Insulin Resistance Is Associated With Abnormal Dephosphorylation of a Synthetic Phosphopeptide Corresponding to the Major Autophosphorylation Sites of the Insulin Receptor

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Insulin resistance in the ob/ob mouse model is associated with a reduction in insulin-induced protein-tyrosine phosphorylation in tissues such as liver. To ascertain whether this decrease in phosphorylation may be due to increased phosphatase activity, protein-tyrosine phosphatase (PTPase) activity was determined in particulate and soluble fractions from livers of 5- to 23-week-old ob/ob mice and age-matched lean littermates. PTPase activity was measured using a synthetic phosphopeptide, TRDIY(P)ETDY(P)Y(P)RK, as the substrate, corresponding to residues 1142 to 1153 of the insulin receptor and containing the major autophosphorylation sites of the regulatory domain. The ob/ob mice were hyperinsulinemic across all age groups, but only the youngest mice (aged 5 to 7 weeks) were hyperglycemic. Most PTPase activity was present in the liver particulate fraction and was 19% to 114% greater in ob/ob mice as compared with controls. PTPase activity in the liver soluble fraction was 26% less than control values in the youngest ob/ob mice (5 to 7 weeks), but increased with age and was 41% and 131% above control values at 21 to 23 and 25 to 27 weeks of age, respectively. Oral administration of the PTPase inhibitor sodium orthovanadate (0.6 mg/mL in drinking water for 2 weeks) to young ob/ob mice caused a significant reduction in the elevated particulate PTPase activity, with concomitant decreases in plasma insulin and plasma glucose. Assessment of PTPase activity with a monophosphate form of the same synthetic peptide, TRDIY(P)ETDYYRK, showed lower PTPase activities as compared with the triphosphate form and no significant differences between ob/ob and control preparations. In conclusion, these findings demonstrate that the mechanism of dephosphorylation of insulin receptor autophosphorylation sites is altered in the liver of the ob/ob mouse. This abnormal PTPase activity may contribute to the decrease in insulin action characteristic of this insulin-resistant model.

INSULIN-MEDIATED protein-tyrosine phosphorylation events appear to be essential for all the biologic properties of insulin.[1] Insulin binding to the α-subunit of the insulin receptor produces activation of the receptor β-subunit as a tyrosine kinase. The receptor kinase autophosphorylates on at least six tyrosine residues: tyrosine 960 in the juxtamembrane domain; tyrosines 1146, 1150, and 1151 in the regulatory domain; and tyrosines 1316 and 1322 in the C-terminal domain.[2] The receptor is fully activated when tyrosines 1146, 1150, and 1151 in the regulatory domain are phosphorylated.[3,4] Receptor kinase activity and presumably insulin's actions are attenuated when these tyrosines are dephosphorylated by protein-tyrosine phosphatases (PTPases).[5,6]

In insulin resistance of type II or non–insulin-dependent diabetes mellitus (NIDDM), insulin binding is normal but the metabolic actions of insulin are subnormal (for review, see Häring[7]). Consequently, hyperglycemia occurs. Although the exact molecular mechanism(s) underlying insulin resistance is unknown, studies in cell models, rodent models, and human diabetics have shown that the metabolic defects consistent with insulin resistance are associated with a decrease in insulin-stimulated protein-tyrosine phosphorylation. This decrease does not appear to be caused by a defect in the receptor kinase, but instead seems to be caused by a postreceptor defect.

The ob/ob mouse, a typical model of NIDDM, is characterized by obesity, hyperglycemia, hyperinsulinemia, and insulin resistance in tissues such as liver, adipocytes, and skeletal muscle.[8-10] A decrease in insulin-induced early protein-tyrosine phosphorylation events in the ob/ob liver and its impact on other protein components of the insulin signal-transduction cascade was recently described.[11,12] In the ob/ob liver, a reduction in insulin receptor phosphorylation caused a decline in insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation that reduced the association of IRS-1 with phosphatidylinositol-3 (PI-3) kinase and ultimately caused a decline in PI-3 kinase activity. Although the role of PI-3 kinase in the insulin signal-transduction cascade is unknown, the study demonstrated a relationship between decreased tyrosine phosphorylation and decreased postreceptor enzyme activation. Since the overall state of protein phosphorylation is dependent on the balance between kinase and phosphatase activity,[13-15] it is likely that abnormal PTPase activity contributes to this decline in phosphotyrosine-mediated events.

