Immobilization depresses insulin signaling in skeletal muscle

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Am J Physiol Endocrinol Metab 279: E1235–E1241, 2000. — Prolonged immobilization depresses insulin-induced glucose transport in skeletal muscle and leads to a catabolic state in the affected areas, with resultant muscle wasting. To elucidate the altered intracellular mechanisms involved in the insulin resistance, we examined insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit (IR-β) and insulin receptor substrate (IRS)-1 and activation of its further downstream molecule, phosphatidylinositol 3-kinase (PI 3-K), after unilateral hindlimb immobilization in the rat. The contralateral hindlimb served as control. After 7 days of immobilization of the rat, insulin was injected into the portal vein, and tibialis anterior muscles on both sides were extracted. Immobilization reduced insulin-stimulated tyrosine phosphorylation of IR-β and IRS-1. Insulin-stimulated binding of IRS-1 to p85, the regulatory subunit of PI 3-K, and IRS-1-associated PI 3-K activity were also decreased in the immobilized hindlimb. Although IR-β and p85 protein levels were unchanged, IRS-1 protein expression was downregulated by immobilization. Thus prolonged immobilization may cause depression of insulin-stimulated glucose transport in skeletal muscle by altering insulin action at multiple points, including the tyrosine phosphorylation, protein expression, and activation of essential components of insulin signaling pathways.

insulin receptor; insulin resistance; insulin receptor substrate-1; muscle wasting; phosphatidylinositol 3-kinase

INSULIN ACTIVATES MULTIPLE signaling pathways, leading to diverse effects on cellular metabolism and mitogenesis. These actions of insulin are initiated by autophosphorylation of specific tyrosine residues of the intracellular portion of the insulin receptor β-subunit (IR-β). Activated IR-β transduces the signal to downstream components by phosphorylating endogenous substrate proteins, such as insulin receptor substrates (IRSs) and Shc (22). A further downstream key molecule of IRS-1 is phosphatidylinositol 3-kinase (PI 3-K), which consists of regulatory (p85) and catalytic (p110) subunits. After phosphorylation by IR-β, IRS proteins bind to the p85 regulatory subunit of PI 3-K, leading to the activation of PI 3-K (34). This induces a diverse range of cellular responses, including glucose transporter translocation, cell growth and proliferation, and synthesis of carbohydrates, lipids, and proteins (8, 18, 25, 27). Thus dimerization and autophosphorylation of IR, tyrosine phosphorylation of IRSs by IR, and subsequent binding of PI 3-K to IRSs are essential initial steps for the metabolic action of insulin (42).

Chronic muscle disuse, produced by the conditions of prolonged bed rest, casting or pinning of limbs, and microgravity, induces insulin resistance and a catabolic state in the affected skeletal muscle in humans (9, 32, 35, 37). Animal studies, including those in rats, have confirmed that immobilization is associated with resistance to insulin-induced glucose uptake and protein synthesis (4, 29). Insulin resistance is also a major problem in critically ill patients, who are invariably physically inactive and/or immobilized. These critical conditions include sepsis (26, 44), burn injury (17), and surgical trauma/stress (26, 36, 40). In all of these conditions, including immobilization and critical illness, the effect of insulin on potassium uptake by the cell seems unaltered. Exercise, in contrast to immobilization, increases insulin sensitivity in humans and rodents (12, 21). Although short-term muscle contraction or electrical stimulation (for ~60 min) increases glucose uptake through insulin-independent mechanisms and has no effect on basal and insulin-stimulated tyrosine phosphorylation of IR-β and IRS-1 (12, 43), long-term exercise is associated with improved insulin sensitivity (7, 14). One week of exercise leads to increased insulin sensitivity with enhanced PI 3-K activity in humans (14), and 6 h of swimming per day for 1 or 5 days results in increases in insulin-stimulated IRS-1 phosphorylation and PI 3-K activation in rats (7). Exercise for 9 wk increases protein or mRNA levels of IR, IRS-1, and the p85 regulatory subunit of PI 3-K in rats (21). It is unclear, however, whether or how the converse, namely, chronic muscle disuse and/or immobilization, alters insulin signaling, al-

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though immobilization and muscle disuse are known to decrease insulin-stimulated glucose uptake.

