Protein-Tyrosine Phosphatase Activity in Human Adipocytes Is Strongly Correlated With Insulin-Stimulated Glucose Uptake and Is a Target of Insulin-Induced Oxidative Inhibition

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Protein-tyrosine phosphatases (PTPases), in particular PTP1B, have been shown to modulate insulin signal transduction in liver and skeletal muscle in animal models; however, their role in human adipose tissue remains unclear. The uptake of ^{14}C-D-glucose in response to 10 or 100 nmol/L insulin was measured in isolated subcutaneous adipocytes from subjects with a mean age of 44 years (range, 26 to 58) and mean body mass index (BMI) of 35.6 (range, 29.7 to 45.5). The endogenous activity of total PTPases and specifically of PTP1B in immunoprecipitates was measured in cell lysates under an inert atmosphere with and without added reducing agents. Using nonlinear regression analysis, higher BMI was significantly correlated with lower adipocyte glucose uptake ($r = 0.73$, $P = .01$) and with increased endogenous total PTPase activity ($r = 0.64$, $P = .04$). Correlation with waist circumference gave similar results. The endogenous total PTPase activity also strongly correlated with insulin-stimulated glucose uptake ($R = .89$, $P < .0001$); however, the activity of PTP1B was unrelated to the level of glucose uptake. Consistent with the insulin-stimulated oxidative inhibition of thiol-dependent PTPases reported for 3T3-L1 adipocytes and hepatoma cells, treatment of human adipocytes with 100 nmol/L insulin for 5 minutes lowered endogenous PTPase activity to 37% of control ($P < .001$), which was increased 25% by subsequent treatment with dithiothreitol in vitro. Cellular treatment with diphenyleneiodonium (DPI), an NADPH oxidase inhibitor that blocks the cellular generation of H_2O_2 and reduces the insulin-induced reduction of cellular PTPase activity, also diminished insulin-stimulated glucose uptake by 82% ($P = .001$). These data suggest that total cellular PTPase activity, but not the activity of PTP1B, is higher in more obese subjects and is negatively associated with insulin-stimulated glucose transport. The insulin-stimulated oxidative inhibition of PTPases may also have an important permissive role in the transmission of the insulin signal to glucose transport in human adipocytes.

INSULIN HAS MAJOR effects on glucose and lipid metabolism in adipocytes.[1] Insulin signaling is initiated by the phosphorylation of specific tyrosyl residues in the cytoplasmic domain of the cell surface insulin receptor, which activates the receptor kinase activity and promotes the tyrosine phosphorylation of IRS proteins and other cellular substrates.[2] In turn, protein-tyrosine phosphatases (PTPases) regulate the steady-state level of tyrosine phosphorylation of proteins in the insulin action pathway and determine the net initiation and propagation of the insulin signal.[3]

Progress has also been made recently in identifying some of the mechanisms involved in the regulation of PTPases in insulin-sensitive cells. We have previously shown that PTPase protein expression and activity is increased in adipose tissue of obese human subjects and is associated with insulin resistance.[4,5] In addition to changes in enzyme mass, cellular reactive oxygen species, which have been recognized for years to increase rapidly in adipose cells in response to stimulation with insulin or other growth factors,[6] have also recently been demonstrated to regulate PTPase enzyme activity in cultured cell systems, since these enzymes are dependent on the reduced state of the catalytic thiol residue.[7] While we have reported that air exposure of human adipose tissue lysates leads to oxidative inhibition of PTPases,[8] the effects of insulin itself on overall PTPase activity in intact, isolated human adipocytes has not been previously examined.

The intracellular enzyme PTP1B has been recently shown by several laboratories to have important effects on the regulation of insulin signaling in a variety of cell types (reviewed by Goldstein[9]). Knock-out mouse models generated by 2 research groups have provided evidence that PTP1B regulates the insulin action pathway in a tissue-specific manner, significantly affecting liver and skeletal muscle but not adipose tissue.[10,11] PTP1B knock-out mice exhibit lower fasting insulin and glucose levels and an accentuated drop in blood glucose during insulin tolerance testing, associated with enhanced insulin-stimulated tyrosine phosphorylation in skeletal muscle and liver, and increased glucose uptake into skeletal muscle with little change in insulin signaling in adipose tissue.[10,11]

Prior studies in adipose cells using transfection techniques have also suggested that PTP1B may have attenuated effects on insulin signaling compared to other insulin-sensitive cell types. For example, primary cultures of rat adipose cells transiently transfected with catalytically active PTP1B demonstrated significantly reduced GLUT4 translocation to the cell surface, as might be expected with overexpression of a highly active PTPase.[12] However, cells overexpressing a catalytically inactive site-directed mutant of PTP1B, which has been shown in other cell types to act in a dominant negative fashion and enhance insulin signaling,[13] did not affect insulin action on

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GLUT4 translocation in the mature adipose cells.[12] Also, in the 3T3L1 adipocyte model, overexpression of active PTP1B inhibited insulin-stimulated tyrosyl phosphorylation of the insulin receptor and IRS-1 and phosphoinositide 3-kinase (PI3K) activity associated with IRS-1 or with phosphotyrosine; however, the activation of Akt was unchanged and PTP1B overexpression had no effect on insulin-stimulated glucose transport.[14] Altogether, these data suggest that the effects of PTP1B on insulin signaling in adipocytes may be limited and may not include an impairment of glucose transport.