Abnormal PTPase activity has been observed in insulin-responsive tissues from diabetic rodent models and diabetic man. Elevated tissue PTPase activity was reported for insulinopenic diabetic rats,[16-18] aged rats,[19] and insulin-resistant humans.[20,21] In contrast, some studies have reported a decline[22-25] or no change[26] in PTPase activity with insulin resistance. The precise explanation for these disparate findings is unknown, but it may involve the type of rodent model, age, duration of diabetes, tissue subcellular preparation, and substrates used to assess PTPase activity. Substrates used in these early studies included the intact insulin receptor, insulin receptor peptides, or artificial substrates, all of which were phosphorylated on tyrosines by tyrosine kinase preparations. Although tyrosine kinases have amino acid sequence preferences, the method of phosphorylating with kinases renders substrates with low stoichiometry.[27-29] Additionally, the exact sites of phosphor-

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ylation on the multiple tyrosine-containing substrates are never certain without additional analyses. Undoubtedly, a homogenous substrate is preferable.

Rat liver membrane PTPase preparations and purified PTPases cloned from the liver have been shown to prefer as a substrate the fully activated insulin receptor, autophosphorylated on tyrosines 1146, 1150, and 1151.[5,6,29-31] Conversion of this triphospho form of the receptor to the bis form by PTPases is correlated with a loss in receptor kinase activity.[5,6] Additionally, purified recombinant PTPases have shown preferential dephosphorylation of specific tyrosine residues in the receptor. Recombinant protein tyrosine phosphatase 1B (PTP-1B) preferred residues 1150 and 1151, whereas leukocyte common antigen–related phosphatase (LAR) preferred residue 1146.[31,32] Collectively, these findings suggest that a purified triphosphotyrosyl form of the insulin receptor regulatory domain would be the most appropriate substrate to evaluate those PTPases affecting the insulin receptor. Accordingly, in the present study PTPase activity was measured in liver preparations from insulin-resistant ob/ob mice using the synthetic triphosphotyrosyl peptide, TRDIY(P)ETDY(P)Y(P)RK, corresponding to the major autophosphorylation sites of the insulin receptor regulatory domain (residues 1142 to 1153).[2] A monophosphotyrosyl form of the same peptide, TRDIY(P)ETDYYRK, was also evaluated as a potential substrate. Since expression of the diabetes syndrome in the ob/ob mouse changes with age,[8-10] a longitudinal assessment of PTPase activity in ob/ob mice from 5 to 27 weeks of age was conducted. Finally, the effect of oral administration of the PTPase inhibitor vanadate on hepatic PTPase activity was evaluated.

MATERIALS AND METHODS

Materials

Ammonium molybdate was supplied by Aldrich Chemical (Milwaukee, WI). Sucrose was from EM Science (Cherry Hill, NJ). TRIS hydrochloride was from JT Baker (Phillipsburg, NJ). β-Mercaptoethanol was from Pierce (Rockford, IL). Okadaic acid was from Upstate Biotechnology (Lake Placid, NY). [4-(2-Aminoethyl)-benzenesulfonylfluoride, HCl] (AEBSF) was from Calbiochem (San Diego, CA). The monophospho peptide, TRDIY(P)ETDYYRK, and the triphospho peptide, TRDIY(P)ETDY(P)Y(P)RK, were synthesized and purified to greater than 98% purity by Dr Anita Hong of AnaSpec (San Jose, CA) using solid-phase peptide synthesis and F-*moc* chemistry. All other chemicals were from Sigma Chemical (St Louis, MO).

Animals

Male obese mice (C57BL/6J-ob/ob) and lean littermates (C57BL/6J-?/+) ages 5 to 7 weeks were obtained from Jackson Laboratory (Bar Harbor, ME). All animals were maintained on Purina Rodent Chow 5001 (Purina Mills, Richmond, IN) and had free access to drinking water.

The first study, longitudinal assessment, evaluated liver PTPase activity in ob/ob mice and lean control littermates from 5 to 27 weeks of age. Four ob/ob mice and four lean controls were killed in the morning by decapitation at 5 to 7, 13 to 15, 21 to 23, and 25 to 27 weeks of age. Blood was collected into NaF-containing tubes (Sarstedt, Nümbrecht, Germany), and plasma was isolated by centrifugation at 8,000 × *g* for 5 minutes. The livers were removed, weighed, rinsed in cold saline, and immediately processed for PTPase measurements.

The second study examined the effect of orally administering the PTPase inhibitor vanadate to ob/ob mice. Ob/ob mice (6 to 8 weeks of age) were tail-bled and randomized into two groups (n = 12) according to plasma glucose concentration. One group of ob/ob mice received sodium orthovanadate (0.6 mg/mL) in a vehicle of 85 mmol/L NaCl supplied as drinking water. The vanadate/NaCl solution was adjusted to pH 7.0 with citric acid.[33] The second group of untreated ob/ob mice and the group of lean control mice (?/+) received vehicle alone. The vehicle consisted of 85 mmol/L NaCl plus a volume of citric acid equivalent to that used in the above-mentioned vanadate solution and was adjusted to pH 7.0 with NaOH. Drinking solutions were prepared fresh every 3 days, and water consumption was measured. The study was concluded after 14 days of treatment. After decapitation, blood was collected and livers were immediately processed for PTPase measurements.

