# Increased Abundance of Specific Skeletal Muscle Protein-Tyrosine Phosphatases in a Genetic Model of Insulin-Resistant Obesity and Diabetes Mellitus

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**Resistance to the biological action of insulin in its target tissues is a cardinal feature of non–insulin-dependent diabetes mellitus. Protein-tyrosine phosphatases (PTPases) have been postulated to play a key role in the regulation of the insulin action pathway, especially in skeletal muscle, the major site of insulin-mediated glucose disposal in vivo. To evaluate whether changes in the activity and/or abundance of candidate skeletal muscle PTPases is associated with severe resistance to insulin in an animal model, we measured PTPase enzyme activity and PTPase protein level by immunoblotting in subcellular fractions of skeletal muscle in lean (+/?), insulin-resistant obese (*fa/fa*), and diabetic (ZDF/Drt-*fa/fa*) Zucker rats. Using a phosphotyrosyl-myelin basic protein substrate, the solubilized-particulate fraction PTPase activity was increased by 65% and 74% ($P < .05$) and in vitro dephosphorylation of a recombinant rat insulin receptor kinase domain was increased by 104% and 114% in obese and diabetic animals, respectively ($P < .01$). These changes in PTPase activity were associated with an increase in specific immunoreactivity of leukocyte common antigen-related PTPase ([LAR] by 42% and 50%), PTPase 1B (by 61% and 69%), and the SHZ domain containing PTPase (SH-PTP2) (by 44% and 48%) in the solubilized-particulate fraction of obese and diabetic animals, respectively ($P < .05$). In diabetic muscle, increased SH-PTP2 abundance was also associated with a shift of SH-PTP2 to a plasma membrane component, which may have important consequences for the activation of this enzyme in the insulin-resistant state. These results provide evidence that specific PTPases play a role in the insulin resistance of this genetic model of obesity and non–insulin-dependent diabetes.**



DEFECTIVE INSULIN SIGNALING in its target tissues is a major feature of the pathophysiology of obesity and non–insulin-dependent diabetes mellitus in humans and animal models for these diseases. Although major advances have been made in our understanding of the central role for reversible tyrosine phosphorylation in the mechanism of cellular insulin action,[1] we still do not have a clear picture of how these events are regulated, or of molecular defects that exist in many insulin-resistant disease states.

Several recent studies have provided evidence that protein-tyrosine phosphatases (PTPases) have an integral role in the regulation of insulin signal transduction.[2] In the insulin action pathway, PTPases can dephosphorylate and attenuate the active (autophosphorylated) form of the insulin receptor tyrosine kinase. In addition, PTPases may modulate postreceptor signaling by dephosphorylating the phosphotyrosyl form of cellular substrate proteins for the insulin receptor such as insulin receptor substrate-1 (IRS-1), which signals to downstream enzymes by its phosphotyrosyl domains as a "docking" protein that binds and activates a number of *src*-homology domain 2 (SH2)-containing signaling proteins.[1,3] The opposing effects of phosphorylation by the insulin receptor kinase and dephosphorylation by cellular PTPases will thus determine the steady-state regulation of signaling through the insulin action pathway. Several laboratories have provided evidence that alterations in the amount or regulation of PTPase activities in the cell may have an important role in the abnormal regulation of insulin action in insulin-resistant disease states (reviewed in Goldstein[2]). In recent studies of induced diabetes or aging in rodents, complex alterations in tissue PTPase activities have been demonstrated.[4-9] Moreover, compelling data for a potential pathogenetic role for skeletal muscle PTPases in insulin-resistant disease states have been provided by the demonstration of abnormal PTPase regulation and increases in particulate-fraction PTPase activity in muscle biopsies from nondiabetic insulin-resistant human subjects.[10]

Recently, we have identified three predominant PTPase homologs in skeletal muscle[2] that are candidates for regulation of the insulin action pathway in this important tissue, the predominant site of insulin-mediated glucose disposal in vivo and the clinical insulin resistance associated with diabetes mellitus.[11] The muscle enzymes include the transmembrane enzyme leukocyte common antigen-related PTPase (LAR)[12] and two intracellular enzymes, PTPase 1B[13,14] and the SH2 domain containing PTPase (SH-PTP2; also termed PTP1D, Syp, or PTP2C)[15,16] (F. Ahmad and B.J. Goldstein, Biochim Biophys Acta, in press). In the present study, we evaluated whether changes in the activity and/or amount of these PTPases are associated with severe insulin resistance in an animal model of obesity and non–insulin-dependent diabetes, the Zucker obese rat and a diabetic strain of this rat, the ZDF/Drt-*fa/fa*. PTPase enzyme activities and PTPase protein abundance were measured by immunoblotting in subcellular fractions of skeletal muscle in lean (+/?) control littermates, insulin-resistant obese Zucker (*fa/fa*) rats, and diabetic Zucker (ZDF/Drt-*fa/fa*) rats. The results demonstrate that an increase in PTPase enzyme activity of subcellular muscle fractions was associated with increases in the abundance of candidate PTPases in insulin-resistant obese and diabetic animals, suggesting that specific PTPases may play a central

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role in the pathophysiology of insulin-resistant disease states, particularly in the genetically determined phenotype of this line of obese and diabetic rats.

## MATERIALS AND METHODS

### *Animals and Materials*

Male Zucker diabetic fatty rats (ZDF/Drt-*fa*/*fa*) and lean male littermates (+/?) were purchased from Genetic Models (Indianapolis, IN). Obese nondiabetic Zucker rats (*fa*/*fa*) were obtained from Charles River (Wilmington, MA). Animals were fed ad libitum and studied at 10 to 12 weeks of age; animal maintenance and euthanasia were performed by procedures approved by the Thomas Jefferson University Animal Care and Use Committee. Studies with lean control, obese, and diabetic rats used four animals in each group. For experiments with insulin treatment of diabetic animals, a new set of rats was examined consisting of three animals in each group (lean, diabetic, and insulin-treated). Blood glucose levels were measured in the rats either by a hand-held glucometer device or by a glucose oxidase kit (Sigma, St Louis, MO). Serum insulin level was measured by radioimmunoassay using a kit from Diagnostic Products (Los Angeles, CA) and rat insulin standards from Bios Pacific (Emeryville, CA). Other materials were obtained as indicated and were of the highest grade available.