To help clarify the potential role of PTPases and PTP1B in insulin signaling in human adipocytes, we related the level of insulin-stimulated glucose uptake to the endogenous level of overall PTPase activity as well as that of PTP1B after immunoabsorption from the cell lysates. To preserve the endogenous level of PTPase catalytic activity, the cell lysates and isolated enzymes were handled under an inert atmosphere to avoid air oxidation of the catalytic thiol residue. Enzyme activity was also measured with and without added reducing agents to determine the level of endogenous, partially oxidized PTPase activity that is activatable by biochemical reduction.[8] We further examined how blocking the insulin-stimulated burst of cellular H_2O_2 with diphenyleneiodonium (DPI), an inhibitor of cellular NADPH oxidase activity,[7] affected cellular PTPase activity as well as insulin-stimulated glucose transport in the human cells. Interestingly, our data show that the level of total cellular PTPase activity, but not the endogenous activity of PTP1B, is higher in more obese subjects and is negatively associated with insulin-stimulated glucose transport. The insulin-stimulated generation of H_2O_2 via NADPH oxidase leading to oxidative inhibition of PTPases may also have an important permissive role in the transmission of the insulin signal to glucose transport in human adipocytes.

MATERIALS AND METHODS

Materials

General reagents were of the highest available grade and obtained from Sigma Chemical Co (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). The BCA dye reagent for protein assay was from Bio-Rad Laboratories (Hercules, CA). Wheat germ agglutinin was from Vector Laboratories, Inc (Burlingame, CA). U-^{14}C-D-glucose and γ-^{32}P-adenosine triphosphate (ATP) were obtained from AP Biotech (Piscataway, NJ). Dinonylphthalate oil was from Pfaltz & Bauer (Waterbury, CT).

Study Subjects

All procedures were approved by the Institutional Review Board of Thomas Jefferson University and informed consent was obtained by the investigators prior to adipose tissue biopsy. The overall group of participants included 11 subjects with a mean age of 44 years (range, 26 to 58) and a mean body mass index (BMI) 35.6 kg/m^2 (range, 29.7 to 45.5). Adipocytes from one additional subject who did not have a BMI recorded were used for analyses of glucose transport and PTPase activity. Prior to adipocyte sampling, the subjects were fasted overnight and they did not take any of their daily medications that morning.

Adipocyte Isolation

Following institutionally approved informed consent procedures, 6- to 8-g samples of subcutaneous adipose tissue were obtained by aspiration according to established methods.[15] Isolated adipocytes were obtained from freshly aspirated adipose tissue as described.[16] Briefly, the tissue fragments were digested in Dulbecco's modified Eagles medium (DMEM):F-12 (50:50) medium containing 1% bovine serum albumin (BSA), 1 mg/mL collagenase (type I) at 37°C, for 40 to 60 minutes, then filtered sequentially through sterile nylon mesh 500 μm followed by 250 μm. Adipocytes were washed twice in warmed DMEM/F-12 medium containing antibiotics. After centrifugation at 800 $\times$ *g* for 5 minutes, adipocytes were transferred to a fresh DMEM/F-12 medium.

Glucose Uptake

Glucose transport was measured according to published procedures.[17] Briefly, adipocytes were washed twice in KRH buffer (140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1.0 mmol/L $CaCl_2$ in 20 mmol/L Hepes buffer, pH 7.4), containing 5% (wt/vol) BSA. Cells were resuspended in the same buffer at 10% cytocrit in 50-mL tubes. Aliquots of 210 μL cell suspension were transferred to small polypropylene scintillation vials in triplicate, and equilibrated at 37°C for 30 minutes in a water bath. Where indicated, the cells were then treated with either 10 or 100 nmol/L insulin for 30 minutes prior to the addition of ^{14}C-D-glucose (1 μCi) to each sample. Incubation was then continued at 37°C for 1 hour. The reactions were terminated by transferring 300 μL of the cell suspension to a 0.4-mL polypropylene microcentrifuge tube (Fisher Scientific, #05-407-9) and rapid centrifugation at 8,000 rpm for 5 minutes through a 75-μL cushion of dinonylphthalate oil. The long tubes were cut with a nail clipper, and the top adipocyte layer was dropped into scintillation vials containing 5 mL of scintillation fluid. The final count was normalized to the amount of cell protein estimated in an aliquot of the adipocyte suspension using the BCA reagent as described by the manufacturer (Bio-Rad).