Preparation of Liver Particulate and Soluble Fractions

After rinsing in cold saline, livers were further processed according to the method reported by Meyerovitch et al[16] with slight modifications. Livers were homogenized using a Potter-Elvehjem homogenizer in 10 vol cold buffer A (20 mmol/L TRIS hydrochloride, 50 mmol/L β-mercaptoethanol, 250 mmol/L sucrose, 2 mmol/L EDTA, 10 mmol/L EGTA, 2 mmol/L AEBSF, 0.1 mmol/L Nα-tosyl-lys chloromethyl ketone (TLCK), 0.1 mmol/L Nα-tosyl-Phe chloromethyl ketone (TPCK), 0.5 mmol/L benzamidine, 10 μg/mL aprotinin, 25 μg/mL leupeptin, and 5 μg/mL each of pepstain A, antipain, and chymostatin, pH 7.4). The homogenates, filtered through nylon mesh (250 μm), were centrifuged at 10,000 × *g* for 20 minutes. The supernatant was designated as the soluble fraction. The resulting pellet was resuspended in 2 vol buffer A by homogenization and was designated the particulate fraction. All preparations were stored at −20°C.

Measurement of PTPase Activity

To assess PTPase activity, dephosphorylation of an insulin receptor phosphopeptide was measured. Nanomolar amounts of inorganic phosphate released from the phosphopeptide were determined with a malachite green–ammonium molybdate assay[32,34] adapted for small-volume analysis in a 96-well platereader. Particulate and soluble liver fractions were resuspended in 53 mmol/L HEPES and 8.32 mmol/L β-mercaptoethanol, pH 7.4, at a final protein concentration of 42 and 125 μg/mL, respectively. The assay also contained 0.32 mmol/L EDTA and 41.6 mmol/L sucrose. The dephosphorylation reaction proceeded at 37°C for 30 minutes after addition of 100 μmol/L phosphopeptide substrate, and was terminated with a malachite green–ammonium molybdate–Tween 20 stopping reagent consisting of three parts 0.45% malachite green hydrochloride, one part 4.2% ammonium molybdate tetrahydrate in 4N HCl, and 0.5% Tween 20. Blanks were prepared by addition of malachite green–ammonium molybdate–Tween 20 to the phosphopeptide substrate and appropriate amounts of liver preparation. The color was allowed to develop at room temperature for 30 minutes, and absorbance was determined at 650 nm using a platereader (Molecular Devices, Menlo Park, CA). A potassium phosphate standard curve was used as a reference. Under the assay conditions, only 5% to 30% of the substrate was dephosphorylated and phosphate release was linear with time and amount of enzyme preparation. PTPase activity was expressed as nanomoles of phosphate released per minute per milligram protein.

Analytical Methods

Plasma glucose concentration was determined by a hexokinase method on an Abbott VP Analyzer (Abbott Laboratories, Irving, TX), and plasma insulin level was measured by radioimmunoassay. Protein determinations were made using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA) with crystalline bovine serum albumin as the standard.

Statistical Analysis

Data are presented as the mean ± SEM. Groups were compared by ANOVA followed by a Dunnett's T test. Significance level was set at P less than .05.

RESULTS

Animal Characteristics in the Longitudinal Study

The ob/ob mouse is known to be transiently hyperglycemic and transiently hyperinsulinemic.[8-10] During the first month of life, the ob/ob mouse is asymptomatic, after which plasma glucose and insulin concentrations increase with age and then decline to normal levels in old mice. Plasma glucose concentration peaks at 3 months, and plasma insulin concentration peaks at 7 months. The evolution of this syndrome indicates that development and expression of insulin resistance and diabetes are limited to a specific age period. Accordingly, it was of interest to examine PTPase activity in liver preparations from ob/ob mice of various ages to determine if hepatic PTPase activity varied with age and degree of hyperglycemia and hyperinsulinemia. Liver fractions were prepared from littermate mice that were 5 to 7, 13 to 15, 21 to 23, and 25 to 27 weeks of age.

Characteristics of the mice in Table 1 and Fig 1 are consistent with the description of the ob/ob mouse as a model of obesity, hyperglycemia, and severe hyperinsulinemia. All ob/ob mice had significantly greater body weights and greater liver weights as compared with age-matched controls. The ob/ob mice showed a steady increase in body mass until 21 to 23 weeks of age, after which there was a slight decline in weight. Plasma glucose concentration (Fig 1) was significantly elevated above control levels in only the youngest ob/ob mice (302 ± 44 mg/dL for ob/ob v 159 ± 5 for control, $P < .01$). At 13 to 15 weeks of age, ob/ob mouse plasma glucose concentration (251 ± 58 mg/dL) was greater than the age-matched control level (142 ± 8 mg/dL), but the difference was not statistically significant. Plasma insulin also varied with age in the ob/ob mouse (Fig 1) but was always extremely high, 22- to 73-fold higher than age-matched control levels. The ob/ob plasma insulin concentration peaked at 21 to 23 weeks (735 ± 108 μIU/mL in ob/ob v 12 ± 1 in control).