### *Preparation of Rat Skeletal Muscle Subcellular Fractions*

Separation of normal rat skeletal muscle tissue into particulate and cytosol fractions was performed using the method reported by Douen et al.[17] Hindlimb skeletal muscle (25 to 50 g) was minced into less than 1-μL pieces in cold homogenization buffer on ice (250 mmol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L EDTA, 5 mmol/L benzamidine, 0.05 TIU/mL aprotinin, and 1 mmol/L dithiothreitol [DTT], in 20 mmol/L $NaHCO_3$, pH 7.0); all subsequent steps were performed at 4°C. After dilution to 1 g tissue/10 mL buffer, the tissue was homogenized with 4 up/down strokes at low setting (4-5) in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 1,200 × *g* for 10 minutes in a Beckman JA-18 rotor (Beckman Instruments, Fullerton, CA). The supernatant was filtered through 4-ply gauze and reserved. The pellets were resuspended with the Polytron with a small amount of homogenization buffer and then centrifuged again at 1,200 × *g* for 10 minutes. The supernatant was filtered and combined with the reserved supernatant; the pellet was discarded. Combined supernatants were centrifuged at 9,000 × *g* for 10 minutes, the pellet was discarded, and the supernatant was centrifuged at 190,000 × *g* for 60 minutes. The cytosol fraction was taken as the supernatant from this ultracentrifugation step.

The pellet from the ultracentrifugation step (representing the crude membrane fraction) was solubilized in homogenization buffer containing 1% Triton X-100 and 0.6 mmol/L KCl by gentle stirring for 45 minutes. The solubilized–crude membrane fraction was then recovered as the supernatant after centrifugation at 15,000 × *g* for 20 minutes. The supernatant was then dialyzed overnight at 4°C against 20 mmol/L HEPES, pH 7.2, containing 0.1% (vol/vol) β-mercaptoethanol. Protein level was measured using the method reported by Bradford[18] with bovine serum albumin as standard.

In some experiments, fractions enriched in plasma membranes and internal membranes were prepared from the crude membrane fraction by layering 4 to 5 mL resuspended crude membranes in homogenization buffer on a discontinuous sucrose gradient consisting of (wt/wt) 25%, 30%, and 35% sucrose.[17] After centrifugation at 150,000 × *g* for 4 hours in a Beckman SW-40 Ti rotor, membrane fractions visible at the interfaces between the sucrose cushions were isolated, solubilized with Triton X-100 as described earlier, and stored at −85°C after resuspension in the original homogenization buffer. Marker enzyme assays for membrane subfractions included the plasma membrane enzyme 5′-nucleotidase[19] and the microsomal marker glucose-6-phosphatase.[20] Inorganic phosphate from the marker enzyme assays was measured as previously described.[21]

### *Phosphatase Assays*

To survey tissue fractions for PTPase activity, we used three substrates, including the autophosphorylated insulin receptor kinase domain and two artificial substrates, myelin basic protein and reduced, carboxamidomethylated, maleyated–lysozyme (RCM = lysozyme).

*Insulin receptor kinase domain dephosphorylation.* The 48-kd recombinant rat insulin receptor cytoplasmic domain was expressed in Sf9 cells from a baculovirus vector containing the entire cytoplasmic coding domain of the rat insulin receptor cDNA[22] and purified as previously described.[23] The insulin receptor cytoplasmic domain was autophosphorylated by incubating 5 mg protein in a 0.45 mL reaction containing 5 mmol/L $MnCl_2$, 0.1 mmol/L adenosine triphosphate (ATP), 180 μCi g-[$^{32}$P]-ATP (3,000 Ci/mmol), and 0.1% (vol/vol) Triton X-100 in 50 mmol/L HEPES, pH 7.6, at 4°C for 120 minutes. Unincorporated [$^{32}$P]-ATP was removed by a Bio-Gel P6 spin column (Bio-Rad, Hercules, CA), and 25-mL aliquots of labeled receptors were incubated with solubilized–muscle particulate fractions in a 125 mL reaction containing 1 mmol/L DTT and 2 mmol/L EDTA in 50 mmol/L HEPES, pH 7.6, at 30°C. The reactions were terminated by addition of 0.5 mL chilled stop solution containing 10 mmol/L ATP, 10 mmol/L sodium pyrophosphate, 4 mmol/L EDTA, 100 mmol/L NaF, 2 mmol/L sodium vanadate, 0.1 mg/mL aprotinin, and 2 mmol/L phenylmethylsulfonyl fluoride in 50 mmol/L HEPES buffer, pH 7.6. After boiling in gel sample buffer containing 100 mmol/L DTT, samples were subjected to electrophoresis in gels containing sodium dodecyl sulfate and 10% polyacrylamide.[24] Dephosphorylation of the 48-kd recombinant cytoplasmic domain was assessed by phosphorimager analysis of the dried gel (Molecular Dynamics, Sunnyvale, CA).