Cell Treatment and Anaerobic Sample Handling

Where indicated, adipocytes were treated with recombinant human insulin (Sigma) and incubated for the indicated period of time under an atmosphere of 5% CO_2 in air at 37°C. For anaerobic assay of endogenous PTPase activity, cell homogenization and enzyme assay were performed in an enclosed anaerobic work station (Forma Scientific, model #901024), as we recently described.[8] The chamber uses a palladium catalyst and desiccant wafers to maintain a strict anaerobic atmosphere ($<$10 ppm O_2). High purity N_2 is used for purging the chamber and the working anaerobic gas mixture is N_2:H_2:CO_2 proportioned at 85:10:5. For lysis under anaerobic conditions, the cell samples were snap frozen in liquid nitrogen after the indicated treatments and introduced into the chamber in a frozen state. All buffers and apparatus exposed to the samples were deoxygenated overnight in the chamber prior to use.

Preparation of Adipose Tissue Homogenates

Approximately 4 g of adipose tissue from each subject was homogenized in 16 mL of ice-cold deoxygenated homogenization buffer containing 10% glycerol, 150 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 25 mmol/L benzamidine, 10 μmol/L leupeptin, 2.5 μmol/L pepstatin A, and 50 U/mL aprotinin in 10 mmol/L Tris-HCl (pH 7.0), with a 4 up/down strokes at setting no. 3 using a Polytron (Brinkmann Instruments, Westbury NY). The crude homogenate was centrifuged at 3,000 $\times$ *g* for 15 minutes, and fat cake was discarded; the infranate was made up to 1% (vol/vol) Triton X-100 to solubilize PTPase enzymes from the particulate compartment into the tissue homogenate. The supernatant resulting from centrifugation at 15,000 $\times$ *g* for 20 minutes at 4°C was stored at $-$80°C in aliquots prior to use. The protein concentration of each sample was measured as described above.

Specific Activity of PTP1B

Under strictly anaerobic conditions, PTP1B was immunoprecipitated from cell lysates with a monoclonal antibody directed at a C-terminal epitope that preserves its enzymatic activity (Oncogene Research Products, San Diego, CA; Ab-2) followed by adsorption to trisacryl protein G (Pierce, Rockford, IL). Aliquots containing 200 μg of adipose cell lysate protein were first pre-cleared by incubation with 2 μL mouse serum and 30 μL protein G beads at 4°C for 1 hour to adsorb nonspecifically binding proteins. Following centrifugation, the protein G beads were discarded. The supernatant was incubated with 2.0 μg monoclonal anti-human PTP1B antibody at 4°C for 12 hours, followed by incubation with 40 μL protein G beads at 4°C for 2 hours. After washing 3 times in the homogenization buffer, the protein G beads were used for enzyme activity assay within the anaerobic chamber using the *p*NPP hydrolysis assay described below. Control samples using non-immune mouse IgG showed minimal background PTPase activity (<5% of the activity with Ab-2).

PTPase Enzyme Activity Using [^{32}P]-RCM-Lysozyme as Substrate

Recombinant human insulin receptors from transfected CHO cells[18] were partially purified on wheat germ lectin-agarose as described[19] and used to phosphorylate reduced, carboxamidomethylated, maleyated (RCM) lysozyme.[20] The reaction was initiated with the addition of 1 mg of RCM-lysozyme in the reaction buffer and incubated at 25°C for 12 to 18 hours. The reaction buffer included 40 mmol/L imidazole hydrochloride, 50 mmol/L NaCl, 12 mmol/L magnesium acetate, 4 mmol/L $MnCl_2$, 0.2 mmol/L EGTA, 0.05% (vol/vol) Triton X-100, 3% (vol/vol) glycerol, 100 μmol/L ammonium molybdate, 0.1 mmol/L Na vanadate, 30 mmol/L *N*-acetylglucosamine, 0.2% (wt/vol) deoxycholate, 2 mmol/L dithiothreitol (DTT), 4 mmol/L ATP, 300 nmol/L insulin, 400 μg wheat germ agglutinin-purified protein enriched in insulin receptors, and [γ-^{32}P]ATP. The reaction was terminated with the addition of trichloroacetic acid (TCA) to a final concentration of 20% (wt/vol) and centrifuged at 27,000 $\times$ *g* for 15 minutes at 4°C. The pellet was washed 3 times with 20% TCA, suspended in 2 mol/L Tris base and dialyzed overnight against 50 mmol/L imidazole-HCl, pH 7.2.

