Insulin Receptor Substrate-1 Phosphorylation and Phosphatidylinositol 3-Kinase Activity in Skeletal Muscle From NIDDM Subjects After In Vivo Insulin Stimulation

Marie Björnholm, Yuichi Kawano, Mikael Lehtihet, and Juleen R. Zierath

We examined the effect of physiological hyperinsulinemia on insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and phosphatidylinositol (PI) 3-kinase activity in skeletal muscle from six lean–to–moderately obese NIDDM patients and six healthy subjects. A rise in serum insulin levels from ~60 to ~650 pmol/l increased IRS-1 tyrosine phosphorylation sixfold over basal levels in control muscle ($P < 0.01$), whereas no significant increase was noted in NIDDM muscle. The reduced IRS-1 phosphorylation in the NIDDM muscle was not related to changes in IRS-1 protein content, since IRS-1 protein expression was similar between control and NIDDM subjects (16.0 ± 1.7 vs. 22.9 ± 4.0 arbitrary units/mg protein for control and NIDDM, respectively; NS). Physiological hyperinsulinemia increased PI 3-kinase activity in control muscle twofold ($P < 0.01$), whereas no increase in insulin-stimulated PI 3-kinase activity was noted in the NIDDM muscle. Furthermore, in vitro insulin-stimulated (600 pmol/l) 3-*O*-methylglucose transport was 40% lower in isolated muscle from NIDDM subjects ($P < 0.05$). The present findings couple both reduced insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity to the impaired insulin-stimulated glucose transport in skeletal muscle from lean–to–moderately obese NIDDM subjects. *Diabetes* 46:524–527, 1997

Intense interest has focused on whether the reduced insulin-mediated glucose transport in muscle from NIDDM patients results from alterations in the insulin signal transduction pathway (1–12) or from alterations in the traffic and/or translocation of GLUT4 to the plasma membrane (12–18). Recently, we have shown that an increase in fasting serum insulin levels from ~50 to ~600 pmol/l induces a translocation of GLUT4 from an intracellular storage site to the plasma membrane in skeletal muscle from healthy individuals (18,19). Conversely, a similar increase in insulin levels did not alter the plasma membrane GLUT4 content in muscle biopsies from NIDDM patients (18), providing evidence for a defect in insulin signaling and/or GLUT4 translocation in muscle from NIDDM patients.

Evidence from animal studies suggests that insulin signaling defects in muscle are associated with altered whole-body glucose homeostasis (2–5). In morbidly obese humans (6) and obese rodents (2–5), impaired insulin-stimulated glucose transport in skeletal muscle is associated with decreased insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) protein content, decreased IR and IRS-1 phosphorylation, and reduced PI 3-kinase activity. Tumor necrosis factor (TNF)-α has been implicated to play a role in development of insulin resistance in obesity by increasing the serine phosphorylation of IR and IRS-1 (10). In contrast to the obese state, in hyperglycemic-hypoinsulinemic states, such as in the streptozotocin-induced diabetic rat, IR and IRS-1 phosphorylation and PI 3-kinase activity are enhanced in skeletal muscle (2,3), despite reduced insulin-stimulated glucose transport (20).

Reduced IR phosphorylation has been observed in skeletal muscle from nonobese NIDDM subjects (7–9). However, whether skeletal muscle insulin resistance in NIDDM is associated with altered IRS-1 phosphorylation and/or PI 3-kinase activity remains to be established. Thus, we examined the effect of physiological hyperinsulinemia on IRS-1 tyrosine phosphorylation and PI 3-kinase activity in skeletal muscle from NIDDM and healthy subjects.

RESEARCH DESIGN AND METHODS

Subjects. The study protocol was reviewed and approved by the institutional ethical committee of the Karolinska Institute, and informed consent was received from all subjects before participation. The clinical characteristics of the subjects are presented in Table 1.

