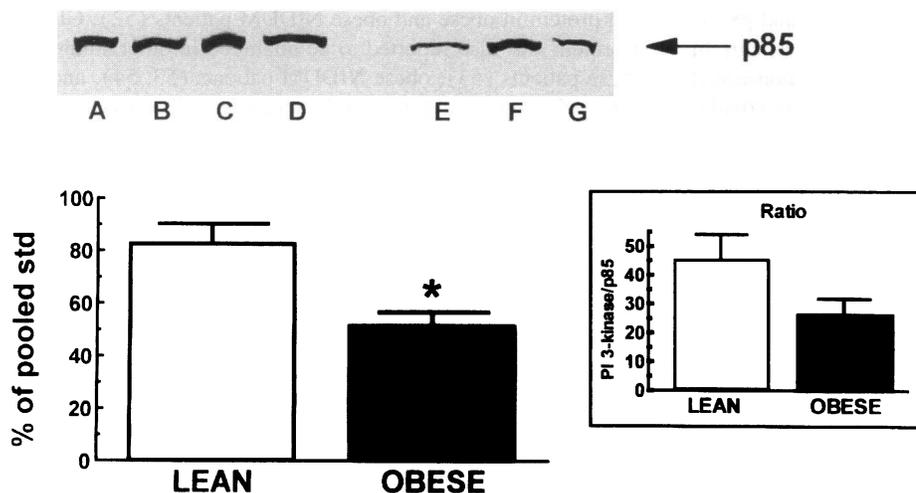


Figure 8. p85 protein content in human skeletal muscle. The image shows immunoreactive IRS-1 from four individual lean subjects (left, A–D) and three individual obese subjects (right, E–G). The bar graph on the left is quantitation from 14 lean and 11 obese subjects. The inset is PI 3-kinase activity (IRS-1 immunoprecipitable) per p85 protein, which was calculated from the peak PI 3-kinase activity from the eight lean and eight obese subjects and expressed as a ratio. Muscle extracts were prepared from frozen muscle samples as described in Methods. Immunoblotting was done as described in Methods using α p85. Data are mean \pm SE. * Significantly different from the lean value ($P < 0.05$).

for the residual amount of insulin-stimulated glucose uptake in the muscle from the obese subjects.

The decrease in the insulin signal could be a consequence of the lower levels of the insulin receptor, IRS-1, and PI 3-kinase proteins in the skeletal muscle from the obese subjects. This decrease in insulin receptor expression in obesity is consistent with previous studies showing significant or modest decreases in insulin binding in the skeletal muscle (25), liver (39), and adipose tissue (40, 41) of obese subjects. Before the results presented in the current study, levels of IRS-1 and PI 3-kinase proteins had not been reported in tissues from insulin-resistant subjects. The concept that decreased insulin signaling is a result of decreased expression of the signaling proteins is based on the mean stoichiometry of insulin receptor phosphorylation, IRS-1 phosphorylation, and PI 3-kinase activity. For example, the similar obesity-associated decreases in tyrosine phosphorylation of the insulin receptor and insulin receptor protein levels suggest that the average amount of tyrosine phosphorylation per receptor is not decreased in the obese subjects.

Although the current study suggests that the obesity-associated decreases in early steps of the insulin signaling pathway are largely due to decreased levels of the insulin receptor, IRS-1, and PI 3-kinase proteins, other forms of insulin resistance may be associated with decreases in both the expression and function of these proteins. In the skeletal muscle from lean patients with NIDDM, autophosphorylation of isolated insulin receptors was reduced by twofold, and kinase activity toward the exogenous substrate poly(Glu4:Tyr1) was decreased by threefold (24). Since these experiments used an *in vitro* assay system that equalizes insulin receptor protein, the defect in receptor kinase activity could not be attributable to an alteration in the number of insulin receptors. In a study of obese subjects with NIDDM, insulin receptor autophosphorylation was not altered, but receptor phosphorylation of poly(Glu4:Tyr1) was decreased by $> 50\%$ (25). However, the defect in receptor kinase activity toward the exogenous substrate was similar in nondiabetic obese patients (25). Understanding the discrepancies among the two *in vitro* studies and the current study is difficult, since the patient population and/or methodologies are very different. Overall, however, all three studies demonstrate that insulin-resistant skeletal muscle is characterized by some degree of defect in insulin receptor tyrosine phosphorylation.