Table 1. Characteristics of the Mice

Age (wk)	Body Weight (g)	Liver Weight (g)
5-7		
Lean control	21.5 ± 0.6	1.32 ± 0.04
ob/ob	40.2 ± 2.6*	3.31 ± 0.36*
13-15		
Lean control	28.3 ± 1.0	1.64 ± 0.05
ob/ob	59.2 ± 0.9*	4.70 ± 0.28*
21-23		
Lean control	31.7 ± 1.6	1.68 ± 0.10
ob/ob	66.2 ± 1.4*	5.56 ± 0.23*
25-27		
Lean control	33.3 ± 1.1	1.83 ± 0.12
ob/ob	65.2 ± 0.7*	5.11 ± 0.27*

NOTE. Mean ± SEM; n = 4 per group.
*$P < .01$ v lean control.

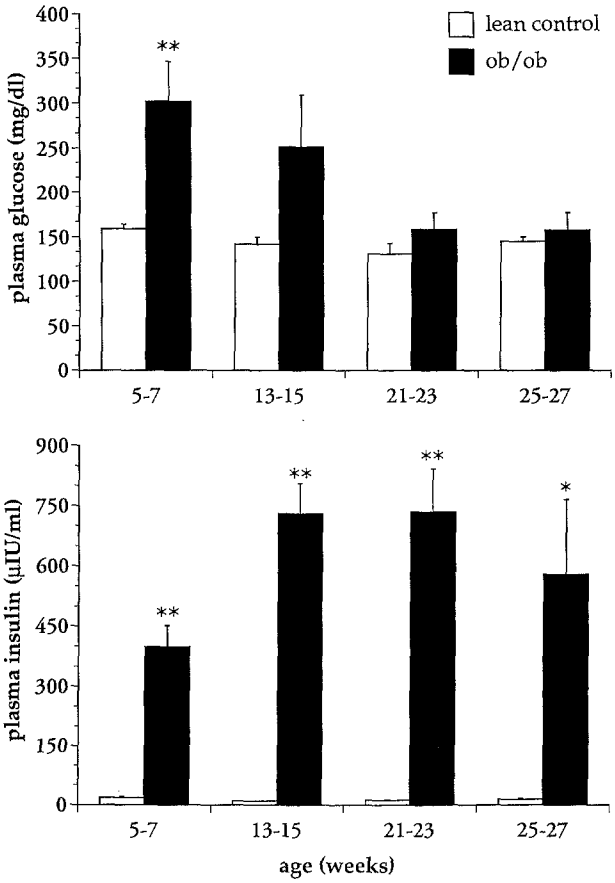

Fig 1. Plasma glucose and insulin in ob/ob and lean control mice of various ages. **$P < .01$, *$P < .05$: v lean control.

Comparison of PTPase Peptide Substrates

Since the region of the insulin receptor regulatory domain containing residues 1142 to 1153 has been shown to be the preferred phosphotyrosyl substrate for PTPases in liver,[5,6,29,31] synthetic peptides homologous to this region were chosen as substrates to assess PTPase activity in the present study. Initially, it was of interest to determine if the tyrosine phosphorylation state of the peptide influenced its effectiveness as a PTPase substrate. Thus, an experiment was conducted with liver preparations from 5- to 7-week-old ob/ob and control mice to compare PTPase activities measured with the triphospho form, TRDIY(P)ETDY(P)Y(P)RK, and the monophospho form, TRDIY(P)ETDYYRK.

Most PTPase activity in the liver was confined to the particulate fraction, consistent with previous studies.[16,22,30] There was a difference in PTPase activity measured with the two phosphorylated forms of the peptide. In all prepara-

Table 2. Comparison of Phosphopeptide Substrates for Measurement of PTPase Activity in Hepatic Particulate and Soluble Preparations

Substrate	Dephosphorylation Rate (nmol/min/mg protein)			
	Lean Control		ob/ob	
	Particulate	Soluble	Particulate	Soluble
TRDIY(P)ETDYYRK	5.18 ± 0.19	0.44 ± 0.02	5.19 ± 0.11	0.38 ± 0.02
TRDIY(P)ETDY(P)Y(P)RK	8.60 ± 0.32	0.70 ± 0.01	10.24 ± 0.33*	0.52 ± 0.05*

NOTE. Mean ± SEM; n = 4 per group. All mice were 5 to 7 weeks of age.
*$P < .01$ *v* lean control.

tions, the triphospho peptide resulted in higher PTPase activities as compared with the monophospho form. Moreover, only the triphospho peptide showed statistically significant differences between ob/ob and control preparations (Table 2).

