# Long-Term Denervation Impairs Insulin Receptor Substrate-1–Mediated Insulin Signaling in Skeletal Muscle

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**Long-term denervation is associated with insulin resistance. To investigate the molecular bases of insulin resistance, the downstream signaling molecules of insulin receptor including insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI 3-K) were examined in skeletal muscle of rats after 7 days of denervation. Long-term denervation attenuated insulin-stimulated activation of the initial steps of the intracellular signaling pathway. Insulin-stimulated tyrosine phosphorylation of insulin receptor was reduced to 36% ($P < .005$), as was the phosphorylation of IRS-1 to 34% ($P < .0001$) of control. While insulin receptor protein level was unchanged, the protein expression of IRS-1 was significantly decreased in denervated muscles. Insulin-stimulated percent tyrosine phosphorylation of IRS-1, normalized to the IRS-1 protein expression, was also reduced to 55% ($P < .01$) of control in denervated muscle. Denervation caused a decline in the insulin-induced binding of p85 regulatory subunit of PI 3-K to IRS-1 to 61% ($P < .001$) and IRS-1–associated PI 3-K activity to 57% ($P < .01$). These results provide evidence that long-term denervation results in insulin resistance because of derangements at multiple points, including tyrosine phosphorylation of insulin receptor and its downstream signaling molecule, IRS-1, protein expression of IRS-1, and activation of PI 3-K.**



INSULIN ACTIVATES MULTIPLE signaling pathways through autophosphorylation of specific tyrosine residues of the intracellular portion of insulin receptor $\beta$-subunit (IR$\beta$), leading to diverse effects on cellular metabolism and mitogenesis. Among these insulin-induced signaling pathways, phosphatidylinositol 3-kinase (PI 3-K) and extracellular receptor kinase (ERK) pathways are the 2 major signal transduction cascades. PI 3-K pathway is believed to be central for the metabolic actions of insulin, including glucose transport and protein synthesis.[1-4] The activated IR phosphorylates endogenous substrate proteins, such as insulin receptor substrates (IRSs) and Shc.[5] Further downstream of IRS-1 is another pivotal molecule, PI 3-K. This consists of regulatory and catalytic subunits. The p85 regulatory subunit of PI 3-K binds to multiple YXXM or YMXM motifs of IRS proteins. The binding of IRS proteins to PI 3-K, in turn, activates the latter, which transduces signals to activate the downstream signaling molecules including serine/threonine protein kinase, Akt/PKB.[6]

Denervation of skeletal muscle results in insulin resistance,[7-9] evidenced as decreased glucose uptake in skeletal muscle, in response to insulin. This phenomenon starts as early as 3 to 6 hours after denervation and continues for at least 17 days.[9] However, the molecular mechanisms involved in denervation-induced insulin resistance are not well understood. Elmendorf et al,[10] and Turinsky et al[11] examined the effects of denervation on insulin signaling. One day after denervation, no changes in IR phosphorylation or PI 3-K activity were observed. Three days after denervation insulin-stimulated IR phosphorylation and PI 3-K activation were reduced.[10] Consistent with changes in IR and PI 3-K, Akt1 kinase activity was unchanged at day 1 and depressed at day 3 after denervation.[11] Changes in insulin transduction after longer-term denervation remains to be investigated.

IRS-1, in particular, is a key molecule for insulin action in skeletal muscle. Gene knock out of IRS-1 leads to insulin resistance in mice.[12] IRS-1 is the major tyrosine-phosphorylated protein bound to the regulatory subunit of PI 3-K (p85$\alpha$) in skeletal muscle in mice and IRS-2 and IRS-3 are only weakly associated with PI 3-K.[4] Accordingly, IRS-2 is not necessary for insulin-stimulated glucose transport in skeletal muscle.[13] Recent data obtained by tissue-specific heterozygons knock out of IRS-1 and IRS-2 also showed that IRS-1 plays a prominent role in insulin action in skeletal muscle, in contrast to the minor role of IRS-2 in muscle.[14] IRS-3 and IRS-4 are not expressed in skeletal muscle.[15,16] Therefore, in the present study, we focused on insulin-stimulated signal transduction cascade, IRS-1–mediated signal transduction, and its immediate pre and postsignaling molecules, IR and PI 3-K, respectively, following long-term (7 days) denervation of skeletal muscle.