Experimental protocol. A modification of the hyperinsulinemic clamp procedure was used in conjunction with an open-muscle biopsy to obtain vastus lateralis muscle (18). Muscle biopsies were obtained under local anesthesia (mepivacain chloride 5 mg/ml) 30 min after a glucose priming period (basal). Thereafter, a bolus injection of insulin was administered (17.6 nmol · kg^{-1} · min^{-1}) for 4 min, and hyperinsulinemia was maintained by means of a continuous insulin infusion (5.5 nmol · kg^{-1} · min^{-1}). Forty minutes after the onset of the insulin infusion, a second biopsy (insulin-stimulated) was obtained. Serum insulin levels at the time of the muscle biopsy were 57.0 ± 10.8 and 73.8 ± 26.4 pmol/l under basal conditions (NS) and 638 ± 44 and 729 ± 90 pmol/l (NS) under insulin-stimulated conditions for the control and NIDDM subjects, respectively. Each biopsy was obtained from different muscle bundles from the same incision site by means of a Weil-Blakesley conchotome (12). The muscles were frozen in liquid nitrogen on excision and stored (–80°C) until analysis.

Tyrosine phosphorylation and protein expression of IRS-1. Muscle specimens (20 mg) were homogenized in ice-cold buffer as described previously (4) and

From the Department of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden.

Address correspondence and reprint requests to Dr. Juleen R. Zierath, Department of Clinical Physiology, Karolinska Hospital, S-171 76, Stockholm, Sweden. E-mail: jrz@klinfys.ks.se.

Received for publication 31 October 1996 and accepted 19 December 1996.

IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; TNF, tumor necrosis factor.

TABLE 1
Subject characteristics

	Control	NIDDM
Age (years)	55 ± 3	56 ± 3
BMI (kg/m^2)	27.5 ± 1.2	27.1 ± 2.0
Glucose (mmol/l)	5.8 ± 0.2	10.5 ± 0.9*
Insulin (pmol/l)	49.8 ± 7.4	71.4 ± 21.6
HbA_{1c} (%)	3.7 ± 0.1	6.4 ± 0.8†
TNF-α (pg/ml)	3.2 ± 0.3	3.2 ± 0.7
Duration of diabetes (years)	NA	5 ± 3

Data are means ± SE for six subjects in each group. *P < 0.001, †P < 0.01, significantly different from the control subjects. NA, not applicable.

centrifuged at 150,000*g* for 35 min (4°C). The supernatant (1 mg) was immunoprecipitated overnight (4°C) with an anti–IRS-1 antibody coupled to protein A–sepharose. The immunoprecipitates were washed as indicated (6), resuspended in Laemmli sample buffer with 100 mmol/l dithiothreitol, and heated (95°C) for 6 min. The proteins were separated by SDS-PAGE (6% resolving gel), transferred to nitrocellulose membranes, and blocked as described previously (13). The membranes were incubated with either anti–IRS-1 (total protein expression) or anti-phosphotyrosine (IRS-1 phosphorylation) antibodies. The membranes were washed and incubated with secondary antibodies as previously described (13). IRS-1 was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified by densitometry.

Determination of PI 3-kinase activity in muscle biopsies. Muscle specimens (20 mg) were homogenized in 500 µl lysis buffer, as previously described (6), and solubilized by continuous stirring for 1 h at 4°C. After centrifugation (12,000*g* for 4 min), the supernatant (1 mg) was immunoprecipitated overnight (4°C) with either anti-phosphotyrosine antibody (Signal Transduction Laboratories, Lexington, KY) or anti–IRS-1 antibody (gift from Drs. Martin Myers and Morris White) coupled to protein A–sepharose (Sigma, St. Louis, MO). The immune complexes were washed as described (6) and resuspended in 40 µl of buffer (20 mmol/l HEPES, pH 7.5, 180 mmol/l NaCl). PI 3-kinase activity was assessed directly on the protein A–sepharose beads as previously reported (5,11). The bands corresponding to PI 3-phosphate were quantitated using a PhosphorImager.

Glucose transport measurements in isolated skeletal muscle. Before removal of the "basal" biopsy, an open-muscle biopsy was excised, and isolated muscle strips were prepared for in vitro incubation and assessment of basal and insulin-stimulated (600 pmol/l) 3-*O*-methylglucose transport as previously described (12).