In these young ob/ob mice, PTPase activity measured with the triphospho peptide in the particulate fraction was 19% greater than that of controls, whereas activity of the soluble fraction was 26% less (Table 2). The monophospho form did not show any differences between ob/ob and control particulate preparations, but it did show a slightly lower (14%) soluble PTPase activity in ob/ob mice. Although this lower soluble activity was similar to that observed with the triphospho peptide, the lower activity observed with the monophospho peptide was not statistically significant. Since only the triphospho form of the peptide showed significant differences between ob/ob and control preparations, this peptide was used for all subsequent analyses.

The liver, like most tissues, contains a variety of phosphatases that are capable of dephosphorylating phosphotyrosine-containing peptide substrates under certain experimental conditions.[15,18,22,35] Although the assay in the present study was designed specifically to measure PTPase activity by inclusion of EDTA, EGTA, the reducing agent β-mercaptoethanol, and a pH 7.4 reaction buffer, it was beneficial to examine the effect of a variety of phosphatase inhibitors on the dephosphorylation of the triphospho peptide. Particulate preparations from both ob/ob and control mice were equally sensitive to inhibition by micromolar concentrations of the commonly used PTPase inhibitors vanadate, zinc, and molybdate[15,27,28] (Table 3). The serine-threonine phosphatase inhibitors fluoride and okadaic acid, the acid phosphatase inhibitor tartaric acid, and the alkaline phosphatase inhibitor tetramisole did not appreciably affect PTPase activity. EDTA has been previously shown to activate certain PTPases and inhibit others.[27,28] In this study, addition of 2 mmol/L EDTA to the reaction had no effect, although this could be due to the fact that the initial homogenization buffer contained 2 mmol/L EDTA and 10 mmol/L EGTA. The inhibition of dephosphorylation of the triphospho peptide by the classic PTPase inhibitors and the lack of an effect by the inhibitors of other types of phosphatases provide further evidence that the phosphopeptide assay specifically measures PTPase activity.

Table 3. Effect of Phosphatase Inhibitors on Dephosphorylation of the Triphosphotyrosyl Peptide by Liver Particulate Preparations

Inhibitor	Concentration (mmol/L)	Relative Activity (%)	
		Lean Control	ob/ob
Untreated	0	100	100
Sodium orthovanadate	0.5	6	4
Ammonium molybdate	0.1	9	9
Zinc acetate	0.5	3	6
Sodium fluoride	5.0	88	90
Okadaic acid	0.005	91	99
Tetramisole	5.0	91	87
Tartaric acid	5.0	88	81
EDTA	2.0	90	94

NOTE. Preparations were obtained from 8- to 10-week-old mice.
*Values represent the percent of activity observed *v* untreated samples (100%).

PTPase Activity in the Longitudinal Study

The results shown graphically in Fig 2 indicate that liver particulate PTPase activity varied with age in both control

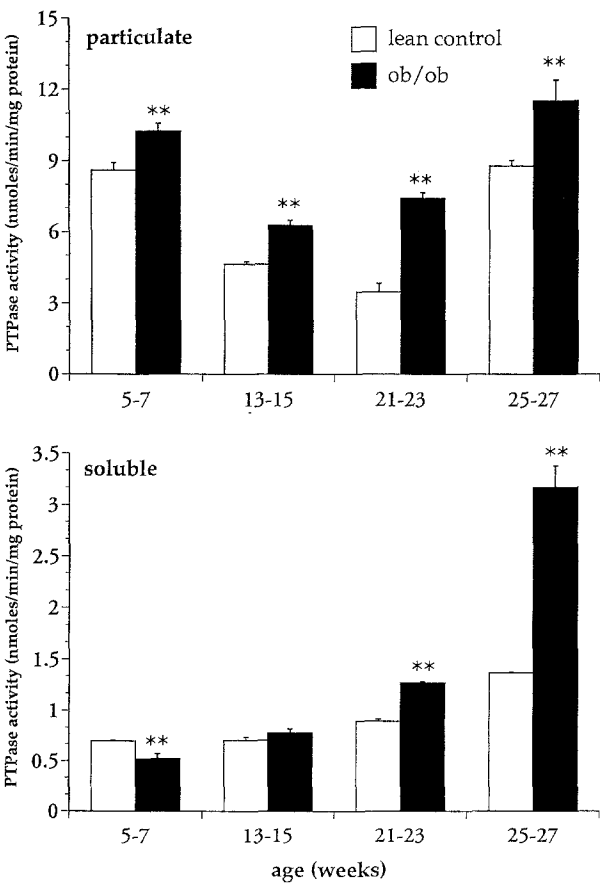

Fig 2. Hepatic particulate and soluble PTPase activity in ob/ob and lean control mice of various ages. **$P < .01$ *v* lean control.