## MATERIALS AND METHODS

### *Animals*

Adult male Sprague-Dawley rats (175 to 200 g) purchased from Taconic Farms (Germantown, NY) were used. The Institutional Animal Care Committee approved the study protocol. 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, illuminated 12:12 hour cycles, and provided with standard rodent chow and water ad libitum. All surgical procedures were performed under anesthesia with 70 mg/kg pentobarbital sodium injected intraperitoneally.

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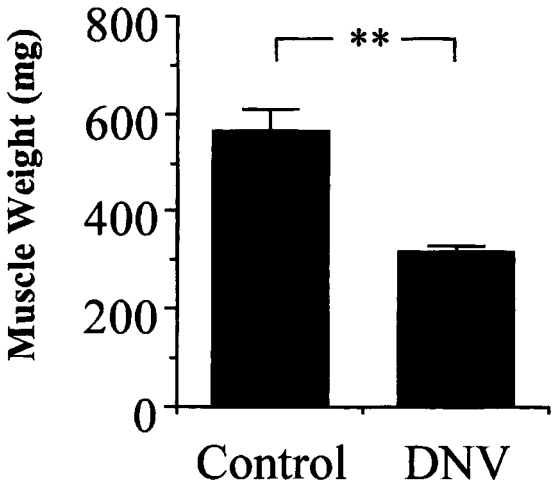


**Fig 1. Weight of tibialis anterior muscle in sham-denervated (Control) and denervated (DNV) rats. The weight of denervated muscle was significantly less than sham-denervated muscle. Values are mean ± SEM, n = 4, **$P < .005$ *v* control.**

### *Denervation*

The left hind limb of each rat was denervated. The sciatic nerve was exposed through a 1 to 2 cm incision on the lateral surface of the thigh and blunt separation of the thigh muscles, and a 5-mm segment of the sciatic nerve was excised. In separate control animals, the sciatic nerve of the left hind limb was exposed in the same way, but the nerve was not cut (sham-denervation). The incision was closed with a 4-0 nylon suture.

On day 7 after denervation or sham-denervation, food was withdrawn for 18 hours, and the rats were anesthetized. Under anesthesia, 2.5 mU/g body weight of human insulin (Humulin R; Eli Lilly, Indianapolis, IN) diluted in saline or saline alone was injected into the portal vein as described previously.[17,18] The tibialis anterior muscle of the left hind limb from the denervated and sham-denervated rats was removed at 4 minutes after insulin or saline injection, and frozen in liquid nitrogen.

### *Detection of Tyrosine Phosphorylation of IRβ and IRS-1*

The frozen muscle tissues were minced in ice-cold lysis buffer (50 mmol/L HEPES/NaOH, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40, 10% glycerol, 10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium pyrophosphate, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5 μg/mL pepstatin), and thereafter were homogenized using Polytron PT-MR 3000 (KINEMATIKA AG, Littau, Switzerland). After centrifugation, aliquots of the supernatant containing equal amounts of protein, as determined using the Bradford protein assay, were subjected to immunoprecipitation for 1 hour at 4°C with anti-IRβ mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti–IRS-1 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY). Following the addition of protein A-sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ), the immunoprecipitates were washed 3 times in washing buffer.