IRS-1, in particular, is a key molecule for insulin action in skeletal muscle (20). Gene knockout of IRS-1 leads to peripheral insulin resistance in mice (2). It is reported that IRS-1 is the major tyrosine-phosphorylated protein bound to the regulatory subunit of PI 3-K (p85) in skeletal muscle in mice, whereas IRS-2 is only weakly associated with PI 3-K (39). Accordingly, IRS-2 is not necessary for insulin-stimulated glucose transport in skeletal muscle (13). IRS-3 and IRS-4 are not expressed in skeletal muscle (23, 24). Therefore, in the present study, we investigated the effect of immobilization on insulin-stimulated activation of IR, IRS-1, and PI 3-K.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (175–200 g), purchased from Taconic Farms (Germantown, NY), were used for this study. The study was approved by the Institutional Animal Care Committee. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The rats were housed in mesh cages in a room maintained at 25°C and illuminated by 12:12-h light-dark cycles. The rats were provided with standard rodent chow and water ad libitum. All surgical procedures were performed under anesthesia with 70 mg/kg pentobarbital sodium injected intraperitoneally.

Immobilization and insulin injection. The left hindlimb was immobilized by pinning the knee at 90° flexion and ankle at 90° dorsiflexion. The ankle and knee joints were immobilized, respectively, by inserting 25-gauge hypodermic needles through the calcaneus into the distal tibia, and through the proximal tibia into the distal femur, as described previously (15, 16). On the sham-immobilized contralateral side, the limb was subjected to the same manipulations, including boring of a hole through the joints, but a pin was not inserted to immobilize the joint. Thus pain was probably similar on both sides. We had previously shown that sham-immobilization of the contralateral knee and ankle joints did not alter muscle function relative to unimmobilized hindlimbs of naive animals (15, 16). In these studies (15, 16), body weight changes and muscle morphology, acetylcholine receptor changes, wet weight, and contraction in the tibialis muscle were not different between the unimmobilized contralateral side and naive separate sham-immobilized animals, indicating that the contralateral side does not undergo compensatory exercise-induced hypertrophy. In the present study, therefore, the contralateral unimmobilized hindlimb served as control.

At 7 days of immobilization, food was withdrawn for 18 h, the rat was then anesthetized, and 2.5 mU/g body weight of human insulin (Humulin R, Eli Lilly, Indianapolis, IN) was diluted with saline, or saline alone, was injected into the portal vein, as described previously (10, 17). The tibialis anterior muscles of both hindlimbs were removed at 4 min after insulin or saline injection and then frozen in liquid nitrogen.

Detection of tyrosine phosphorylation of IR-β and IRS-1. The frozen muscle tissue was minced with surgical scissors for 1 min in ice-cold lysis buffer [50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 10 mM sodium fluoride, 2 mM sodium pyrophosphate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.5 μg/ml pepstatin]. The samples were prepared for SDS-PAGE by a Polytron PT-MR 3000 (KINEMATIKA AG, Littau, Switzerland) at maximum speed for 30 s. The homogenates were kept on ice for 30 min. The insoluble material was removed by centrifugation at 12,000 rpm for 30 min. Aliquots of the supernatants containing equal amounts of protein, as determined by the Bradford protein assay, were subjected to immunoprecipitation for 1 h at 4°C with anti-IR-β mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-IRS-1 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY). After the addition of protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ), the immunoprecipitates were washed three times in 50 mM HEPES-NaOH (pH 7.5) with 150 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 0.5 μg/ml pepstatin. The samples were prepared for SDS-PAGE by addition of Laemmli sample buffer (Boston Bioproducts, Ashland, MA) and boiling for 5 min.

The immunoprecipitates were subjected to SDS-PAGE in 7.5% acrylamide resolving gels and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then blocked in 5% dried milk in PBS containing 0.1% Tween 20 (PBS-Tween) for 2 h at room temperature and immunoblotted with anti-phosphotyrosine (PY99; Santa Cruz Biotechnology), anti-IR-β, or anti-IRS-1 antibody for 1 h at room temperature. After three washes with PBS-Tween, the membrane was incubated for 30 min with anti-mouse or -rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The antigen-antibody complexes were visualized using chemiluminescence luminol reagent (Santa Cruz Biotechnology). Bands of interest were scanned by use of MD-4000 (Alps Electric, San Jose, CA) and were quantified with National Institutes of Health Image 1.61 software (NTIS, Springfield, VA).

Protein expression of IRS-1 and p85. The changes in protein expression of IRS-1 and p85 regulatory subunit of PI 3-K after immobilization were then investigated. Aliquots of the muscle homogenates containing equal amounts of protein were subjected to SDS-PAGE and were immunoblotted with anti-IRS-1 rabbit polyclonal antibody or anti-p85 rabbit polyclonal antibody, respectively (Upstate Biotechnology). The bands of interest were scanned as described above.