Table 4. Effect of Vanadate Treatment on 8- to 10-Week-Old ob/ob Mice

Group	No.	Body Weight (g)	Liver Weight (g)
Lean control	12	23.9 ± 0.5	1.40 ± 0.03
ob/ob	12	41.6 ± 0.7*	2.93 ± 0.11*
ob/ob + vanadate	12	38.7 ± 0.8*†	2.55 ± 0.80*†

NOTE. Mean ± SEM. Sodium orthovanadate was administered in the drinking water (0.6 mg/mL) for 2 weeks.

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

†$P < .01$ *v* ob/ob.

and ob/ob mice and was consistently greater in ob/ob mice of all age groups. At 5 to 7 weeks, particulate PTPase activity was 19% higher in ob/ob mice, and at 21 to 23 weeks, it was 114% higher. Soluble PTPase activity increased gradually with age in both control and ob/ob mice, but the age-dependent increase was more dramatic in ob/ob mice. At 5 to 7 weeks of age, ob/ob soluble activity was 26% less than control activity. At 21 to 23 weeks, ob/ob soluble PTPase activity was 41% higher than control activity, and at 25 to 27 weeks, ob/ob soluble PTPase activity was 131% above control activity.

In an attempt to ascertain if there were changes in subcellular distribution of PTPases with age, soluble specific PTPase activity was compared with total specific PTPase activity, the sum of particulate and soluble activities. Soluble PTPase activity in ob/ob mice increased from 5% in the youngest to 22% in the oldest. In control mice, the percentage of soluble PTPase activity increased from 7.5% in the youngest to 21% in 21- to 23-week-old mice, and then decreased to 14% in the oldest mice. This decrease in the oldest control mice reflected an increase in particulate PTPase activity. Thus, along with the overall elevation of PTPase activity in ob/ob mice, there were age-dependent changes in the distribution of subcellular PTPase activity.

Effect of Oral Vanadate Administration

Since PTPase activity of ob/ob mice was found to be elevated in all particulate and most soluble preparations, it was important to evaluate the effect of administering a PTPase inhibitor. Accordingly, a group of ob/ob mice ages 6 to 8 weeks was treated for 2 weeks with vanadate (0.6 mg/mL in drinking water). Results of this study are shown in Table 4 and Figs 3 and 4.

Treatment of ob/ob mice with vanadate resulted in 7.0% lower body weights (Table 4), 13% lower liver weights, a 47% reduction in plasma insulin (Fig 3), and a normalization of plasma glucose as compared with untreated ob/ob mice. Water consumption measurements (not shown) indicate that the daily intake of vanadate was approximately 50 mg/kg. Figure 4 shows differences that were observed in hepatic PTPase activity after vanadate treatment. In untreated ob/ob mice, particulate PTPase activity was 7% above control levels ($P < .05$) and soluble PTPase activity was 18% less than control levels ($P < .01$). Vanadate treatment reduced particulate PTPase activity of ob/ob mice to 4.66 ± 0.10 nmol/min/mg, decreasing its level to less than that of untreated ob/ob mice (6.35 ± 0.14 nmol/min/mg, $P < .01$) and controls (5.95 ± 0.12 nmol/min/mg, $P < .01$). Soluble PTPase activity of ob/ob mice treated with vanadate appeared to be less than that of untreated mice, but this difference was not statistically significant.

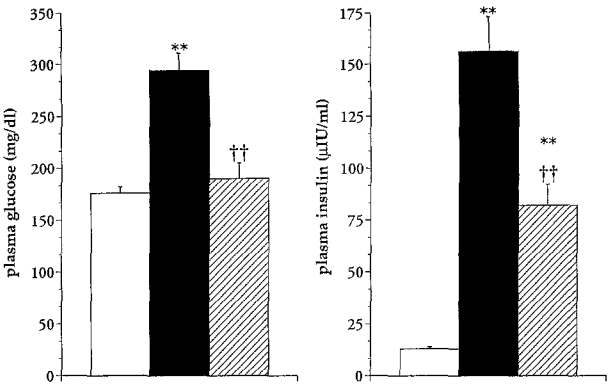

Fig 3. Effect of vanadate treatment on ob/ob plasma glucose and insulin. **$P < .01$ *v* lean control; ††$P < .01$ *v* ob/ob. (□) Lean control; (■) ob/ob; (▨) ob/ob + vanadate.