*Artificial substrate dephosphorylation.* Partially purified rat liver insulin and epidermal growth factor receptor kinases were isolated[25] and used to phosphorylate myelin basic protein (Sigma) and RCM-lysozyme (Sigma) on tyrosyl residues as described by Tonks et al.[26,27] PTPase enzymes were incubated with 24,000 dpm [$^{32}$P]-RCM-lysozyme (specific activity, 42,000 dpm/nmol) or 18,000 dpm [$^{32}$P]-myelin basic protein (35,000 dpm/nmol) in 100 mL assay buffer consisting of 50 mmol/L HEPES, pH 6.0, 2 mmol/L EDTA, and 1 mmol/L DTT. After incubation at 30°C, the amount of $^{32}$P released was measured by addition of 0.90 mL acidic charcoal mixture (0.9 mmol/L HCl, 90 mmol/L sodium pyrophosphate, 2 mmol/L $NaH_2PO_4$, and 4% vol/vol activated, untreated charcoal powder) and sedimentation of the precipitate in a microfuge, and radioactivity in 0.4 mL of the supernatant was determined by Cerenkov counting in a liquid scintillation counter.[28] One unit of PTPase activity was defined as the amount of enzyme releasing 1 nmol phosphate/min.

### *Immunoblotting of PTPases*

Cytosol and solubilized-particulate fractions of skeletal muscle were separated on gels containing sodium dodecyl sulfate and 7.5% (for LAR and SH-PTP2) or 10% (for PTPase 1B) polyacrylamide in a minigel apparatus.[24] Proteins were transferred to nitrocellulose filters (0.45-μm pore size) at 100 V for 3 hours in buffer

containing 20% (vol/vol) methanol, 25 mmol/L Tris base, and 192 mmol/L glycine at pH 8.3.[29] Nitrocellulose membranes were then incubated in blocking buffer containing 150 mmol/L NaCl, 0.05% (vol/vol) Nonidet P-40, 5% (wt/vol) bovine serum albumin, 1% (wt/vol) ovalbumin, 0.01% (wt/vol) sodium azide, and 10 mmol/L Tris, pH 7.4, for 1 hour at room temperature with rocking. The blocking solution was replaced with blocking buffer containing 0.5 mg/mL PTPase antibody (PTPase 1B, LAR, or SH-PTP2), and rocking was continued for 2 hours. Membranes were washed three times for 10 minutes with blocking buffer, followed by incubation with 2 μCi $^{125}$I-protein A (30 mCi/mg; ICN Biomedicals, Irvine, CA) for 1 hour at room temperature and then three additional 10-minute washes with TBST. Immunoreactive proteins were visualized by direct phosphorimager analysis of the immunoblot (Molecular Dynamics). Protein migration was calibrated with prestained molecular size standards from Bio-Rad.

Polyclonal antiserum to the cytoplasmic domain of recombinant rat LAR was obtained by immunization of rabbits with LAR protein purified from a bacterial expression system.[30] In both cases, the antibodies were affinity-purified using Affi-Gel (Bio-Rad) columns containing immobilized, purified LAR cytoplasmic domain.[31] Polyclonal antiserum to PTPase 1B and a monoclonal antibody to SH-PTP2 were obtained from Transduction Laboratories (Lexington, KY). After blotting with the monoclonal antibody, blots were incubated with rabbit antimouse IgG (Sigma) before reaction of the immunoblot with labeled protein A.

### *Statistics*

Except where indicated, the data represent at least three independent experiments and are presented as the mean ± SEM. Statistical calculations were based on ANOVA for comparisons of more than two groups, with Bonferroni's correction for determination of significance. Student's *t* test was used for comparing two samples.

## RESULTS

### *Animal Characteristics*

Three types of rats of the Zucker strain were used in these experiments, including lean littermate control (+/?), obese but nondiabetic Zucker rats (*fa/fa*), and diabetic rats of the ZDF/Drt subline (ZDF/Drt-*fa/fa*). As listed in Table 1, obese animals were on average 160 g heavier than lean littermates, with a mild but significant elevation of blood glucose. Diabetic animals were still obese relative to lean littermates, but had markedly elevated blood glucose values into the 28-mmol/L range. By 10 weeks of age, obese and diabetic animals had significantly elevated levels of circulating insulin, on average 9.3 and 2.7 times higher than lean littermate controls (Table 1), respectively, as a manifestation of the underlying insulin resistance that is characteristic of this model.[32]

**Table 1. Characteristics of Study Animals**

| | Weight (g) | Blood Glucose (mmol/L) | Serum Insulin (ng/mL) |
|---|---|---|---|
| Group A | | | |
| Lean | 308 ± 12 | 4.8 ± 0.1 | 3.2 ± 0.4 |
| Obese | 468 ± 17* | 10 ± 2* | 29.6 ± 3.5† |
| Diabetic | 368 ± 9‡ | ≥27.8‡ | 8.7 ± 0.6† |
| Group B | | | |
| Lean | 329 ± 3 | 6.3 ± 0.9 | 3.2 ± 1.1 |
| Diabetic | 362 ± 6 | 31.3 ± 2.4 | 7.6 ± 1.6 |
| Diabetic-treated | 443 ± 9 | 14.7 ± 4.5 | 93.9 ± 1.7 |

NOTE. Male Zucker rats were studied at 10 to 12 weeks of age including lean (+/?), obese (*fa/fa*), and ZDF/Drt-*fa/fa* diabetic animals. Group A contained 4 rats per group, and group B, 3 per group.

*$P < .01$ *v* lean.

†$P < .001$ *v* lean.

‡$P < .05$ *v* obese.

**Table 2. PTPase Activity in Skeletal Muscle Subcellular Fractions**

| | RCM-Lysozyme Assay (mU/mg) | | Myelin Basic Protein Assay (mU/mg) | |
|---|---|---|---|---|
| | Cytosol | Solubilized Particulate | Cytosol | Solubilized Particulate |
| Lean | 170 ± 17 | 610 ± 70 | 190 ± 17 | 780 ± 64 |
| Obese | 189 ± 23 | 640 ± 35 | 203 ± 12 | 1,290 ± 52* |
| Diabetic | 175 ± 9 | 690 ± 52 | 197 ± 23 | 1,360 ± 75† |

*$P < .05$ *v* lean.

†$P < .01$ *v* lean.