Detection of p85 associated with IRS-1. The frozen muscle tissue was minced with surgical scissors for 1 min in ice-cold lysis buffer and was then homogenized, as described previously. After sonication (Sonic disembrator, MODEL300, Fisher, Pittsburgh, PA), the samples were kept on ice for 30 min. Insoluble material was removed by centrifugation at 12,000 rpm for 30 min. Aliquots of the supernatants containing equal amounts of protein were subjected to immunoprecipitation for 1 h at 4°C with anti-IRS-1 rabbit polyclonal antibody, provided by Drs. K. Yonezawa and K. Hara (see Ref. 46). The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After blocking in 5% dried milk in PBS-Tween, the membranes were incubated with anti-PI 3-K p85 rabbit polyclonal antibody (Upstate Biotechnology) followed by incubation with anti-rabbit IgG antibody conjugated with horseradish peroxidase and visualization by chemiluminescence. The bands of interest were scanned as described above.

PI 3-K activity assay. PI 3-K activity in the immunoprecipitates obtained using anti-IRS-1 rabbit polyclonal antibody (46) was measured in vitro by its ability to phosphorylate exogenous phosphatidylinositol (Sigma, St. Louis, MO) to phosphatidylinositol monophosphate, as described previously (17), with minor modifications. Briefly, 10 μl of 100 mM MgCl₂ and 10 μl of PI (2 mg/ml) dissolved in 10 mM Tris·HCl...
RESULTS

The weight of the tibialis anterior muscle was significantly \( n = 8, P < 0.01 \) smaller in the 7-day-immobilized hindlimb \( (366 \pm 30 \text{ mg}) \) compared with the contralateral control hindlimb \( (531 \pm 27 \text{ mg}) \). To assess tyrosine phosphorylation of IR-\( \beta \) or IRS-1, muscle homogenates from immobilized and contralateral limbs were immunoprecipitated with anti-IR-\( \beta \) or anti-IRS-1 antibodies followed by immunoblotting with anti-phosphotyrosine antibody. The basal level of tyrosine phosphorylation of IR-\( \beta \) was higher in immobilized muscle than in control muscle (Fig. 1A; \( P < 0.05 \)). By contrast, insulin-stimulated tyrosine phosphorylation of IR-\( \beta \) was attenuated in immobilized muscle (Fig. 1A; \( P < 0.02 \)). IR protein level did not differ between the immobilized and control muscles (Fig. 1B). This indicates that the quantitative decrease in tyrosine phosphorylation of IR-\( \beta \) after insulin treatment in the immobilized muscle was not due to decreased IR-\( \beta \) protein expression but resulted from the impaired activation of IR.

Consistent with the attenuation of phosphorylation of IR-\( \beta \), tyrosine phosphorylation of IRS-1 after insulin treatment was also decreased in the immobilized hindlimb (Fig. 2A; \( P < 0.02 \)). However, in contrast to IR-\( \beta \), the recovery of IRS-1 protein after immunoprecipitation was also decreased significantly in the immobilized hindlimb compared with the contralateral control hindlimb (Fig. 2B; \( P < 0.02 \)). Simple Western blotting of the homogenates with anti-IRS-1 antibody also confirmed the reduced expression of IRS-1 in immobilized hindlimb (Fig. 3A; \( P < 0.01 \)). Thus the apparent decrease in phosphorylation of IRS-1 may be due to decreased protein expression and/or decreased activation of IR-\( \beta \), the activation of IR-\( \beta \) being a crucial initial step for subsequent phosphorylation of IRS-1 (22).

To assess the relative contributions of the decrease in IRS-1 protein expression and the decrease in the kinase activation of IR-\( \beta \) to the decrease in phosphorylation of IRS-1, the percent decline in tyrosine phosphorylation of each protein was normalized to the level of the recovery of the corresponding protein after immunoprecipitation. Insulin-stimulated tyrosine phosphorylation of IR-\( \beta \) and IRS-1 was reduced in immobilized muscle to 54.7% \( (P < 0.02) \) and 35.3% \( (P < 0.02) \), respectively.
respectively. Although IR-β protein level did not change significantly in immobilized muscle (90.0% of contralateral control muscle), IRS-1 protein level decreased to 66.3% \( (P < 0.02) \) after immobilization. Thus the insulin-stimulated percent tyrosine phosphorylation of IR-β and IRS-1 in the tibialis anterior muscle, when normalized to the IR-β and IRS-1 protein levels, was downregulated to 61.9% (Fig. 1, \( P < 0.02 \)) and 53.1% (Fig. 2; \( P < 0.02 \)), respectively. These results indicate that the apparent reduction of IRS-1 tyrosine phosphorylation derives from a decline in both protein expression of IRS-1 and tyrosine phosphorylation of the remaining IRS-1 protein, the latter probably resulting from impaired kinase activation of IR.