The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide solving gels and transferred to nitrocellulose membrane (Bio-Rad, Herculus, CA). After blocking with in 5% nonfat dried milk, the

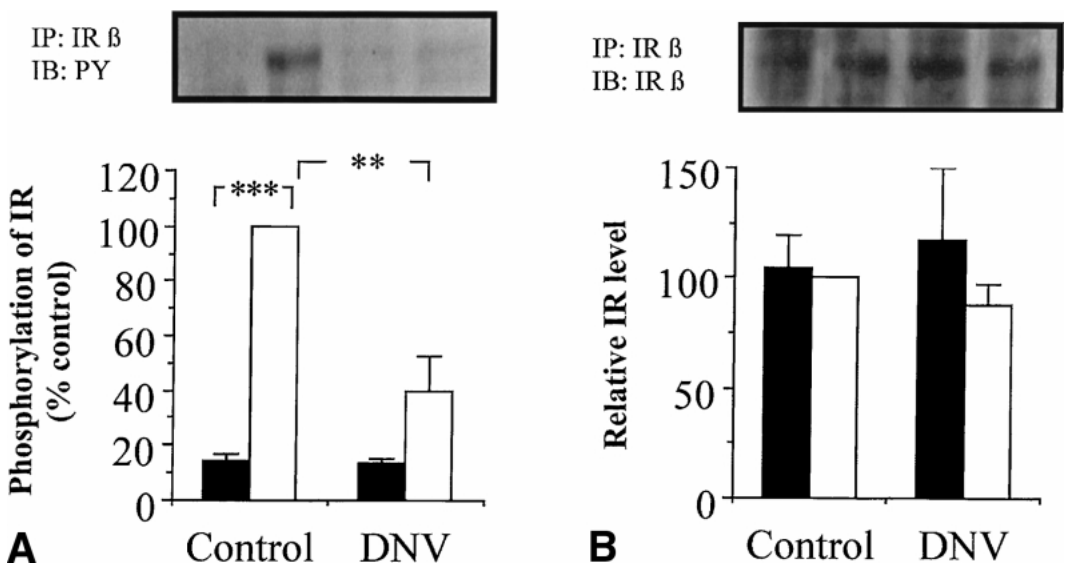


**Fig 2. Levels of (A) tyrosine phosphorylation of insulin receptor (IR) β and (B) IRβ protein expression in the denervated (DNV) and sham-denervated (Control) muscle. Equal amounts of protein, extracted from sham-denervated and denervated muscles were subjected to immunoprecipitation (IP) with anti-IRβ antibody and subjected to immunoblotting (IB) with antiphosphotyrosine (PY) shown in (A) or anti-IRβ (IRβ) antibody shown in (B). Phosphorylation of each protein was normalized to the level of the corresponding protein and expressed as percent phosphorylation. The solid and open bars on lower panel represent basal and insulin-stimulated levels, respectively. Intensities of the bands of interest in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. (A) Insulin-stimulated tyrosine phosphorylation of IRβ was attenuated in the DNV compared with the control muscle. (B) IRβ protein content was not altered, however, following denervation. The results displayed on the upper panels of (A) and (B) represent typical immunoblots. Values are mean ± SEM, n = 4 for each group, **$P < .005$, ***$P < .0001$.**

membranes were immunoblotted with antiphosphotyrosine (PY99) (Santa Cruz Biotechnology), anti-IR$\beta$ or anti–IRS-1 antibodies for 1 hour at room temperature. After washing 3 times with PBS-Tween buffer (phosphate-buffered saline containing 0.1% Tween 20), the membrane was further incubated with antimouse IgG or antirabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 30 minutes. Blots were detected by Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology). The bands of interest were scanned by using MD-4000 (Alps Electric, San Jose, CA) and were quantified by using National Institutes of Health Image 1.61 software (NTIS, Springfield, VA).

### *Protein Expression of IRS-1*

To confirm the change in IRS-1 protein expression after denervation, the muscle homogenates containing equal amounts of protein were subjected to SDS-PAGE for immunoblotting with anti–IRS-1 rabbit polyclonal antibody (Upstate Biotechnology). The bands of interest were scanned as described above.