### *PTPase Activity in Muscle Subcellular Fractions*

To examine whether the level of overall PTPase activity was altered in obese and diabetic animals, we tested cytosol and solubilized-particulate fractions for PTPase activity using two artificial substrates and the autophosphorylated cytoplasmic domain of the insulin receptor as substrate.

In lean animals, both fractions contained significant PTPase activity (Table 2). The solubilized-particulate fraction had a threefold to fourfold greater specific activity than the cytosol with either of the artificial substrates. Using the myelin basic protein substrate, PTPase specific activity in the solubilized-particulate fraction was increased in obese animals by 65% over the value observed in lean animals ($P < .05$), and in diabetic animals by 74% of the control value ($P < .01$). With the RCM-lysozyme substrate, on average, there was a smaller increase in specific PTPase activity in the solubilized-particulate fraction of affected animals, which was not statistically significant. Interestingly, no significant change was noted in the cytosol for either artificial substrate (Table 2).

Alterations in PTPase activity that paralleled the results obtained with the myelin basic protein substrate were also evident in experiments using the insulin receptor cytoplasmic domain as substrate (Fig 1). The solubilized-particulate fraction from obese animals dephosphorylated the insulin receptor kinase domain 104% more than that from lean control animals. This effect persisted in diabetic littermates, where insulin receptor PTPase activity was 114% greater than the control value.

### *Alterations in PTPase Enzyme Abundance in fa/fa Obese and Diabetic Animals*

Using immunoblotting techniques, we assayed the abundance of candidate PTPases for the insulin action pathway in skeletal muscle, including PTPase 1B, LAR, and SH-PTP2, from lean control and insulin-resistant obese and diabetic aninmals. We recently identified these enzymes as major PTPase homologs in skeletal muscle and found that LAR was restricted to the particulate fraction, whereas SH-PTP2 and PTPase 1B had a characteristic distribution

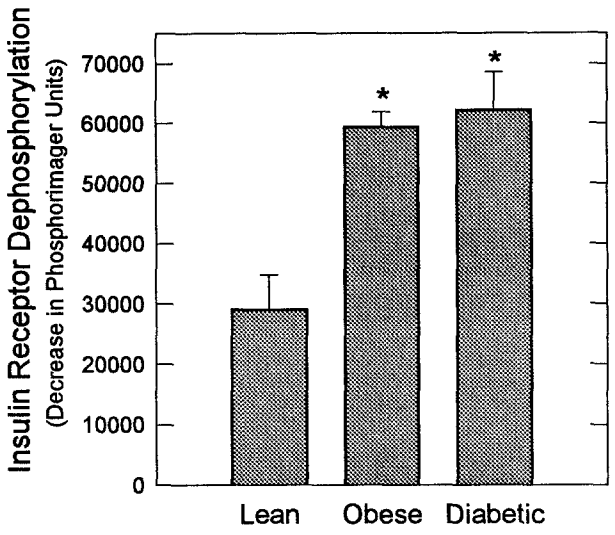


**Fig 1. Dephosphorylation of a recombinant rat insulin receptor kinase domain by the solubilized-particulate fraction of skeletal muscle from lean, obese, and diabetic Zucker rats (4 animals per group). *$P < .01$ *v* lean.**

in both the membrane and cytosol fractions. (F. Ahmad and B.J. Goldstein, Biochem Biophys Acta, in press) No differences were observed in apparent molecular mass of each of the PTPases on immunoblots between lean, obese, and diabetic animals (Figs 2 and 3). Using the antibody to the LAR cytoplasmic domain, the 80-kd transmembrane subunit of the processed enzyme is detected in the solubilized-particulate fraction. SH-PTP2 is observed as a 67-kd protein in the cytosol and the solubilized-particulate fraction. PTPase 1B is isolated as either the full-length 48-kd enzyme or a truncated 37-kd form in which a labile C-terminal portion has been cleaved.[33,34]

In the solubilized-membrane fraction, significant increases in PTPase protein abundance were observed in obese and diabetic animals for each of the major enzymes found in muscle tissue, in accordance with the increased PTPase activity measured in the particulate fraction (Fig 2 and Table 3). PTPase 1B was increased to the greatest extent, ie, 60% and 70% over the level observed in lean animals, in obese and diabetic rats, respectively. LAR and SH-PTP2 were increased to a similar magnitude, 53% and 42% over control levels for LAR and 48% and 50% over control levels for SH-PTP2, in obese and diabetic rats, respectively. Minor differences between levels observed in obese and diabetic animals were not statistically significant by ANOVA with Bonferroni's correction for multiple comparisons.

In the cytosol fraction, where PTPase activity toward the artificial substrates was unchanged in obese and diabetic animals (Table 2), there were no significant alterations in abundance of PTPase 1B or SH-PTP2 (Fig 3). When quantified by phosphorimager analysis, abundance of SH-PTP2 was increased slightly in obese animals by 14%.

To test the potential effect of insulin treatment on the increased amount of PTPases in affected animals, we studied an additional group of lean and diabetic (ZDF) rats, in which diabetic rats were treated with a single nocturnal dose of 10 to 12 U long-acting Ultralente insulin (Eli Lilly and Co, Indianapolis, IN) for a 2-week period. Compared with diabetic rats, insulin-treated rats had gained weight to a mean of 443 g and had a 53% decrease in blood glucose (Table 1). However, insulin resistance had persisted in these animals, since they manifested a significant hyperglycemia even in the face of serum insulin values that were 29 times the levels in lean controls. This insulin resistance was associated with continued elevation of PTPase protein in the solubilized-particulate fraction, including