To assess the consequence of the hypophosphorylation of IRS-1 on the further downstream signal transduction components, the association of IRS-1 with PI 3-K and IRS-1-associated PI 3-K activity was examined. The immuno-precipitates obtained with anti-IRS-1 antibody were subjected to immunoblotting with anti-PI 3-K p85 antibody. In the contralateral control hindlimb, insulin stimulation resulted in a marked increase in the amount of p85 bound to IRS-1. However, insulin-stimulated binding of p85 to IRS-1 was reduced to 49.8% \( (P < 0.02) \) in immobilized muscle (Fig. 4). In accordance with the attenuated binding of PI 3-K to IRS-1, insulin-stimulated PI 3-K activity was impaired in immobilized muscle compared with the contralateral control (Fig. 5, \( P < 0.02 \)). This decrease in binding of IRS-1 to p85 and in PI 3-K activity was not due to decreased protein expression of p85; the protein expression assessed by simple Western blotting was not different between immobilized and contralateral sides (Fig. 3B).

**DISCUSSION**

The present study clearly demonstrates that immobilization per se attenuated insulin-stimulated tyrosine phosphorylation of IR-β and IRS-1, the binding of IRS-1 to PI 3-K, and IRS-1-associated PI 3-K activation in skeletal muscle. IRS-1 protein expression was also downregulated in immobilized muscle (Fig. 2), whereas IR-β and p85 protein levels were not altered (Figs. 1B and 3B). Not only was the total amount of phosphorylated IRS-1 decreased to 35.3%, but also the percent phosphorylation of IRS-1, normalized to IRS-1 protein level, was reduced to 53.1% in the immobilized limb relative to contralateral control muscle. This indicates that reduction in insulin-stimulated IRS-1 phosphorylation in immobilized muscle is attributable to the decreases in both protein expression of IRS-1.
reported previously in patients with type 2 diabetes and a reduction in IRS-1 protein expression have been observed on both sides. Holes and excepting maintenance of pins, were similar the same, because all procedures, including boring of the immobilized and contralateral sides was probably localized side and separate naive controls (15, 16). Pain in the differences between the contralateral sham-immobilized and control sides; previous studies of immobilization, therefore cannot account for the decreased binding of IRS-1 to PI 3-K and decreased activation of PI 3-K. The finding of unaltered expression of p85 is consistent with previous studies of insulin resistance with burn (17). Altered expression of the p110 catalytic subunit of PI 3-K to IRS-1 is a key event for insulin-stimulated PI 3-K activation, and this binding is dependent on tyrosine phosphorylation of IRS-1. A decrease in tyrosine phosphorylation of IRS-1 may thus account for reductions in insulin-stimulated binding of p85 to IRS-1 (Fig. 4) and also IRS-1-associated PI 3-K activation (Fig. 5). A decreased protein expression of p85 was not observed and therefore cannot account for the decreased binding of IRS-1 to PI 3-K and decreased activation of PI 3-K. The decreased protein expression of IRS-1, phosphorylation of IRS-1, and activation of PI 3-K after immobilization, therefore, contrast with the decreases seen in exercise (12, 21). Long-term exercise was associated with increases in protein or mRNA levels of IR, IRS-1, and p85 (21), together with increases in insulin-stimulated IRS-1 phosphorylation and PI 3-K activation (7). Increased expression of p110, however, has also not been observed after prolonged exercise. In our study, the differences between the immobilized and contralateral sides cannot be attributed to overuse (exercise) of the contralateral side; previous studies of function and morphology, which included measurements of fiber size, contraction, fade with tetanus, wet weights, and receptor expression, did not show any differences between the contralateral sham-immobilized side and separate naive controls (15, 16). Pain in the immobilized and contralateral sides was probably the same, because all procedures, including boring of holes and excepting maintenance of pins, were similar on both sides.

A decrease in the phosphorylation capability of IR and a reduction in IRS-1 protein expression have been reported previously in patients with type 2 diabetes (31) and in genetically obese diabetic (ob/ob) mice (19). We have also shown that the insulin resistance after thermal injury was associated with attenuated tyrosine phosphorylation of IR-β and IRS-1 (17). A previous study also revealed that mice with heterozygous knockouts of both IR and IRS-1 exhibit insulin resistance and diabetes, whereas heterozygous targeting of either one of these genes was associated with no obvious phenotype of diabetes (5). Taken together, these findings are supportive of the notion that the combination of functional defects in IR and IRS-1 may be important in the pathogenesis of insulin resistance. They also suggest that a common molecular mechanism, involving IRS-1-mediated signaling, may underlie the insulin resistance of obesity, burn injury, and immobilization. Recent studies have also suggested that other IRS-1-like docking proteins, including Gab1 and p62 (Dok), may play an important role in insulin signaling (2, 22, 42). These molecules, when tyrosine phosphorylated in response to insulin, bind to PI 3-K. Thus it would be of interest also to analyze, in future studies, the roles of these signaling molecules in the altered insulin signaling after immobilization (and exercise).