### *Detection of Binding of p85 to IRS-1*

The frozen tissues were minced in ice-cold lysis buffer, and then homogenized. After sonication (Sonic dismembrator, MODEL 300; Fisher, Pittsburgh, PA), the samples were kept on ice for 30 minutes. After centrifugation, the supernatant was collected and equal amounts of protein were subjected to immunoprecipitation for 1 hour at 4°C with anti–IRS-1 polyclonal antibody, provided by Drs K. Yonezawa and K. Hara,[19] followed by immunoblotting with anti-PI 3-K p85 rabbit polyclonal antibody (Upstate Biotechnology). After treatment with anti-IgG antibody with horseradish peroxidase, the bands of interest were scanned as described above.

### *PI 3-K Activity Assay*

PI 3-K activity in the immunoprecipitates obtained with anti–IRS-1 rabbit polyclonal antibody[18] was measured in vitro by its ability to phosphorylate exogenous phosphatidylinositol (Sigma, St Louis, MO), to phosphatidylinositol monophosphate, as described previously.[16] Briefly, 10 $\mu$L of 100 mmol/L $MgCl_2$ and 10 $\mu$L of PI (2 mg/mL) dissolved in 10 mmol/L Tris/HCl pH 7.5 containing 1 mmol/L EGTA were added to the immunoprecipitates. PI 3-K reaction was initiated by the addition of 10 $\mu$L of 440 mmol/L adenosine triphosphate (ATP) containing 20 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. After 10 minutes at 37°C, the reaction was stopped by the addition of 20 $\mu$L of 8 N HCl and 160 $\mu$L of CHCl3/methanol (1:1). The samples were centrifuged (at 13,000 for 10 minutes), and the lower organic phase was applied to a silica gel TLC plate (Whatman, Clifton, NJ). The plate was developed in CHCl3/CH3OH/H2O/NH4OH (60:47:11:3.2), dried, and visualized by autoradiography. The bands of interest were scanned as described above.

### *Statistical Analysis*

The levels of tyrosine phosphorylation of IR$\beta$ and IRS-1, the protein expression of IR$\beta$ and IRS-1, the binding of p85 subunit of PI 3-K to IRS-1, and the IRS-1–associated PI 3-K activity in each muscle were expressed as a percent of the corresponding level in the insulin-stimulated control muscle. Tyrosine phosphorylation of IR$\beta$ and IRS-1, protein expression of IRS-1, the binding of p85 to IRS-1 and IRS-1–associated PI 3-K activity were compared using 1-way analysis of