TNF-α determinations. Fasting TNF-α levels were assessed in serum, using a commercially available kit (Promega, Madison, WI). Human recombinant TNF-α was used as a standard.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed using the unpaired Student's *t* test.

Materials. PI was from Avanti Polar Lipids (Alabaster, AL), and the aluminum backed Silica Gel 60 thin-layer chromatography plates were from EM Separations (Gibbstown, NJ). All other chemicals were purchased from Sigma or Merck (Rahway, NJ). Protein was determined using a kit from Bio-Rad (Richmond, CA).

RESULTS

IRS-1 tyrosine phosphorylation and protein content. Physiological hyperinsulinemia increased IRS-1 tyrosine phosphorylation approximately sixfold (P < 0.01) in skeletal muscle from the healthy subjects (Fig. 1). Conversely, insulin infusion did not significantly alter IRS-1 tyrosine phosphorylation in skeletal muscle from NIDDM subjects. Interestingly, basal IRS-1 tyrosine phosphorylation tended to be higher in NIDDM muscle. We next assessed IRS-1 protein expression to determine whether the lack of IRS-1 phosphorylation in NIDDM muscle was associated with altered IRS-

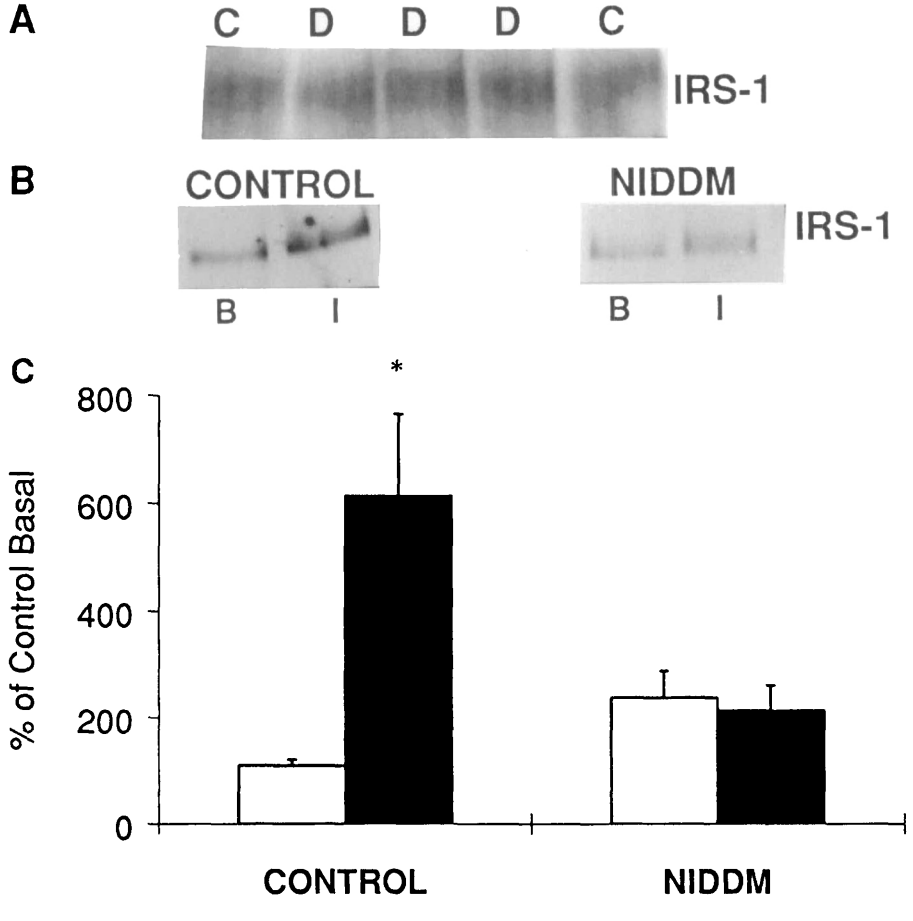

FIG. 1. In vivo stimulation of IRS-1 in skeletal muscle from control and NIDDM subjects. Muscle biopsies were obtained under fasting conditions (basal; □) and after a 40-min insulin infusion to raise plasma levels to 638 ± 44 or 729 ± 90 pmol/l (insulin-stimulated; ■) for control or NIDDM subjects, respectively. *A*: representative autoradiogram of immunoreactive IRS-1 in basal muscle; C, control; D, NIDDM. *B*: representative autoradiogram of tyrosine phosphorylated IRS-1 from basal (B) or insulin-stimulated (I) muscle. *C*: tyrosine phosphorylated IRS-1 in control (basal n = 3 and insulin n = 6) and NIDDM (basal n = 4 and insulin n = 6) muscle. Results are expressed as means ± SE. Tyrosine phosphorylated IRS-1 was determined in IRS-1 immunoprecipitates as described in METHODS. *P < 0.01, significantly different from basal.

