Improved Sensitivity to Insulin in Obese Subjects Following Weight Loss Is Accompanied by Reduced Protein-Tyrosine Phosphatases in Adipose Tissue

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Insulin resistance in adipose tissue in human obesity is associated with increased protein-tyrosine phosphatase (PTPase) activity and elevated levels of the PTPases leukocyte common antigen–related PTPase (LAR) and PTP1B. To determine whether the improved insulin sensitivity associated with weight loss in obese subjects is accompanied by reversible changes in PTPases, we obtained subcutaneous adipose tissue from seven obese subjects (mean body mass index [BMI], 40.4 kg/m^2) before and after a loss of 10% of body weight and again after a 4-week maintenance period. Weight loss was accompanied by an 18.5% decrease in overall adipose tissue PTPase activity ($P = .015$) that was further reduced to 22.3% of the control value ($P = .005$) at the end of the maintenance period. By immunoblot analysis, the abundance of LAR was decreased by 21% ($P = .04$) and abundance of PTP1B was decreased by 40% ($P < .004$) after the initial weight loss, and the decreases persisted during the maintenance period. Enhanced insulin sensitivity following weight loss, evident from a 26% decrease in fasting insulin levels ($P < .05$), was also closely correlated with the reduction in the abundance of both LAR ($R^2 = .80$, $P < .01$) and PTP1B ($R^2 = .64$, $P = .03$). These results support the hypothesis that LAR and PTP1B may be reversibly involved in the pathogenesis of insulin resistance, and may be therapeutic targets in insulin-resistant states.

OBESITY, a major cause of morbidity and a contributor to mortality in a variety of chronic illnesses, has been increasing in prevalence in the United States, where it now affects over 30% of the adult population.[1] Defects in the insulin signaling pathway in its target tissues are recognized to be a major feature of the pathophysiology of human obesity that contributes to the increased incidence of non–insulin-dependent diabetes mellitus in overweight individuals.[2-4] The central role of reversible tyrosine phosphorylation of the insulin receptor and its substrate proteins is now well established,[5] although the mechanisms involved in the regulation of these pathways and derangements that occur in disease states have not been elucidated.

A number of recent studies have provided evidence that protein-tyrosine phosphatases (PTPases) play an integral role in the regulation of insulin signal transduction (reviewed in Goldstein[6]). The action of cellular PTPases can balance the autophosphorylation state of the insulin receptor kinase and regulate its enzymatic activity. Also, PTPases may modulate postreceptor signaling by dephosphorylating the phosphotyrosyl form of cellular proteins that are substrates for the insulin receptor kinase such as IRS-1, IRS-2, and Shc, which signal to downstream enzymes as "docking" proteins that bind and activate a number of *src*-homology 2 (SH2) domain–containing signaling proteins.[5]

We recently reported that nondiabetic obese human subjects had significantly increased PTPase activity in adipose tissue (1.74-fold higher than in lean controls) that correlated positively with body mass index (BMI).[7] Of candidate insulin receptor PTPases examined in the adipose tissue samples, significant increases were observed in the transmembrane PTPase leukocyte common antigen–related PTPase (LAR) and the intracellular enzyme PTP1B, both of which have been shown to act as negative regulators of the insulin action pathway at a proximal site in intact cells, where they affect the autophosphorylation state of the insulin receptor itself and activation of its kinase activity.[8-11] Further significance was attributed to the increase in LAR protein mass, since only immunodepletion of LAR from the tissue homogenates with neutralizing antibodies resulted in normalization of the PTPase activity toward the insulin receptor.[7] This result provided evidence that the increase in LAR was responsible for the enhanced PTPase activity in adipose tissue from obese subjects.

The present study was undertaken to explore whether the improved insulin sensitivity associated with weight loss in obese subjects is accompanied by reversible changes in tissue PTPase activity or in the abundance of specific PTPase enzymes that have been implicated in the regulation of the insulin signaling pathway. Subcutaneous adipose tissue was obtained by percutaneous aspiration from seven nondiabetic obese subjects before and after a loss of 10% body weight by caloric restriction and after a 4-week maintenance period at the target weight. Tissue total PTPase activity and the abundance of LAR and PTP1B protein were assessed in the adipose tissue samples.