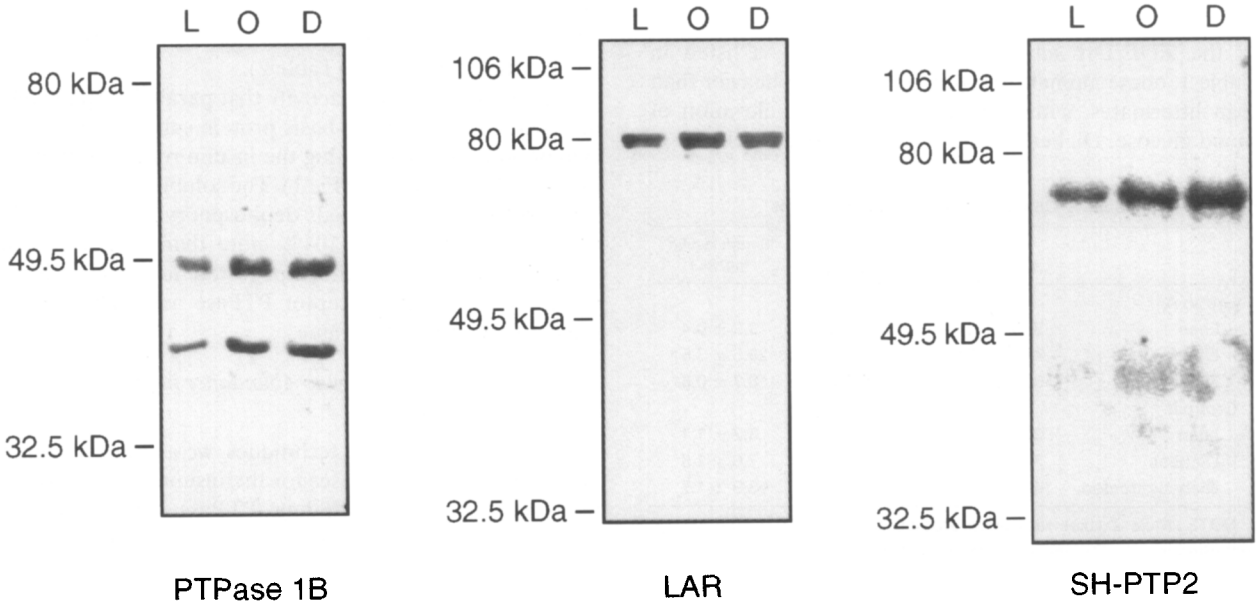


**Fig 2. Immunoblot analysis of abundance of PTPase 1B, LAR, and SH-PTP2 in the solubilized-particulate fraction of skeletal muscle from lean (L), obese (O), and diabetic (D) Zucker rats.**

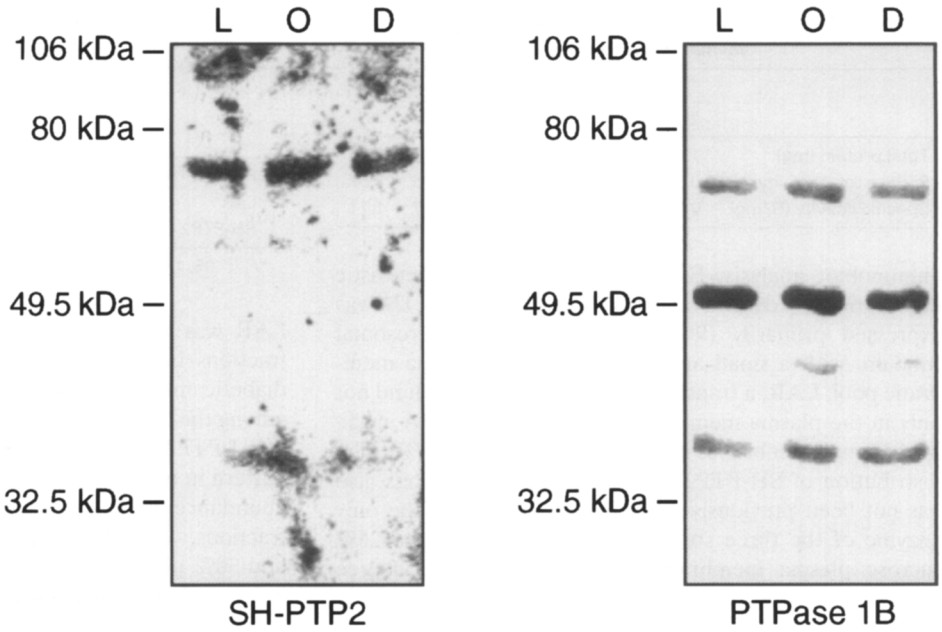


**Fig 3. Immunoblot analysis of abundance of SH-PTP2 and PTPase 1B in the cytosol fraction of skeletal muscle from lean (L), obese (O), and diabetic (D) Zucker rats.**

PTPase 1B (mean, 86% and 83% > lean controls), LAR (28% and 68% > lean controls), and SH-PTP2 (39% and 65% > lean controls) in diabetic and insulin-treated diabetic animals, respectively (Table 3). In addition, there was no significant change in the elevated PTPase activity in the solubilized-particulate fraction toward the insulin receptor cytoplasmic domain in insulin-treated animals (data not shown).

*PTPase Abundance in Membrane Subfractions*

Since subcellular compartmentalization of various PTPases has been postulated to affect their overall activity and direct their accessibility to cellular substrates, we studied the localization of the three membrane-associated PTPases to skeletal muscle membrane subfractions. Klip and Marette[35] have described discrete fractions of skeletal muscle membranes enriched in plasma membrane elements and internal membranes that have been used to study glucose transporter translocation in response to insulin treatment. These fractions are isolated by ultracentrifugation of crude muscle membranes over sucrose density step-gradients of 25%, 30%, and 35% (wt/wt). Membrane elements that undergo sedimentation at 25% and 30% are enriched in plasma membrane markers with the highest specific activity of 5′-nucleotidase; the membranes in the 35% sucrose fraction consist of an internal microsomal pool enriched in the marker enzyme, glucose-6-phosphatase (Table 4).