In future research it will be important to study patients with NIDDM using the intact human skeletal muscle preparation.

The obesity-associated decreases in insulin receptor signaling and PI 3-kinase activity in human skeletal muscle are generally similar to findings reported in animal models of obesity (27, 42). In the obese, hyperglycemic, and hyperinsulinemic *ob/ob* mouse, *in vivo* insulin-stimulated tyrosine phosphorylation of the receptor and IRS-1 was decreased by ~ 40 and 50% , respectively (27). In contrast to our findings in human skeletal muscle, in which both insulin receptor and IRS-1 protein levels were decreased, in the skeletal muscle of this mouse strain there was a decrease only in insulin receptor protein levels. Thus, in the obese mouse, the decrease in IRS-1 phosphorylation may be more a consequence of impaired insulin receptor kinase activity, whereas in human skeletal muscle, the decrease may be a function of both reduced receptor kinase activity and decreased IRS-1 protein levels. In mice made obese by goldthiogluconate, there was a 40–60% decrease in insulin-stimulated PI 3-kinase activity immunoprecipitated with an anti-phosphotyrosine antibody (42). Although obesity did not result in a decrease in “total” (anti-p85 immunoprecipitable) PI 3-kinase activity, PI 3-kinase protein amounts were not measured. Thus, it is difficult to determine whether, as with human skeletal muscle, the decreased IRS-1-associated PI 3-kinase activity in the obese mice is a function of decreased expression of p85.

The mechanism for decreased expression of signaling proteins in insulin-resistant patients could be a function of elevations in blood insulin or glucose. In the current study the obesity-associated decreased expression of the insulin receptor, IRS-1, and p85 occurred in the absence of statistically significant elevations in blood glucose and insulin. However, it is important to recognize that although not statistically significant, insulin concentrations were 88% higher in the obese group, similar to our previous findings (36, 43). Furthermore, since glucose tolerance testing was not performed, it is possible that some or all of the obese subjects had elevated postprandial glucose and/or insulin concentrations (44). Nevertheless, our data demonstrate that defects in insulin signaling components and insulin-stimulated glucose uptake in skeletal muscle from severely obese subjects can occur before the onset of frank hyperglycemia.

One possible mechanism to explain the obesity-associated

decreased expression of the insulin receptor, IRS-1, and p85 is that lower levels of these proteins are caused by a shift in muscle fiber type composition. We have recently demonstrated a significantly lower percentage of slow twitch fibers (oxidative) and a tendency for an increase in type IIB fibers (fast, glycolytic) in a similar group of obese subjects (44a). In rats, slow twitch fibers have higher levels of insulin receptor binding compared with fast twitch fibers (45, 46). Thus, lower levels of type I fibers in the obese subjects may result in an overall decrease in insulin receptor expression. The alteration in fiber type composition may also result in the lower levels of IRS-1 and p85 protein, although this is speculative, since to our knowledge there have been no published reports comparing IRS-1 and p85 levels in slow and fast twitch muscles. It is important to note that the obesity-associated decrease in the expression of these specific signaling proteins probably does not reflect a generalized decrease in all muscle proteins. In comparing the lean and obese subjects in the current study, there was no difference in the estimated recovery of total muscle protein between the two groups. Furthermore, in preliminary studies of a subset of the subjects in the current study, we did not detect an alteration in the expression of two isoforms of microtubule-associated protein kinase (Markuns, J. F., and L. J. Goodyear, unpublished observations).