SUBJECTS AND METHODS

Subjects

Seven obese subjects (six females and one male) with a stable body weight of at least 120% of the ideal value and who were not taking any medications and had no evidence of other metabolic disease besides obesity entered the study (Table 1). Blood samples were taken from the subjects while fasting, and were frozen at −80°C before analysis. To study the effect of weight loss, the subjects were fed a liquid diet providing 800 kcal/d (Optifast 800; Sandoz Nutrition, Minneapolis, MN) for a period of 8 to 12 weeks. Blood and tissue samples were then obtained after each subject had lost 10% of the initial body weight, and

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Table 1. Characteristics of the Obese Subjects Before and After Weight Loss

Variable	Baseline Value	After Loss of 10% Body Weight	After 4-Week Maintenance Period
BMI (kg/m^2)	40.4 ± 5.2	35.1 ± 4.5*	34.7 ± 4.5*
Fasting serum insulin (μU/mL)	16.1 ± 4.5	11.9 ± 5.0*	11.6 ± 3.9*
Fasting serum glucose (mg/dL)	84 ± 7	82 ± 6	85 ± 8

NOTE. Data are presented as the mean ± SD.
*P = .05 *v* baseline.

again after a 4-week maintenance period at the reduced weight. Informed consent was obtained before the study, and the procedures used were approved by the Institutional Review Board of Thomas Jefferson University. These subjects were studied in a parallel investigation to determine the effect of changes in body weight on serum leptin levels.[12] Plasma insulin and glucose levels were determined as described previously.[12]

Preparation of Adipose Tissue Homogenates

Adipocytes were obtained by aspiration of subcutaneous tissue from human volunteers by published methods.[13] Samples were rapidly frozen in liquid nitrogen and stored at −85°C before use. Approximately 10 to 12 g adipose tissue from each patient was homogenized in 50 mL ice-cold 10-mmol/L Tris hydrochloride buffer (pH 7.0) containing 0.25 mol/L sucrose, 2 mmol/L EDTA, 1 mmol/L PMSF, 25 mmol/L benzamidine, 10 μmol/L leupeptin, and 50 U/mL aprotinin, with 4 up/down strokes at setting no. 3 on a Polytron (Brinkmann Instruments, Westbury, NY). The crude homogenate was centrifuged at 3,000 × *g*, and the infranate below the fat cake was removed. To solubilize PTPase enzymes from the particulate compartment into the tissue homogenate, the infranate was made up to 1% (vol/vol) Triton X-100 and 0.6 mol/L KCl, stirred for 45 minutes at 4°C, and centrifuged at 15,000 × *g* for 20 minutes. The resulting supernatant was dialyzed overnight at 4°C against the homogenization buffer. Protein was assayed by the method of Bradford.[14]