Since the solubilized-particulate fraction from diabetic animals exhibited increased PTPase activity, we localized the increase in this activity in membrane subfractions from the affected animals. The distribution of recovered protein in the three membrane fractions was not significantly different between lean and diabetic animals (Table 5). PTPase specific activity and total activity were increased in each of the three membrane fractions from diabetic animals. Similar increases of PTPase specific activity of 46% and 53%, respectively, relative to control levels were seen in the plasma membrane fraction (30% sucrose) and the microsomal pool (35% sucrose). The 25% sucrose plasma membrane fraction exhibited a small increase in PTPase specific activity of 5% in diabetic animals (Table 5).

Distribution of enzyme protein for each of the PTPases among these membrane fractions in lean control animals and alterations in the diabetic state were determined by

**Table 3. Abundance of Specific PTPase Enzymes (% of lean) in the Solubilized-Particulate Fraction by Immunoblotting**

| | PTPase 1B | LAR | SH-PTP2 |
|---|---|---|---|
| Group A | | | |
| Lean | 100 | 100 | 100 |
| Obese | 160 ± 2* | 153 ± 10* | 148 ± 11† |
| Diabetic | 170 ± 11* | 142 ± 8† | 150 ± 9† |
| Group B | | | |
| Lean | 100 | 100 | 100 |
| Diabetic | 167,205 | 131,125 | 139,144 |
| Diabetic-treated | 146,221 | 172,164 | 165,146 |

NOTE. For group B diabetic and insulin-treated animals, results from 2 representative animals are shown.

*$P < .01$ *v* lean.

†$P < .05$ *v* lean.

**Table 4. Marker Enzyme Specific Activities for Skeletal Muscle Subcellular Fractions**

| Sucrose Fraction (wt/wt) | 5′-Nucleotidase (nmol $P_i$/min/mg) | Glucose-6-phosphatase (nmol $P_i$/min/mg) |
|---|---|---|
| Lean | | |
| 25% | 315 ± 26 | 460 ± 210 |
| 30% | 257 ± 32 | 960 ± 130 |
| 35% | 63 ± 17 | 4,160 ± 310 |
| Diabetic | | |
| 25% | 351 ± 21 | 320 ± 230 |
| 30% | 243 ± 27 | 830 ± 190 |
| 35% | 79 ± 19 | 3,870 ± 210 |

**Table 5. PTPase Activity and Protein Content of Skeletal Muscle Membrane Subfractions**

| | Lean | | | Diabetic | | |
|---|---|---|---|---|---|---|
| | 25% | 30% | 35% | 25% | 30% | 35% |
| Total protein (mg) | 1.13 | 0.79 | 1.97 | 1.26 | 0.63 | 2.09 |
| PTPase total activity (U) | 0.85 | 0.74 | 2.21 | 1.00 | 0.86 | 3.57 |
| Specific activity (U/mg) | 0.75 | 0.94 | 1.12 | 0.79 | 1.37 | 1.71 |

immunoblot analysis. Each PTPase had a characteristic distribution in control animals (Fig 4). PTPase 1B was expressed primarily (96%) in the internal microsomal fraction, with a small amount (4%) in the plasma membrane pool. LAR, a transmembrane PTPase, was found not only in the plasma membrane pool (30%), but also in the microsomal membranes in substantial amounts (70%). The distribution of SH-PTP2 among membrane fractions also has not been previously studied. This PTPase is the only enzyme of the three studied that was found in the 25% sucrose plasma membrane fraction, to a small degree (~3% of the total). However, the bulk of SH-PTP2 activity was found distributed between the 30% sucrose plasma membrane fraction (39%) and the internal microsomal pool fraction (58%).

We compared immunoblots from control and diabetic animals to assess any changes in specific protein immunoreactivity in the discrete membrane subfractions between lean control and diabetic animals. The distribution of enzyme protein among the three fractions was also determined and compared with that of control animals. The increase in PTPase 1B protein observed with the crude-particulate fraction was limited to the internal microsomal membrane fraction, where this enzyme is almost exclusively localized (Table 6). Since the abundance of PTPase 1B is negligible in the other membrane subfractions, the increase in PTPase 1B protein had no significant effect on the overall distribution of this enzyme among the three membrane fractions in diabetic animals (Fig 4). The abundance of

**Table 6. Effect of Diabetes on Abundance of PTPases in Muscle Membrane Subfractions**

| | PTPase Abundance in Sucrose Frations (% of lean) | | |
|---|---|---|---|
| | 25% | 30% | 35% |
| PTPase 1B | NS | NS | 198 ± 19† |
| LAR | NS | 127 ± 16 | 159 ± 2† |
| SH-PTP2 | 281 ± 75* | 223 ± 57* | 112 ± 1.5 |

*$P < .05$, †$P < .01$: diabetic *v* lean.

LAR was increased in both the 30% and 35% sucrose fractions in diabetic animals (Table 6); this increase in diabetic animals also did not alter the distribution of LAR among the three membrane elements (Fig 4).

SH-PTP2, in contrast, exhibited an altered distribution pattern in membrane subfractions of diabetic animals. The abundance of SH-PTP2 was increased in each of the three fractions, with the greatest change occurring in the 25% and 30% plasma membrane fraction pools, and a smaller increase in the microsomal fraction (Table 6). When the distribution of SH-PTP2 is analyzed among the three membrane fractions in the individual experiments, a significant shift of the bulk of SH-PTP2 protein to the plasma membrane elements and a relative decrease in the portion of SH-PTP2 protein found in the internal microsomal membrane pool are evident in diabetic animals (Fig 4).