Although the decreased expression of the insulin receptor, IRS-1, and PI 3-kinase proteins may largely explain the obesity-associated decreases in receptor/IRS-1 phosphorylation and PI 3-kinase activity, it is possible that other cellular regulatory factors contribute to the down-regulation of these signaling reactions. For example, increased serine phosphorylation of the insulin receptor has been shown to decrease receptor tyrosine kinase activity (47), and serine phosphorylation of the p85 regulatory subunit of PI 3-kinase significantly decreases enzyme activity (48, 49). Serine phosphorylation of p85 by PI 3-kinase in vitro decreases enzyme activity by three- to sevenfold, an effect that can be reversed by dephosphorylation with phosphoprotein phosphatase 2A (49). Thus, the obese state may result in an increase in PI 3-kinase kinase, a decrease in phosphoprotein 2A activity, or both. The down-regulation of the insulin signal could also be a function of an increase in protein tyrosine phosphatase (PTPase) activity. The findings of one study support this concept, showing a 33% increase in muscle PTPase activity in obese insulin-resistant subjects (50). On the other hand, another recent report has demonstrated a 21–22% decrease in muscle PTPase activity in obese nondiabetic and NIDDM subjects (51). A putative role for cellular phosphatases in the regulation of insulin signaling reactions and skeletal muscle insulin resistance will require further investigation.

Our data, taken together with studies in cultured cells (21–23, 34–36), suggest that decreased activity of early steps in insulin receptor signaling with obesity is a factor in the lower rates of skeletal muscle glucose uptake. A blunted insulin signal may lead to a block in the activation of glucose transporter function, or may occur in parallel with an independent effect of obesity on the glucose transport system. Any effect of obesity on the glucose transport system is probably at the level of transporter translocation, since effects on the expression of glucose transporter proteins appear to be minimal (43, 52–54). In severely obese patients, we have seen only a modest, 23% decrease in skeletal muscle GLUT-4 protein (43), whereas another report showed no change in GLUT-1 and GLUT-4 mRNA

and protein in obese and obese NIDDM patients (52). GLUT-4 protein was also reported to be unaltered in other studies on obese patients (53), obese NIDDM patients (53, 54), and patients with impaired glucose tolerance (53). The effect of obesity on skeletal muscle glucose transporter translocation has not been determined in human skeletal muscle. However, in the obese Zucker rat, in which both transporter expression and function can be measured, GLUT-4 levels were not altered (55, 56) but insulin-stimulated glucose transporter translocation was impaired (56). Thus, human obesity may also be associated with a defect in GLUT-4 translocation.

Given the consistent findings of decreased levels of signaling proteins in subjects with obesity (25, 39–41, current study), the recent advances in understanding the insulin signaling pathways (57), and the current knowledge of glucose transporter physiology (56, 58), it is possible to propose a series of events that lead to the impaired insulin-stimulated glucose uptake in human skeletal muscle. First, the obese state is associated with a mild to severe decrease in the muscle content of various signaling proteins, including the insulin receptor, IRS-1, and the p85 subunit of PI 3-kinase. Decreased levels of these proteins could lead to diminished phosphorylation reactions and decreased insulin-stimulated activation of PI 3-kinase. The blunting of PI 3-kinase activity could then lead to decreased signaling via specific branches of the insulin signaling pathway, including the transmission of the signal that activates the movement of glucose transporters to the plasma membrane. Finally, the defect in transporter translocation would result in decreased glucose uptake.

In summary, we have developed a procedure to assess both glucose uptake and initial steps in insulin signaling in intact human skeletal muscle. Among the advantages of these methods is the ability to study intact tissue rather than partially purified receptor preparations. We have shown that in the skeletal muscle from obese subjects, there is decreased content and tyrosine phosphorylation of the insulin receptor β subunit, decreased content and tyrosine phosphorylation of IRS-1, decreased content of the p85 subunit of PI 3-kinase, and decreased PI 3-kinase activity associated with IRS-1. Future studies in human skeletal muscle will focus on determining whether this down-regulation of the insulin signaling pathway with obesity plays a role in decreased glucose uptake by altering the functional properties of glucose transporter proteins.

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