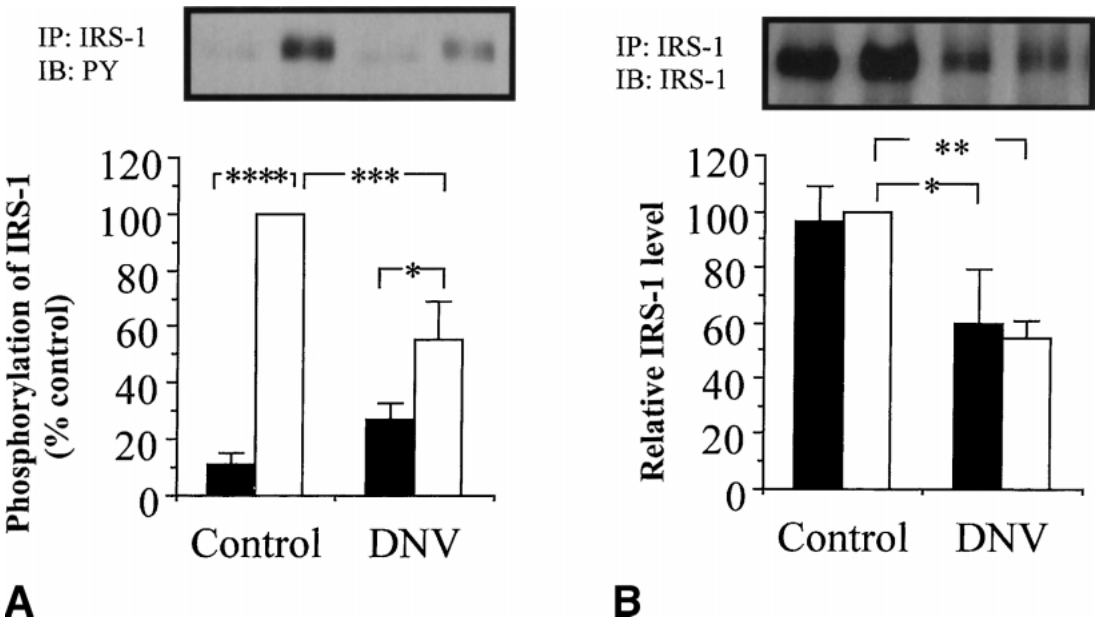


**Fig 3. Levels of (A) tyrosine phosphorylation of IRS-1 and (B) IRS-1 protein expression in the denervated (DNV) and sham-denervated (Control) muscle. Equal amounts of protein, extracted from sham-denervated and denervated muscle, were subjected to immunoprecipitation (IP) with IRS-1 antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblotting (IB) with antiphosphotyrosine (PY) or anti–IRS-1 antibody (IRS). The solid and open bars on the lower panel represent basal and insulin-stimulated levels, respectively. Intensities of the bands of interest in the autoradiograms were normalized to the levels in the insulin-stimulated sham-denervated muscles, denoted as 100%. Denervation was associated with the reductions in both insulin-stimulated tyrosine phosphorylation of IRS-1, (A) and also IRS-1 protein expression (B). The results displayed on the upper panels of (A) and (B) represent typical immunoblots. Values are mean ± SEM, n = 4 for each group, *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.**

variance (ANOVA) followed by Scheffe's F-test. The null hypothesis was rejected when $P < .05$. All values are reported as means ± SEM.

## RESULTS

The body weight of sham-denervated rats increased from 188.5 ± 4.2 g to 205.5 ± 6.2 g during the 7 days. Denervated rats also showed an increase in body weight from 185.5 ± 3.5 to 197.5 ± 7.8 g. Although denervated rats tended to show a blunted increase in body weight, there was no significant difference in body weight between the 2 groups. In contrast, the weight of the tibialis anterior muscle was significantly lower in the denervated compared with sham-denervated leg (Fig 1).

To assess tyrosine phosphorylation of IR$\beta$ and IRS-1, homogenates of tibialis muscles from the sham-denervated and denervated legs were subjected to immunoprecipitation with anti-IR$\beta$ or anti–IRS-1 antibodies followed by immunoblotting with antiphosphotyrosine antibody. Intraportal injection of insulin resulted in a significant increase in tyrosine phosphorylation of IR$\beta$ in tibialis of sham-denervated animals (Fig 2A). This effect of insulin on tyrosine phosphorylation of IR$\beta$ was attenuated in denervated animals. IR$\beta$ protein levels, however, were unaltered between sham and experimental groups (Fig 2B). This latter finding indicates that the decreased tyrosine kinase activation of IR following insulin treatment after long-term denervation is unrelated to altered protein quantities. These results are in agreement with a previous report of attenuated IR$\beta$ phosphorylation at 3 days after denervation.[10]

Consistent with the resistance to insulin-induced IR$\beta$ phosphorylation, tyrosine phosphorylation of IRS-1 following insulin treatment was also decreased in denervated rats (Fig 3). However, in contrast to IR$\beta$, the recovery of IRS-1 after immunoprecipitation was also decreased significantly in denervated muscle compared with sham-denervated muscle (Fig 3B; $P < .01$). Simple Western blotting of the homogenates with anti–IRS-1 antibody confirmed the reduced expression of IRS-1 in denervated muscle (Fig 4; $P < .02$). Thus, the apparent decrease in phosphorylation of IRS-1 may be due to decreased protein expression and/or to decreased activation of IR$\beta$, the activation of IR$\beta$ being a crucial initial step for subsequent phosphorylation of IRS-1.[5]