It is important to reiterate that protein expression of the insulin receptor, as assessed by immunoblots, was not altered (Fig. 1B). Thus the decreased phosphorylation of IR-β seems to be related to an intrinsic mechanism inhibiting IR-β phosphorylation. Numerous factors, including epinephrine and cytokines, particularly tumor necrosis factor (TNF), are potential candidates that may play an inhibitory role on insulin signaling (30, 34). Stress of immobilization itself can result in release of catecholamines and steroidal hormones. Because these mediators are released systemically, the effects would have been evident not only on the immobilized side but also on the contralateral control side. The differences observed in our study between the immobilized and contralateral sides, in the same animals, are therefore not consistent with a systemic effect.

Recently, TNF has been shown to play a central role in insulin resistance (34). Neutralization of TNF alleviated insulin resistance. Interestingly, this effect was attributed to TNF produced locally in muscle and adipose tissues, because serum concentrations of TNF in both lean and obese diabetic mice is low. This suggests that TNF acts in a paracrine and/or an autocrine manner. TNF can convert IRS-1 to hyperserine-phosphorylated form and render it an inhibitory molecule to insulin receptor kinase (34). Increased levels of TNF are also associated with concomitant upregulation of inducible nitric oxide synthase (iNOS) and insulin resistance. Furthermore, the iNOS inhibitor aminoguanidine reversed the TNF-induced impaired insulin-stimulated glucose transport in cultured muscle cells (3). Evidence for local expression of TNF in muscle after immobilization has not been demonstrated previously. However, in hindlimb unloading, a form of immobilization, the administration of the iNOS inhibitor Nω-nitro-L-arginine (L-NAME) decreased the inflammatory response associated with muscle disuse (28), suggesting that iNOS expression may be increased in immobilization. Thus the relationship between local
expression of TNF, iNOS, and insulin resistance after immobilization deserves further study.

Among the effects of insulin in muscle, uptake of glucose and protein synthesis are cardinal. Increased glucose uptake and glycogen synthesis occur through translocation of the insulin-sensitive glucose transport GLUT-4 and activation of glycogen synthase (34). Protein synthesis is regulated by phosphorylation of eukaryotic initiation factor 4E-binding proteins. Many of the metabolic actions of insulin, except its effect on potassium, have been documented to be mediated by PI 3-K via the activation of its downstream serine/threonine protein kinases, Akt/protein kinase B (PKB), and an atypical isoform of protein kinase C. Hence, wortmannin, a specific inhibitor of PI 3-K, inhibits insulin-induced protein synthesis. Furthermore, PI 3-K and Akt/PKB are pivotal in a pathway that conveys survival signals. Specific inhibition of PI 3-K by wortmannin or LY-294002 enhances apoptosis (6). Thus activation of PI 3-K by insulin and/or insulin-like growth factor I enhances protein synthesis and blocks apoptosis (6, 11).

The overall rate of protein synthesis and degradation in tissue tightly controls muscle mass (8, 25, 27). Insulin is known not only to stimulate protein synthesis but also to inhibit protein degradation in skeletal muscle (18). Many conditions associated with muscle wasting, including immobilization, burns, sepsis, and autoimmunodefiency syndrome (AIDS), are also associated with decreased insulin signaling (insulin resistance), despite normal or elevated plasma insulin levels (29, 33, 36, 37, 40). In these instances, protein catabolism outweighs protein synthesis, regardless of the fact that protein synthesis itself can sometimes be enhanced in these conditions (32, 33, 44). Furthermore, apoptosis is associated with muscle wasting during hindlimb unweighting (1) and also after burn injury (45). As indicated previously, PI 3-K activation is a pivotal antiapoptotic signaling molecule (6, 11, 41). Taken together, the decreased glucose uptake, decreased protein synthesis, increased protein degradation, muscle atrophy, and apoptosis previously observed after muscle disuse or immobilization might be related to decreased insulin action and defective insulin signaling via PI 3-K. Therefore, correction of insulin resistance may retard the immobilization-induced muscle wasting, including apoptosis and the associated muscle weakness.

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