Tissue PTPase Activity

Reduced, carboxamidomethylated, and maleyated (RCM) lysozyme (Sigma, St Louis, MO) was phosphorylated on tyrosine by partially purified rat liver insulin and epidermal growth factor (EGF) receptor kinases[15] in a 0.5-mL reaction mixture containing 35 mmol/L imidazole-HCl, pH 7.2, 50 mmol/L NaCl, 12 mmol/L magnesium acetate, 4 mmol/L manganese chloride, 0.2 mmol/L EGTA, 3% (vol/vol) glycerol, 30 mmol/L *N*-acetylglucosamine, 2 mmol/L DTT, 300 μmol/L insulin, 200 μmol/L EGF, and 4 mmol/L adenosine triphosphate (ATP) containing 200 cpm/pmol [γ-^{32}P]ATP as previously described.[16] The reaction was initiated with the addition of 0.5 mg RCM-lysozyme and incubated at 25°C for 12 to 18 hours. Na_3VO_4 (1 mmol/L) and ammonium molybdate (100 mmol/L) were added as inhibitors of PTPase activity contaminating the receptor kinase preparations. The reaction was terminated with the addition of trichloroacetic acid (TCA) to a final concentration of 20% (wt/vol) and centrifuged at 30,000 × *g* for 15 minutes at 4°C. The pellet was washed three times with 20% TCA and dialyzed against 50 mmol/L imidazole-HCl, pH 7.2. PTPase activity was assayed using 20 μL tissue extract (2.5 mg/mL protein) preincubated for 5 minutes at 30°C. The reaction was initiated by the addition of 20 μL phosphotyrosyl RGM-lysozyme (10 μmol/L) and terminated at 30 minutes by the addition of 0.9 mL acidic charcoal mixture (0.9 m NaCl, 90 mmol/L sodium pyrophosphate, 2 mmol/L NaH_2PO_4, and 4% vol/vol Norit A). After centrifugation in a microfuge, the amount of radioactivity in 0.4 mL supernatant was measured by Cerenkov counting in a liquid scintillation counter. Less than 10% of the added PTPase substrate was hydrolyzed during the reaction period, allowing a measurement of the initial reaction rate for tissue PTPase activity.

Immunoblot Analysis of PTPase Abundance

The adipose tissue homogenates (40 μg protein) were fractionated on gels containing sodium dodecyl sulfate and 7.5% (for LAR) or 10% (for PTP1B) polyacrylamide in a minigel apparatus.[17] 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.[18] Nitrocellulose membranes were then incubated in blocking buffer containing 150 mmol/L NaCl, 0.05% (vol/vol) NP-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 150 mmol/L NaCl, 0.05% (vol/vol) Tween 20, 5% (wt/vol) bovine serum albumin, 1% (wt/vol) ovalbumin, 0.01% (wt/vol) sodium azide, and 10 mmol/L Tris, pH 7.4, containing individual PTPase antibodies (1.0 μg/mL for PTP1B or 0.5 μg/mL for LAR), and rocking was continued for 2 hours. 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 and affinity-purified using Affi-Gel (Bio-Rad, Melville, NY) columns containing immobilized purified LAR cytoplasmic domain.[19,20] Polyclonal antiserum to PTP1B was obtained from Transduction Laboratories (Lexington, KY). Membranes were washed three times for 10 minutes with blotting buffer alone, 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-min washes with TBST. Immunoreactive proteins were visualized by direct phosphorimager analysis of the immunoblot (Molecular Dynamics, Sunnyvale, CA). Protein migration was calibrated with prestained molecular-size standards from Bio-Rad.

Statistical Methods

Experimental assays were performed in duplicate, and the data are presented as the mean ± SEM for results from tissue samples representing each of the study patients. Each of the experimental groups (after weight loss and during the weight-maintenance period) were compared with the control sample using a paired Student's *t* test. Correlations were analyzed by simple linear regression analysis and by Pearson product-moment analysis. Calculations were performed with the SigmaStat PC computing software (Jandel Scientific, San Rafael, CA).

RESULTS

In obese subjects, an improvement in metabolic status was evident after a loss of 10% body weight, since there was a significant (26% to 28%) decrease in fasting insulin that was maintained throughout the 4-week maintenance period at the reduced weight (Table 1). Since there was no change in the glucose level, the reduction in steady-state plasma insulin levels is an indirect indicator suggesting that weight loss was accompanied by an improvement in insulin action.

To determine whether the increase in tissue PTPase activity in obese versus lean individuals observed in our previous study was reversible after a significant loss of body weight, we determined the dephosphorylation of a synthetic PTPase substrate (phosphotyrosyl-RCM-lysozyme) that has been shown in our previous study to parallel tissue PTPase activity toward the insulin receptor β-subunit.[21,22] Following the period of weight loss, adipose tissue PTPase enzyme activity was decreased 18.5% (P = .015); at the end of the maintenance period, tissue

PTPase activity was further reduced by 22.3% of the control value ($P = .005$; Fig 1).