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 percentage 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 denervated muscle to 35.7% ($P < .005$) and 33.6% ($P < .0001$), respectively. While IR$\beta$ protein level was not altered significantly in denervated muscle (87.4% of sham-denervated muscle), IRS-1 protein level decreased to 54.7% ($P < .01$) following denervation. Thus, the insulin-stimulated percent tyrosine phosphorylation of IR$\beta$ and IRS-1 in the tibialis anterior muscle, when normalized to the IR$\beta$ and IRS-1 protein levels, were downregulated to 39.7% (Fig 2, $P < .005$) and 55.3% (Fig 3; $P < .001$), respectively. These results indicate that the apparent reduction of IRS-1 tyrosine phosphorylation derives both from a decline in protein expression of IRS-1, as well as a decline in tyrosine phosphorylation of the remaining IRS-1 protein, the latter probably resulting from impaired kinase activation of IR.

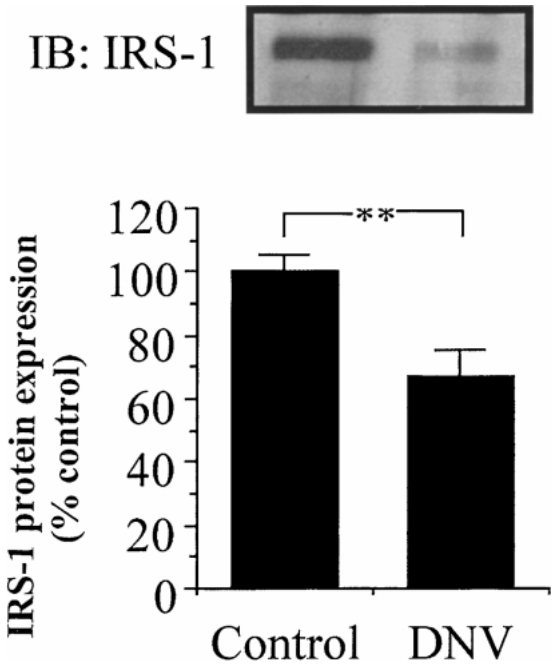


**Fig 4. Protein expression of IRS-1 following denervation. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to Western blot analysis with anti–IRS-1 antibody (IB). Intensities of the bands of interest in the autoradiograms were normalized to the levels in the insulin-stimulated sham-denervated muscles, denoted as 100%. Simple Western blotting showed reduced IRS-1 expression following denervation. The results displayed on the upper panel represents a typical immunoblot. Values are mean ± SEM, **$P < .02$, n = 6 for each group.**

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 immunoprecipitates with anti–IRS-1 antibody were subjected to immunoblotting with anti-PI 3-K p85 antibody. In the sham-denervated muscle, 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 61.0% ($P < .001$) at 7 days after denervation (Fig 5). In accordance with this attenuated binding of PI 3-K to IRS-1, insulin-stimulated PI 3-K activation was also impaired in denervated muscles compared with sham-denervated muscles (Fig 6; $P < .01$).

## DISCUSSION

The present study clearly shows that denervation of the tibialis anterior for 7 days impaired insulin-stimulated tyrosine phosphorylation of IR$\beta$ and IRS-1, binding of IRS-1 to PI 3-K, and IRS-1 associated PI 3-K activation, all essential steps in intracellular insulin signaling. While IR$\beta$ protein level was not altered (Fig 2), IRS-1 protein expression was downregulated in