## DISCUSSION

Resistance to the biological action of insulin is one of the central pathophysiologic features of human obesity and non–insulin-dependent diabetes mellitus. Recent advances in our understanding of cellular pathways of insulin action have provided some insight into specific signaling steps involving reversible protein-tyrosine phosphorylation that may be altered in these insulin-resistant disease states. Insulin-stimulated autophosphorylation of its receptor and activation of receptor kinase activity occurs as a sequential

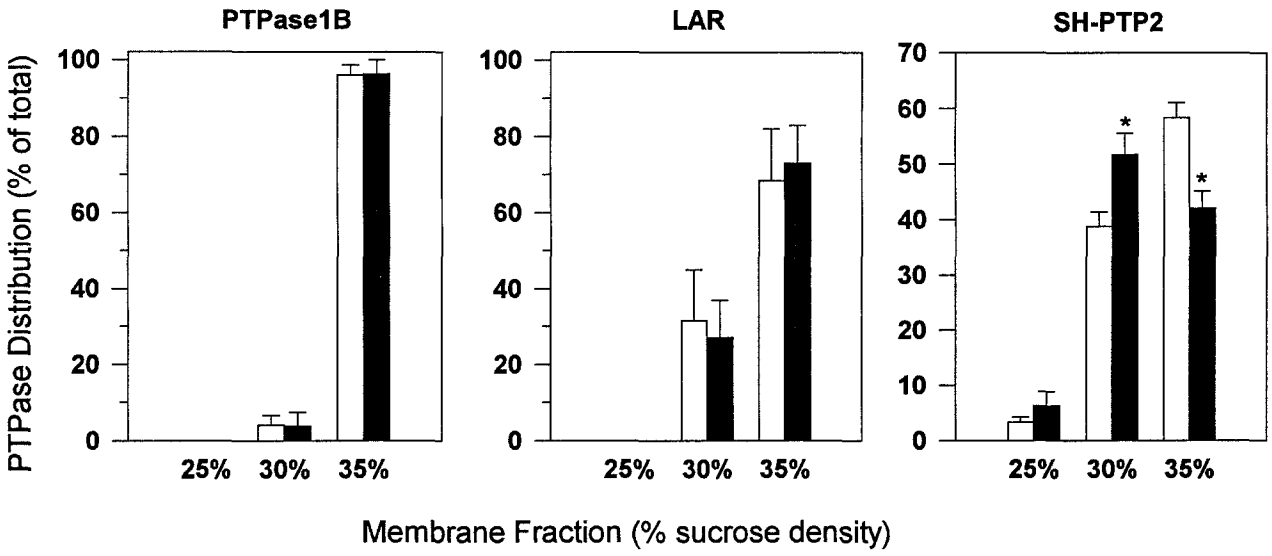


**Fig 4. Distribution of PTPase 1B, LAR, and SH-PTP2 among skeletal muscle subfractions in lean and diabetic Zucker rats. Distribution of the relative abundance of each PTPase was determined by phosphorimager analysis and plotted as a percent of the total signal present in each sucrose fraction. (□) Lean control animals; (■) diabetic animals. *$P < .05$ *v* lean.**

cascade involving a series of tyrosyl residues in the receptor cytoplasmic domain.[1] Tyrosyl phosphorylation of cellular substrates by the insulin receptor kinase, especially the widely expressed IRS-1, has been shown to transduce the postreceptor insulin signal.[36,37] Phosphorylated IRS-1 acts as an adapter or docking protein for binding and activation of a variety of SH2 domain–containing signaling proteins, which form a close noncovalent association with the phosphotyrosyl domains of IRS-1. Two examples of this mode of signaling in insulin action are the binding of phosphatidylinositol-3′-kinase to IRS-1 by SH2 domains in its noncatalytic subunit that activates the enzyme,[1] and the association of SH-PTP2, a widely expressed PTPase that contains two SH2 domains, with IRS-1 and other tyrosine phosphorylated membrane receptors such as platelet-derived growth factor.[3,38] The association of SH-PTP2 with the platelet-derived growth factor receptor and certain consensus phosphopeptides has also been shown to activate its PTPase activity.[39,40]

Despite many recent advances in our understanding of specific elements involved in the activation of insulin signaling in target cells, we still lack a significant appreciation of the cellular mechanisms that reverse or suppress insulin signaling and are responsible for insulin resistance. However, since reversible tyrosyl phosphorylation has been shown to play a fundamental role in these processes, much attention in several laboratories has recently been directed at protein-tyrosine phosphatase enzymes that, in concert with receptor kinase activation, act to determine the steady-state balance of tyrosyl phosphorylation in the insulin action pathway.[2]

The insulin-resistant Zucker fatty rat has been used as a model for human insulin-resistant obesity and non–insulin-dependent diabetes.[41] In these animals, obesity is transmitted by an autosomal recessive mutation in the *fa* gene. Zucker fatty rats inherit many features of type II diabetes in humans, including insulin resistance with hyperinsulinemia, glucose intolerance, and hyperlipidemia.[42] Insulin resistance in liver and muscle due to defects in insulin receptor signaling at both receptor and postreceptor sites has been described.[43-46] The underlying basis of these abnormalities in insulin action has not been determined. The ZDF/Drt-*fa*/*fa* rat strain is a partially inbred derivative of the Zucker fatty rat that predictably develops frank hyperglycemia and diabetes.[32] These animals show a pattern of development of disease similar to their nondiabetic obese counterparts, with the early development of hyperinsulinemia and insulin resistance, and after 7 to 10 weeks, development of hyperglycemia with the concomitant failure of the pancreas to support circulating insulin levels, which decrease thereafter. The similarity of the development of diabetes in these animals to human type II diabetes[11] has made this an excellent model system for the human disease.