Weight loss was also associated with a reduced adipose tissue abundance of two PTPases that have been implicated in the regulation of the insulin action pathway (Fig 2). LAR and PTP1B were quantified by immunoblot analysis after polyacrylamide gel electrophoresis in which they migrated as the 85-kd processed PTPase subunit of LAR and the full-length, approximately 50-kd form of PTP1B, respectively. The mass of LAR was decreased by 21% after the loss of 10% body weight ($P = .04$); and the decrease expanded to 30% at the end of the maintenance period ($P = .004$). Similarly, PTP1B protein mass was reduced by 40% after the initial period of weight loss ($P = .004$). At the end of the maintenance period, PTP1B protein mass was decreased 29% compared with the control level. This latter result was not significantly different from the control value ($P = .08$), primarily because of a return to the baseline level of PTP1B protein in one individual.

As an indicator of improvement in insulin sensitivity, the change in fasting insulin level for each of the individuals in the study was also found to be correlated with the change in PTPase protein mass for both LAR and PTP1B (Fig 3). The correlation between these variables was highly significant, with R^2 values of .80 ($P < .01$) and .64 ($P = .03$) for LAR and PTP1B, respectively.

DISCUSSION

The association between obesity and defective insulin signaling in human subjects is well documented, but the cellular mechanisms involved remain poorly understood.[23-25] Defects in both insulin binding capacity and postbinding signaling in adipocytes from obese subjects have been reported.[3,25-27] In obese subjects, especially those with non–insulin-dependent diabetes mellitus, defects in adipocyte insulin receptor autophosphorylation and kinase activity have been observed, indicating a cellular defect affecting the steady-state balance of insulin receptor tyrosine phosphorylation and receptor activation.[28,29]

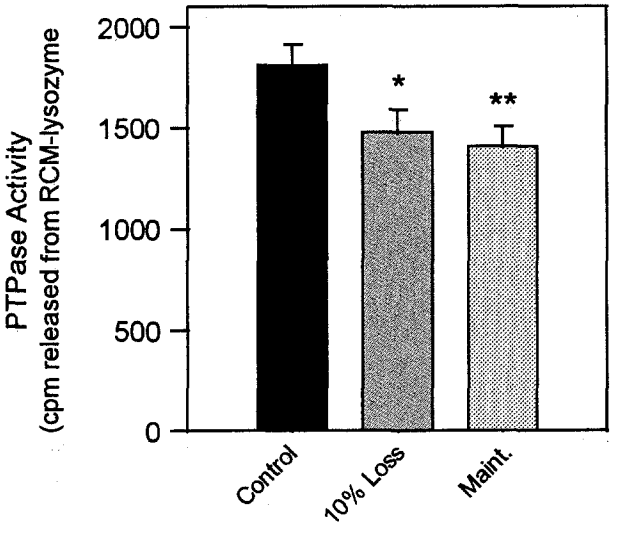

Fig 1. Effect of weight loss on PTPase activity in subcutaneous adipose tissue. Homogenates were prepared from subcutaneous adipose tissue obtained by aspiration biopsy, and PTPase activity was assayed by incubation with phosphotyrosyl RCM-lysozyme. Results are reported as cpm released from RCM-lysozyme (mean ± SE) for tissue samples from all of the individuals in each group as indicated. *$P < .015$, **$P < .005$. Maint., maintenance.