DISCUSSION

The present study demonstrates that the obese, hyperinsulinemic ob/ob mouse, a typical rodent model of NIDDM, has elevated hepatic PTPase activity directed against a synthetic peptide that is homologous to the major autophosphorylation sites of the insulin receptor, residues 1142 to 1153. Significant differences in liver PTPase activity between ob/ob and control mice were observed with the triphospho form of the peptide, TRDIY(P)ETDY(P)Y(P)RK, but not with the monophospho form, TRDIY(P)ETDYYRK. Oral administration of the PTPase inhibitor vanadate to ob/ob mice reduced the elevated liver PTPase activity, normalized plasma glucose, and caused a significant reduction in the inordinate hyperinsulinemia observed in this model. Results from this study suggest that the elevated hepatic PTPase activity may cause or contribute to the decline in insulin-induced protein phosphorylation that has been reported for this insulin-resistant model.[11,12]

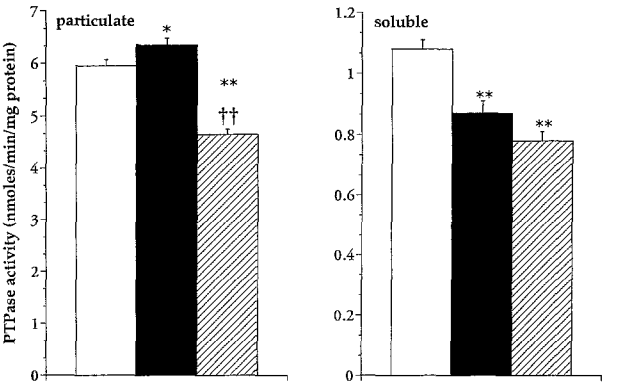

Fig 4. Effect of vanadate treatment on ob/ob hepatic particulate and soluble PTPase activity. **$P < .01$, *$P < .05$: *v* lean control; ††$P < .01$ *v* ob/ob. (□) Lean control; (■) ob/ob; (▨) ob/ob + vanadate.

Selecting an appropriate substrate for accurate assessment of PTPase activity has been a common problem,[27] especially in tissue extracts, since they contain a mixture of PTPases.[18,28] Consistent with this issue, the present study shows that the results and study conclusions were substrate-dependent. The triphospho form of the insulin receptor regulatory domain peptide showed significant differences between ob/ob and control preparations, whereas the monophospho form of the peptide showed some analogous differences that were not statistically significant. In all liver preparations, PTPase specific activity was higher with the triphospho peptide than with the monophospho form, suggesting that the higher enzyme activity with the triphospho peptide may be due to the stoichiometric difference, with the triphospho peptide offering more potential phosphotyrosine per mole substrate. Alternatively, the higher enzyme activity and the manifestation of significant differences between ob/ob and control liver preparations with the triphospho form only may indicate that the triphospho peptide was a selective substrate for a specific insulin receptor PTPase.

Aging and duration of disease have been associated with changes in hepatic PTPase subcellular activity in the rat.[16,17,19] In the present study, the ob/ob liver particulate fraction was elevated above control levels in all age groups. Enzyme activity in the particulate fraction did vary between age groups, but there was not a consistent age-dependent change. On the other hand, in both control and ob/ob mice, liver soluble PTPase activity increased with age, and the increase in ob/ob mice was dramatic. Initially, ob/ob liver soluble PTPase activity was less than control levels in the youngest mice (5 to 7 weeks) and increased with age, and was 131% higher than control levels at 25 to 27 weeks of age. Furthermore, when compared with total specific PTPase activity, the sum of particulate and soluble activities, the percentage of ob/ob soluble PTPase activity increased with age, from 5% in 5- to 7-week-old mice to 22% in the oldest ob/ob mice (25 to 27 weeks). These data clearly indicate an age-dependent change in soluble PTPase activity. It has been proposed that changes in PTPase subcellular activity may be attributed to a redistribution or translocation of enzyme(s), differential expression of enzyme(s), or increase in intrinsic enzyme activity.[16,17,36] The data in the present study do not entirely support an enzyme redistribution or translocation between subcellular fractions with age, since PTPase activity was higher in both the particulate and soluble fractions in the oldest mice, 25 to 27 weeks of age.

Besides the age-dependent change in hepatic PTPase activity, there were age-dependent changes in plasma glucose and plasma insulin. The ob/ob model has been characterized as being transiently hyperglycemic and transiently hyperinsulinemic.[8,9] Plasma glucose and insulin concentrations have been reported to peak at approximately 3 and 7 months of age, respectively. The evolution of this disorder indicates that the development of insulin resistance and diabetes is limited to a specific age period. In view of this, the influence of aging on metabolic parameters has been extensively studied in this model.[10] For example, increased hepatic glycogenolysis was reported for young but not old ob/ob mice.[37] Consistent with previous studies, the youngest mice in the present study (5 to 7 weeks) had significantly elevated plasma glucose concentrations. These young mice also had high plasma insulin concentrations and elevated particulate PTPase activity. The older mice, aged 21 to 23 weeks, were no longer hyperglycemic but were still extremely hyperinsulinemic and had elevated particulate and soluble PTPase activities. Therefore, the findings in the present longitudinal evaluation indicate that there is not a clear relationship between elevated hepatic PTPase activity and postprandial hyperglycemia.