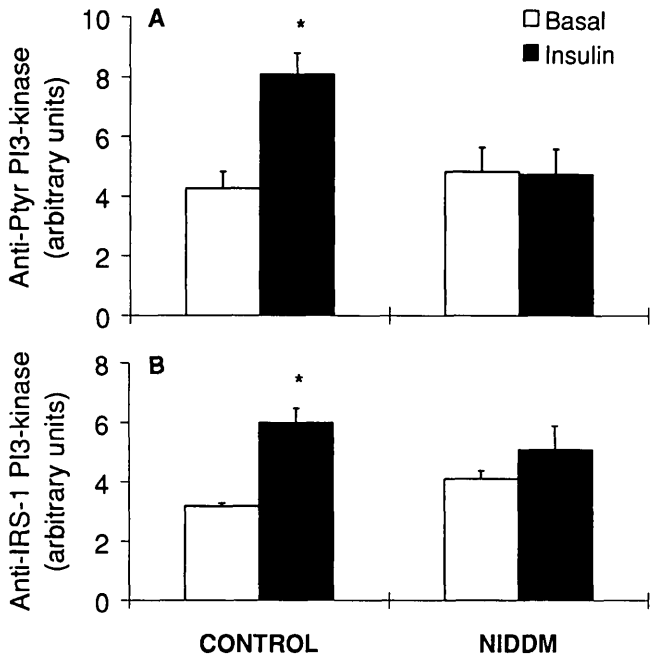

FIG. 2. Effect of physiological hyperinsulinemia on PI 3-kinase in skeletal muscle from control and NIDDM subjects. Basal and insulin-stimulated muscle was obtained as described in Fig. 1, and PI 3-kinase was measured in immunoprecipitates obtained with antibodies to phosphotyrosine (*A*) or to IRS-1 (*B*), as described in METHODS. Values are presented as phosphoimager units determined from quantitation of the phosphoimage of the [^{32}P]PI 3-phosphate products. Results are expressed as means ± SE for control and NIDDM subjects, as indicated in Fig. 1. *P < 0.01, significantly different from basal.

1 protein content. A representative autoradiogram of immunoreactive IRS-1 in skeletal muscle is presented in Fig. 1. Skeletal muscle IRS-1 protein expression was similar between control and NIDDM subjects (16.0 ± 1.7 vs. 22.9 ± 4.0 arbitrary units/mg protein for n = 6 control and n = 6 NIDDM subjects, respectively; NS).

PI 3-kinase activity. PI 3-kinase was assessed in anti-phosphotyrosine or anti–IRS-1 immunoprecipitates of basal or insulin-stimulated muscle from control and NIDDM subjects (Fig. 2). Physiological hyperinsulinemia increased tyrosine-associated PI 3-kinase activity twofold (P < 0.01) in the control muscle. Similarly, insulin increased IRS-1–associated PI 3-kinase activity twofold (P < 0.01) in the control muscle. In contrast, insulin failed to increase either tyrosine- or the IRS-1–associated PI 3-kinase activity in NIDDM muscle.