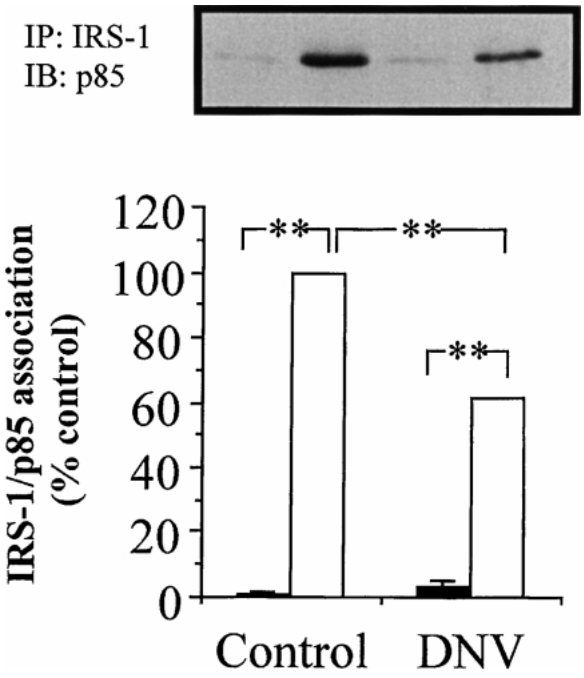


**Fig 5. Insulin-stimulated association of IRS-1 with PI 3-K following denervation. Equal amounts of protein, extracted from sham-denervated (Control) and denervated (DNV) muscle, were subjected to immunoprecipitation (IP) with IRS-1 antibody, and subjected to immunoblotting (IB) with anti-p85 (p85) or anti–IRS-1 antibody (IRS-1). The solid and open bar on the lower panel represents basal and insulin-stimulated levels, respectively. Intensities of the bands of interest in the autoradiograms were normalized to the levels in the insulin-stimulated sham-denervated muscles, denoted as 100%. Denervation caused a reduction in the association of p85 with IRS-1. The results displayed on the upper panel represent a typical immunoblot. Values are mean ± SEM, n = 4 for each group, **$P < .001$.**

denervated muscle (Figs 3 and 4). Not only was the total amount of phosphorylated IRS-1 decreased to 33.6%, but also the percent phosphorylation of IRS-1, normalized to IRS-1 protein level, was reduced to 55.3% in denervated muscle relative to sham-denervated muscle. This is indicative of reduction in insulin-stimulated IRS-1 phosphorylation in denervated muscle is attributable to the decreases in both protein expression of IRS-1 (Figs 3 and 4), and insulin-stimulated tyrosine kinase activation of IR (Fig 2). The binding of p85 regulatory 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 5) and also IRS-1–associated PI 3-K activation (Fig 6). These data together indicate that denervation attenuates insulin-stimulated intracellular signal transduction at multiple points to cause denervation-induced insulin resistance.

The impaired tyrosine phosphorylation of IR after long-term denervation (Fig 2) is in agreement with the previous observation by Elmendorf et al,[10] who observed the same at 3 days after denervation. That study, however, did not examine the effects on insulin signaling at the level of IRSs. Our experiments have expanded on these observations to the level of IRS-1 in terms of both phosphorylation (Fig 3) and protein expression (Figs 3 and 4). Of note, a role for low protein expression of IRS-1 in insulin resistance has recently attracted attention. A low IRS-1 expression seems to be a good marker to predict insulin resistance in type 2 diabetes (non–insulin-dependent diabetes mellitus) in humans.[20] Furthermore, the transfection of IRS-1, but not that of IRS-2 results in an 8-fold increase in tyrosine phosphorylation of IR in mouse 32D myeloid leukemia cells, which express neither IRS-1 nor IRS-2.[21] These data suggest that low expression of IRS-1 per se may play a major role in the impaired activation of the initial steps of insulin signaling.

Combined defects in the initial components of insulin signaling, namely, decreased phosphorylation capability of IR and reduction in IRS-1 protein abundance, have been observed in