We and others have recently demonstrated that PTPase enzymes play an essential role in the steady-state regulation of insulin receptor autophosphorylation and activation and in the phosphorylation state of downstream signaling proteins in the insulin action pathway.[6,8,11,30,31] These findings have led to the hypothesis that aberrant regulation or increased activity of PTPases that impact the insulin action pathway may be implicated in the pathogenesis of insulin-resistant disease states. Also, the clinical effectiveness of vanadate and related compounds, which may act as potent PTPase inhibitors, has been demonstrated, since they enhance insulin signaling in vitro and can effectively reduce hyperglycemia in diabetic animal models[32-34] and human subjects.[35-37]

In human obesity, we have recently shown that specific PTPases may be involved in the pathogenesis of clinical insulin resistance, since obese subjects had a highly significant increase in tissue PTPase activity (1.7-fold) that correlated (by linear regression analysis) with the BMI.[7] This study also implicated an increase in LAR as being responsible for the enhanced PTPase activity in adipose tissue samples in the obese subjects, since it had the most prominent increase in mass (twofold). Furthermore, of three PTPases examined, only immunodepletion of LAR protein from the homogenates with neutralizing antibodies resulted in normalization of PTPase activity toward the insulin receptor.

The occurrence of insulin resistance or its magnitude in obesity appears to be acquired to a large extent, since normal-weight individuals develop insulin resistance with weight gain,[23] and numerous studies have demonstrated an improvement in insulin action in obese individuals following a period of weight loss.[4,25,38-40] Since we have demonstrated an increase in PTPase activity in adipose tissue in obese individuals,[7] the present study was undertaken to explore whether weight loss, which leads to an enhancement of impaired insulin action in obesity, is associated with an alteration in PTPase activity and affects specific PTPase enzymes. Our data, showing decreased PTPase activity in tissue homogenates and a reduction in LAR and PTP1B protein levels, support this hypothesis.

LAR is a major candidate for regulating the insulin action pathway, since it is expressed in the membrane of insulin-sensitive tissues, where rapid dephosphorylation of the insulin receptor has been demonstrated, and in vitro the cytoplasmic domain of LAR has a catalytic preference for the regulatory phosphotyrosines in the insulin receptor kinase domain.[6,41-44] We have also shown by manipulation of LAR abundance and subcellular localization in intact cells that LAR acts as an important negative regulator of insulin receptor activation in situ at the plasma membrane.[9-11] Taken together, these studies provide strong evidence that LAR is a physiological modulator of insulin signaling. The finding in the present study that LAR abundance is decreased after weight loss in obese subjects,