Since a high plasma insulin concentration accompanied elevated hepatic PTPase activity, it is reasonable to propose a relationship between the two. Perhaps the inordinately high plasma insulin concentration caused the increased hepatic PTPase activity. In support of this hypothesis is a report in FAO hepatoma cells in which insulin caused an increase in particulate PTPase activity.[36] An additional study with hepatoma cells showed that insulin treatment increased the abundance of mRNA for PTP-1B but not for LAR or leukocyte common antigen-related phosphatase (LRP).[38] In the present study, statistical analyses were conducted to ascertain the relationship between plasma insulin concentration and hepatic PTPase activity. No significant trends were found. However, statistical analyses may have been influenced by the large variation in plasma insulin concentration in the older mice. Thus, the data in the present study simply suggest that the elevated hepatic PTPase activity may have been caused by the hyperinsulinemia. Future in vivo studies designed to regulate rigorously the plasma insulin concentration should shed light on this issue.

Oral administration of vanadate to young ob/ob mice caused a significant reduction in the elevated hepatic particulate PTPase activity. The reduction in PTPase activity was associated with a reduction in hyperinsulinemia and a normalization of plasma glucose, corroborating an earlier study in ob/ob mice.[33] Numerous studies have shown that treatment of a variety of diabetic rodent models and isolated cells with vanadate increases protein phosphotyrosine content and enhances insulin action (reviewed in Shechter[39]). Vanadate appears to replace insulin in insulin-deficient rats and to improve insulin action in insulin-resistant mice and rats. Although vanadate is a widely used in vitro PTPase inhibitor,[27,28] the precise in vivo glucose-lowering mechanism of vanadate is controversial. Alternative or different activities of vanadate that could directly or indirectly decrease plasma glucose concentrations or improve insulin sensitivity include activation of a cytosolic tyrosine kinase,[40] activation of a mitogen-activated protein kinase,[41] or inhibition of Ca^{2+}/Mg-adenosine triphosphatase.[42] All these effects of vanadate could also indirectly affect liver PTPase activity, since some PTPases have been shown to be regulated by second-messenger systems.[14] Nonetheless, it is possible that the decline in PTPase activity in the present study was caused by a direct inhibitory effect of vanadate on liver PTPases. If the pharmacokinetics of vanadate are similar in rat and mouse, a study in streptozotocin-diabetic rats[43] supports the assumption that

daily intake of vanadate 50 mg/kg by ob/ob mice in the present study produced serum vanadate levels that directly inhibited liver PTPase activity.

The increase in particulate PTPase activity reported in the present study does not appear to be in agreement with an earlier study of the ob/ob mouse that reported a decline in hepatic particulate PTPase activity.[25] Disparate findings between laboratories often reflect differences in rodent age, duration of diabetes, tissue preparations, and assay conditions. A close comparison of methodology suggests that the seemingly opposite findings may be explained by differences in tissue preparations. In the present study, the particulate fraction consisted of a pellet collected by low-speed centrifugation at 10,000 × *g* for 20 minutes. In the earlier study, this fraction was discarded and a fraction designated as the particulate was obtained after detergent extraction of the pellet collected after centrifugation at 100,000 × *g* for 45 minutes. Thus, the fraction for which we observed an elevation in PTPase activity was not examined in the earlier study. The soluble preparation in the present study is most similar to the particulate fraction in the earlier study. Moreover, results of these two preparations are similar, ie, both show a decline in PTPase activity in ob/ob mice that were 5 to 10 weeks of age. In older mice (21 to 25 weeks), an elevation of soluble PTPase activity was observed in the present study, whereas the earlier study did not examine older mice. Since the expression of insulin resistance in this model changes with maturity,[8-10] it is extremely important to relate all findings in this model to a specific age of mouse. Thus, if one compares similar liver preparations from same-aged mice, results between the two studies are alike—just the conclusions differ.

In summary, this is the first study to evaluate hepatic PTPase activity directed against a peptide that corresponds to the fully activated insulin receptor autoregulatory domain. The conclusion from this study is that ob/ob liver has elevated PTPase activity directed against this domain of the insulin receptor. The elevated hepatic PTPase activity may cause or contribute to the reported decline in receptor and postreceptor tyrosine phosphorylation[11,12] and the pathophysiology of insulin resistance reported in this model.[8-10] Although the present study and earlier studies[6,16-26,29,30,35,36] have shown that most of the insulin receptor PTPase activity is confined to the membrane fraction along with its proposed substrate, the insulin receptor, the precise identity of the PTPase that regulates the insulin receptor needs to be determined. Candidate enzymes cloned from the liver, LAR, LRP, and PTP-1B, have been shown to dephosphorylate and regulate insulin receptor tyrosine kinase activity in vitro.[5] The identity of the PTPase that is abnormal in the ob/ob liver is presently unknown, and studies are planned to address this issue.

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