Glucose transport. Insulin-stimulated 3-*O*-methylglucose transport was reduced by 40% in NIDDM skeletal muscle (1.14 ± 0.14 vs. 0.69 ± 0.13 μmol · ml^{-1} · h^{-1}, for control and NIDDM subjects, respectively; P < 0.05). Basal glucose transport was not different between the groups.

TNF-α levels. We next assessed serum levels of TNF-α to determine whether the reduced insulin-stimulated IRS-1 tyrosine phosphorylation in NIDDM muscle was related to increased serum TNF-α levels. Fasting TNF-α levels were not significantly different between control and NIDDM subjects (Table 1).

DISCUSSION

Here we provide the first evidence to show that insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity are reduced in skeletal muscle from lean–to–moderately obese NIDDM subjects. Thus, in addition to reduced IR kinase activity (7–9), defects in insulin signaling through IRS-1 and PI 3-kinase may to contribute to impaired glucose transport (6,14–18) and reduced plasma membrane GLUT4 content in muscle from NIDDM subjects (16,18).

In skeletal muscle from obese rodents and humans, IR kinase activity (3,4,6), IRS-1 tyrosine phosphorylation (2,3), PI 3-kinase activity (3–6,11), and glucose transport (5,6,11) are markedly decreased after insulin stimulation. In muscle from morbidly obese humans, these defects are accompanied by decreased expression of several key proteins in the insulin signaling cascade, including the β-subunit of the IR, IRS-1, and the p85 subunit of PI 3-kinase (6). However in the latter study, the obese subjects had a BMI of ~53 kg/m^2, and consequently, they do not represent the vast majority of the NIDDM population. In the present study, IRS-1 protein content was not significantly altered in skeletal muscle from NIDDM subjects, despite reduced tyrosine phosphorylation. Our results suggest that the decreased IRS-1 tyrosine phosphorylation in muscle from lean–to–moderately obese NIDDM subjects may be a consequence of impaired IR tyrosine kinase activity, whereas defects in muscle from insulin-resistant morbidly obese humans may be related to reduced activity and protein expression of IR and IRS-1 (6).

TNF-α has been suggested to induce peripheral insulin resistance in obesity and diabetes by increasing serine phosphorylation of IRS-1, thus converting IRS-1 to an inhibitor of IR through increased serine phosphorylation of the IR (10). Since serum TNF-α levels were not elevated in the NIDDM subjects, elevations in circulatory TNF-α levels are unlikely to account for the reduced insulin-stimulated tyrosine IRS-1 phosphorylation observed in NIDDM muscle. However, TNF-α may be produced locally in skeletal muscle and may function in an autocrine manner to inhibit insulin signaling and glucose transport (21). Nevertheless, a recent study showed that antibody-mediated neutralization of TNF-α did not improve insulin sensitivity in obese NIDDM subjects (22). Further studies are warranted to determine the role of TNF-α and insulin signaling in insulin-resistant human skeletal muscle.

PI 3-kinase associates with tyrosine phosphorylated IRS-1 after insulin stimulation and acts as an important upstream mediator of signaling to p70 S6 kinase, DNA synthesis, and glycogen synthesis (1). Furthermore, PI 3-kinase has been shown to play a seminal role in insulin-stimulated glucose transport and GLUT4 translocation in skeletal muscle (23). The present finding couples reduced insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity to the reduced glucose transport observed in NIDDM muscle. Furthermore, the present report is the first to show that physiological hyperinsulinemia is sufficient to activate IRS-1 phosphorylation and PI 3-kinase in vivo in human skeletal muscle.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Medical Research Council (9517, and 11823), Novo Nordisk Insulin Foundation, Thuring's Foundation, and the Karolinska Institute Foundation.

We are especially grateful to Dr. Harriet Wallberg-Henriksson for her contributions to the study.