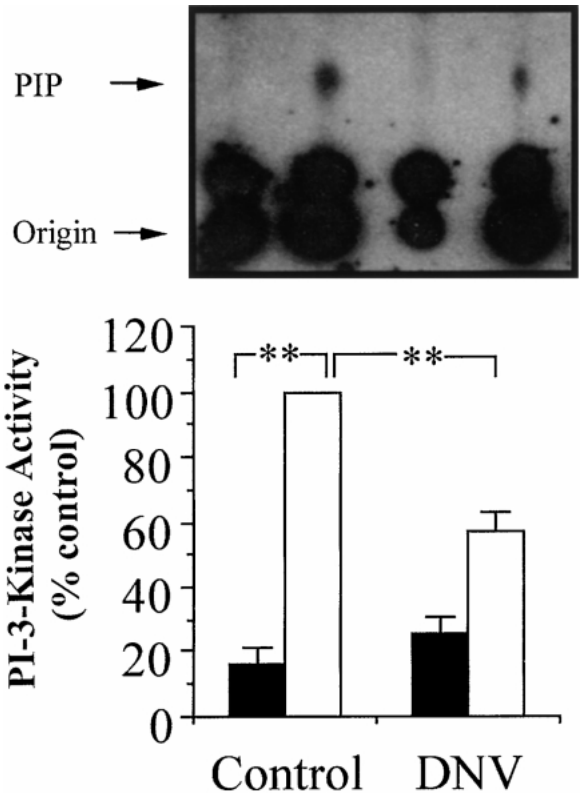


**Fig 6. Insulin-stimulated IRS-1–associated PI 3-K activation following immobilization. Equal amounts of protein were subjected to immunoprecipitation with IRS-1 antibody, and subjected to in vitro PI 3-K reaction. PI 3-K activities were assessed by the phosphorylation of phosphatidylinositol to phosphatidylinositol monophosphate (PIP). The solid and open bars on the lower panel represent basal and insulin-stimulated IRS-associated PI 3-K activity, respectively. Intensities of the bands of interest in the autoradiograms were normalized to the levels in the insulin-stimulated sham-denervated muscles, denoted as 100%. Denervation caused a reduction in insulin-stimulated activation of PI 3-K. The results displayed on the upper panel represent a typical experiment. Values are mean ± SEM, n = 4 for each group, $P < .01$.**

patients with type 2 diabetes[22] and in an animal model of insulin resistance, genetically obese diabetic (*ob*/*ob*) mice,[23] as well. Thus, common molecular mechanism(s) may underlie long-term denervation, obesity-induced insulin resistance, and type 2 diabetes. Ceramide, 1 of the proposed mediators of insulin resistance,[23-27] is known to be upregulated by long-term denervation[28] as is tumor necrosis factor (TNF)-$\alpha$, which induces insulin resistance in vitro[29,30] and in vivo.[31] In addition, protein tyrosine phosphatase activity, which can downregulate insulin sensitivity,[32] is augmented after long-term denervation.[33] Therefore, the upregulation of ceramide production and/or protein tyrosine phosphatase activity might be potential candidates of molecular events involved in insulin resistance observed in the present study. Further studies will be required to clarify this point.

Insulin resistance in diabetes, sepsis, acquired immunodeficiency syndrome (AIDS), burns, and denervation is associated with enhanced protein breakdown and decreased muscle mass.[7-9,34-36] Clinically, denervation produced pharmacologically with neuromuscular relaxants to facilitate mechanical ventilation also results in a denervation-like state with upregulation of acetylcholine receptors, muscle wasting, and persistent muscle weakness.[37] Recognized pathway for accelerated protein breakdown includes the ubiquitin-proteosome system and enhanced gluconeogenesis related to the release of cytokines (TNF-$\alpha$) and catabolic hormones (steroids, epinephrine).[34-36] More recently, apoptosis as a cause of muscle wasting has been observed following denervation, sepsis, and burns.[38-40] Increased expression of proapoptotic cytokines, Fas, or TNF-$\alpha$, and/or their second messengers, including stress activated protein kinase, and ceramide is seen in muscle following denervation and burns.[35,38,40] Increased expression of TNF-$\alpha$ and/or ceramide is also associated with insulin resistance and apoptosis.[24,26,41] The common pathway for insulin sensitization and antiapoptosis is enhanced PI 3-K activation.[6,42,43] Previously, it was demonstrated that postreceptor insulin signaling, including PI 3-K activation, was decreased in burns[17] and was associated with apoptosis.[39] In the present study, we document decreased activation of PI 3-K, which may also be related to increased apoptosis and atrophy in denervated muscle (Fig 1). Further studies should evaluate the cause and effect relationship between insulin signaling, muscle wasting, and apoptosis, and if the apoptosis and muscle wasting of denervation can be attenuated by reversal of insulin resistance.

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