In our analysis of the potential role of alterations in PTPase activity in the insulin resistance characteristic of this disorder, we focused on skeletal muscle, since this tissue is the major site of insulin-mediated glucose disposal in vivo and the clinical insulin resistance associated with diabetes mellitus is predominantly due to defects in insulin action in muscle.[11] Furthermore, defects in the insulin receptor kinase pathway in skeletal muscle have been demonstrated in insulin-resistant human diabetes.[47,48] In skeletal muscle of Zucker rats, we have demonstrated that the insulin-resistant state is accompanied by increases in specific activity of PTPase enzymes measured with artificial substrates and the cytoplasmic domain of the insulin receptor as substrate. These increases were found predominantly in the solubilized-particulate fraction of muscle and were associated with increased abundance of multiple candidate PTPase enzymes. We hypothesize that one or more of these PTPases has a major role in the tissue insulin resistance in these animals, acting either at the insulin receptor in situ or at the level of insulin receptor substrate proteins to affect the balance of reversible tyrosine phosphorylation in muscle and cause an insulin-resistant state. The increased PTPase enzyme activity and proteins were found both in obese, insulin-resistant animals and in diabetic rats, suggesting that they are associated with the *fa*/*fa* genotype as either a primary or secondary alteration.

The change in distribution of SH-PTP2 among skeletal muscle membrane subfractions in diabetic animals is also of interest. Although SH-PTP2 has been shown to be primarily a cytoplasmic enzyme, it can bind with high affinity to tyrosine-phosphorylated membrane proteins via its SH2 domains and be retained in a particulate fraction before solubilization. We have extended these findings in the current study by demonstrating a shift of SH-PTP2 between membrane subfractions in diabetes where SH-PTP2 apparently is translocated between an internal microsomal compartment and the plasma membrane (Fig 4). This redistribution of SH-PTP2 may be caused by association of the SH2 domains of this enzyme and phosphotyrosyl proteins in the plasma membrane of diabetic animals, which may activate its catalytic domain[39,40] and may be an important factor in the physiologic role of this enzyme in insulin-resistant diabetic animals. Furthermore, these data provide further evidence for the dynamic nature of the regulation of SH2-domain–containing PTPases, which may be compartmentalized in discrete skeletal muscle membrane fractions in different physiologic states. A study by Minami et al[49] has shown an interesting redistribution of the CD45 PTPase in response to T-cell activation. Subcellular redistribution may thus be a more generalized phenomenon that helps determine the access of different PTPases to appropriate cellular substrates in various physiologic settings.

To our knowledge, this is the first report demonstrating an increase in PTPase activity in obese and diabetic Zucker rats and implicating PTPases as a potential factor in the genetic insulin resistance of this model. In recent animal and human studies, complex alterations in tissue PTPase activities in insulin-resistant states using a variety of substrates have been demonstrated in several laboratories. Increases in cytosol or particulate fraction PTPase activity have been demonstrated in several[4-7] but not all[9,50,51] studies in livers of rodents with hypoinsulinemic diabetes induced by toxins. In other recent investigations, elevated levels of cytosolic PTPase activity have been demonstrated in the liver of aged rats, associated with defective activation of the receptor kinase in vivo.[8] Complex profiles of PTPase activities have been observed, undoubtedly because of the

variety of substrates used and the different animal models and durations of diabetes. It should be emphasized that enzymatic studies with any tissue fractions will be measuring levels of several PTPases simultaneously, and that variation in the magnitude and direction of changes in the overall PTPase assay will depend on alterations in the mean abundance and activity of different PTPases toward the selected artificial substrates. By examining the abundance of enzyme protein for specific PTPases, we have been able to correlate changes in overall PTPase activity with candidate enzymes that are abundant in muscle tissue. These results enable us to hypothesize that alterations in the amount and distribution of specific enzymes may be involved in the insulin resistance of these diabetic animals.

Only a few previous studies have directly examined PTPase activities in skeletal muscle of animals or patients with insulin resistance. In skeletal muscle biopsies from insulin-resistant Pima Native American subjects, basal PTPase activity in the particulate fraction of muscle was 33% greater than in insulin-sensitive controls.[10] In contrast, a related study in a different patient population recently reported that the particulate fraction PTPase activity was reduced by 21% and 22% in obese nondiabetic and non–insulin-dependent diabetic subjects, respectively.[52] This latter study also demonstrated by immunoblot analysis that PTPase 1B levels were decreased on average by 38% in diabetic subjects. These conflicting data underscore the multiple factors, both genetic and environmental, that determine the heterogeneous pathogenesis of insulin resistance in human diabetes, of which PTPase activities are likely to be one element. As a pathogenetic factor, it is possible that altered PTPase abundance may influence insulin receptor signaling in insulin-resistant Native Americans and in a subset of insulin-resistant kindreds with varied genetic backgrounds among the remainder of the American population. Further studies are essential to distinguish these possibilities.

In summary, this is the first report in a genetic model of insulin-resistant obesity and diabetes of elevations of tissue PTPase activity against the insulin receptor associated with increased abundance of enzyme protein for specific candidate PTPase homologs. It is of interest that Zucker rats are resistant to both insulin and insulin-like growth factor-I, suggesting that elevated PTPase activity may influence common signaling pathways for these two hormones.[53] Since insulin-sensitive tissues, especially liver, contain a complex array of PTPases, studies on crude-cytosol or solubilized-particulate fractions may generate results that are difficult to interpret, at least with regard to PTPases that might impact insulin signaling. It has become important to conduct follow-up evaluations on the initial reports of altered PTPase activities in crude cell extracts with a careful examination of the abundance of individual PTPase homologs that may have a direct role in regulating the insulin action pathway. These enzymes may have important influences on insulin action in situ, which may even be lost in some cases when the enzymes are solubilized.[54] Evaluation of the role of these candidate PTPases in insulin action at a cellular level is currently under way in our laboratory to discern their exact mode of regulation of reversible tyrosine phosphorylation in the insulin signaling pathway. Once the pertinent PTPases are fully established, additional human studies may enable us to identify insulin-resistant patients that may exhibit increased abundance or activity of specific PTPases. These individuals may be candidates for therapy directed at PTPase enzyme activity, since it has been suggested to be a potential mechanism of action for oxidized vanadium and related compounds in ameliorating the insulin-resistant state in animal models, including skeletal muscle of Zucker rats.[55-57]

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