REFERENCES

1. Kahn CR: Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* 43:1066–1084, 1994
2. Saad MJA, Araki E, Miralpeix M, Rothenberg PL, White MF, Kahn CR: Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. *J Clin Invest* 90:1839–1849, 1992
3. Folli F, Saad MJA, Backer JM, Kahn CR: Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. *J Clin Invest* 92:1787–1794, 1993
4. Saad MJA, Folli F, Kahn JA, Kahn CR: Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* 92:2065–2072, 1993
5. Heydrick SJ, Jullien D, Gautier N, Tanti JF, Giorgetti S, Van Obberghen E, Marchand-Brustel Y: Defect in skeletal muscle phosphatidylinositol-3-kinase in obese insulin-resistant mice. *J Clin Invest* 91:1358–1366, 1993
6. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL: Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195–2204, 1995
7. Nolan JJ, Freidenberg G, Henery R, Reichart D, Olefsky JM: Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of non-insulin-dependent diabetes mellitus and obesity. *J Clin Endocrinol Metab* 78:471–477, 1994
8. Nyombia BL, Ossowski VM, Bogardus C, Mott DM: Insulin-sensitive tyrosine kinase relationship with in vivo insulin action in humans. *Am J Physiol* 258:E964–E974, 1990
9. Maegawa H, Shigeta Y, Egawa K, Kobayashi M: Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles in nonobese subjects with NIDDM. *Diabetes* 40:815–819, 1991
10. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spegelman BM: IRS-1 mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α- and obesity-induced insulin resistance. *Science* 271:665–668, 1996
11. Zierath JR, Houseknecht KL, Gnudi L, Kahn BB: High-fat feeding impairs insulin-stimulated GLUT4 recruitment via an early insulin signaling defect. *Diabetes* 46:215–223, 1997
12. Zierath JR: In vitro studies of human skeletal muscle: hormonal and metabolic regulation of glucose transport. *Acta Physiol Scand* 155:1–96, 1995
13. Pedersen O, Bak JF, Andersen PH, Lund S, Moller DE, Flier JS, Kahn BB: Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39:865–870, 1990
14. Andréasson K, Galuska D, Thörne A, Sonnenfeld T, Wallberg-Henriksson H: Decreased insulin-stimulated 3-*O*-methylglucose transport in in vitro incubated muscle strips from type II diabetic subjects. *Acta Physiol Scand* 142:255–260, 1991
15. Zierath JR, Galuska D, Nolte LA, Thörne A, Smedegaard Kristensen J, Wallberg-Henriksson H: Effects of glycaemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance. *Diabetologia* 37:270–277, 1994
16. Kelley DE, Mintun MA, Watkins SC, Simoneau J-A, Jadali F, Fredrickson A, Beattie J, Thériault R: The effect of non-insulin-dependent diabetes mellitus and obesity on glucose transport and phosphorylation in skeletal muscle. *J Clin Invest* 97:2705–2713, 1996
17. Bonadonna RC, Del Prato S, Bonora E, Saccomani MP, Gulli G, Natali A, Frascerra S, Pecori N, Ferrannini E, Bier D, Cobelli C, DeFronzo RA: Roles of glucose transport and glucose phosphorylation in muscle insulin resistance of NIDDM. *Diabetes* 45:915–925, 1996
18. Zierath JR, He L, Gumá A, Odegaard-Wahlström E, Klip A, Wallberg-Henriksson H: Insulin action on glucose transport and plasma membrane content in skeletal muscle from patients with NIDDM. *Diabetologia* 39:1180–1189, 1996
19. Gumà A, Zierath JR, Wallberg-Henriksson H, Klip A: Insulin induces translocation of GLUT-4 glucose transporters in human skeletal muscle. *Am J Physiol* 268:E613–E622, 1995
20. Wallberg-Henriksson H, Zetan N, Henriksson J: Reversibility of decreased insulin-stimulated glucose transport capacity in diabetic muscle with in vitro incubation: insulin is not required. *J Biol Chem* 262:7665–7671, 1987
21. Saghizadeh M, Ong JM, Garvey WT, Henery RR, Kern PA: The expression of TNFα by human muscle: relationship to insulin resistance. *J Clin Invest* 97:1111–1116, 1996
22. Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R: Effects of engineered human anti-TNF-α antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45:881–885, 1996
23. Lund S, Holman GD, Schmitz O, Pedersen O: Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci USA* 92:5817–5821, 1995