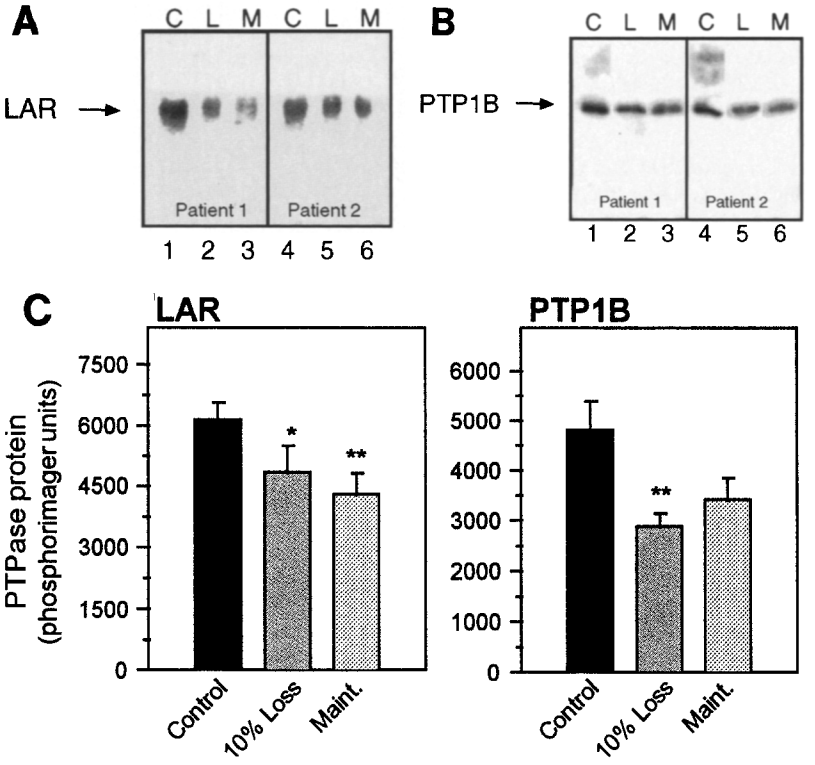

Fig 2. Effect of weight loss on protein abundance of (A) LAR and (B) PTP1B. Samples of tissue homogenates from each study participant at baseline (control), after 10% loss of body weight, and after the maintenance period (Maint.) were separated on polyacrylamide gels, transferred to nitrocellulose filters, and blotted with affinity-purified antibody to the recombinant cytoplasmic domain of LAR and PTP1B. (C) Quantitation of data presented in A and B. Signal density was evaluated by phosphorimager analysis; mean ± SE for each group shown. *$P < .05$, **$P < .01$.

along with the previously observed increase in LAR in obese versus normal-weight individuals, provides compelling evidence of a potential role for this regulatory PTPase in the reversible insulin resistance that occurs in obese subjects.

Other PTPases, particularly PTP1B, have also been implicated as regulators of insulin signaling.[6] PTP1B is a widely expressed enzyme that was first identified as a prominent PTPase in placenta.[45] Cell microinjection and transfection studies have suggested that this PTPase has a potential role in the regulation of insulin signaling and that of other tyrosine kinase receptors.[46-49] We have recently demonstrated by osmotic loading of neutralizing antibodies into insulin-sensitive

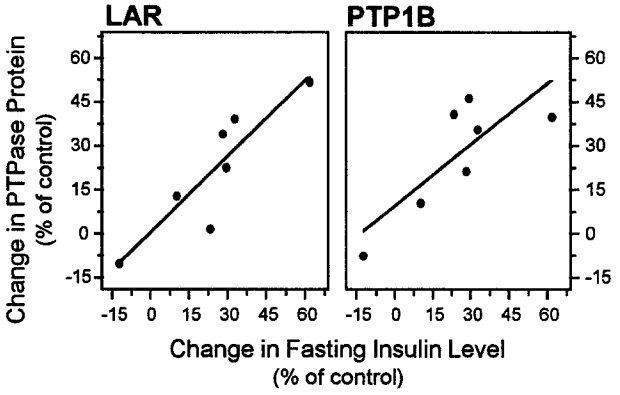

Fig 3. Reduction of fasting insulin level after weight loss correlates with decreased protein abundance of LAR and PTP1B. For each individual, the change in protein level for LAR or PTP1B was plotted against the change in fasting insulin level after the maintenance period compared with the control value. Linear regression lines are drawn through the data points.

hepatoma cells that PTP1B negatively regulates insulin receptor activation and distal transmission of the insulin signal.[8] Since PTP1B has a demonstrated role in the regulation of insulin signaling and acts, at least in part, directly at the level of the receptor kinase, it may function in concert with LAR in physiological regulation of the insulin receptor.

Other cellular PTPase enzymes have been implicated in the insulin action pathway in a few reported studies. LRP (RPTP-α) is a receptor-type PTPase that is widely expressed, including insulin-sensitive tissues, and is localized to the cell membrane. In addition, LRP can catalyze dephosphorylation of the insulin receptor and inactivation of the receptor kinase.[44] Recently, using a novel assay system in transfected cells, Moller et al.[30] provided evidence that LRP and the closely related transmembrane enzyme RPTP-ϵ expressed at high levels could serve as negative regulators of the insulin action pathway. Clearly, additional study will be required to delineate the role of each of these PTPases in the regulation of insulin signaling and their potential role in insulin resistance.

In summary, these studies provide further support for the involvement of specific PTPases, LAR and PTP1B, in the reversible insulin resistance found in adipose tissue in human obesity. Whether these findings can be extended to other insulin-sensitive tissues such as liver and skeletal muscle will be the subject of further studies. Since LAR and PTP1B may be reversibly involved in the pathogenesis of insulin resistance in human obesity, they may be appropriate therapeutic targets to ameliorate insulin-resistant disease states.

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