PTPase activity using radiolabeled RCM lysozyme was measured with 20 μg protein aliquots of adipose tissue lysates in reaction buffer (25 mmol/L imidazole hydrochloride, pH 7.0, containing 1 mg/mL fatty acid and globulin-free BSA). Where indicated, assay samples were incubated with 1 mmol/L DTT on ice for 10 minutes prior to enzyme assay. The reaction was initiated by the addition of phosphotyrosyl RCM-lysozyme (>10,000 dpm) and incubated at 30°C, and terminated by the addition of 0.9 mL of acidic charcoal mixture, consisting of 0.9 mol/L NaCl, 90 mmol/L Na pyrophosphate, 2 mmol/L NaH_2PO_4 and 4% (vol/vol) Norit A.[21] After centrifugation in a microfuge, the amount of radioactivity in 0.4 mL of supernatant was measured by Cerenkov counting in a liquid scintillation counter. The tissue fraction was diluted so that less than 20% of the RCM-lysozyme was hydrolyzed during the reaction period of up to 30 minutes. The initial rate of RCM-lysozyme hydrolysis was estimated from the linear portion of the earliest time points of the enzymatic reaction and reported as CPM ^{32}P released per μg protein per minute.

PTP1B Enzyme Assay Using pNPP as Substrate

Under strict anaerobic conditions, PTP1B activity was determined in the immunoprecipitates in a final volume of 100 μL at 30°C for 30 minutes in reaction buffer containing 10 mmol/L (*p*NPP) and 2 mmol/L EDTA in 20 mmol/L 2-(*N*-Morpholino) ethanesulfonic acid (MES) at pH 6.0. The reaction was stopped by the addition of 50 μL of 1 mol/L NaOH and the absorption was determined at 410 nm.[22] PTPase activity is reported as the OD from hydrolysis of *p*NPP.

Statistical Analyses

Quantitative data are calculated from the mean $\pm$ standard error values from at least 3 separate determinations. One-way analysis of variance (ANOVA) was used for comparison of multiple group means with post-hoc testing for determination of significance. Nonlinear regression and correlation analyses were performed using SigmaPlot and SigmaStat software (SPSS, Inc, Chicago, IL), modeling with an inverse first-order polynomial equation ($y = y_0 + a/x$). Differences were regarded as significant when the *P* value was less then .05.

RESULTS

Association Between Adipocyte Glucose Uptake and BMI of the Study Subjects

Glucose transport was initially measured in the isolated subcutaneous adipocytes and tested whether this action of insulin was related to the BMI of the study subjects. For glucose uptake stimulated by either 10 nmol/L or 100 nmol/L insulin, BMI was significantly correlated in a negative fashion with glucose uptake ($r = 0.73$, $P = .01$; and $r = 0.63$, $P = .04$, respectively; Fig 1A and B). Similar results were obtained using waist circumference in place of BMI, as an index of visceral adiposity. For glucose uptake stimulated by 10 nmol/L insulin, the correlation with waist circumference was significant ($r = 0.61$, $P = .04$). Using 100 nmol/L insulin, the correlation with waist circumference was borderline due to one outlying subject with waist circumference of 99 cm and a very low glucose uptake of 1.05 (Fig 1D). If this point were excluded from the analysis, the correlation becomes significant ($r = 0.64$, $P = .04$). The subject's gender or the presence of type 2 diabetes had no effect on the correlation analyses (Fig 1).

Association Between BMI and PTPase Activity

Previously, we reported that nondiabetic obese subjects have a higher adipose tissue PTPase activity compared to lean subjects.[4,5] In the present work, we applied our updated methodology using anaerobic conditions before and after biochemical reduction of cell lysates, in order to assess the endogenous cellular PTPase activity and determine whether this was also related to the BMI of the study subjects. These results confirmed that BMI was significantly correlated with PTPase activity in the unreduced cell lysates, reflecting the endogenous overall activity level of the cellular PTPase activity ($r = 0.64$, $P = .04$). Interestingly, the subject's BMI was not significantly associated with PTPase activity in cell lysates following biochemical reduction by incubation with 1 mmol/L DTT for 10 minutes prior to enzyme assay ($P > .05$).

We also previously reported that in human subjects with type 2 diabetes, PTPase activity in skeletal muscle was significantly decreased compared to nondiabetic control subjects.[23] In the present data set, endogenous PTPase activity measured anaerobically in the lysates from subcutaneous adipose tissue, either before or after reduction with DTT, did not correlate with the presence or absence of clinical type 2 diabetes.

We also evaluated the specific activity of PTP1B, immunoprecipitated from the adipocyte lysates under anaerobic conditions. The enzyme activity of PTP1B showed a median of 0.19 $\pm$ 0.03 before and 0.18 $\pm$ 0.03 (mean $\pm$ SEM) after treatment of the immunoprecipitated enzyme samples with

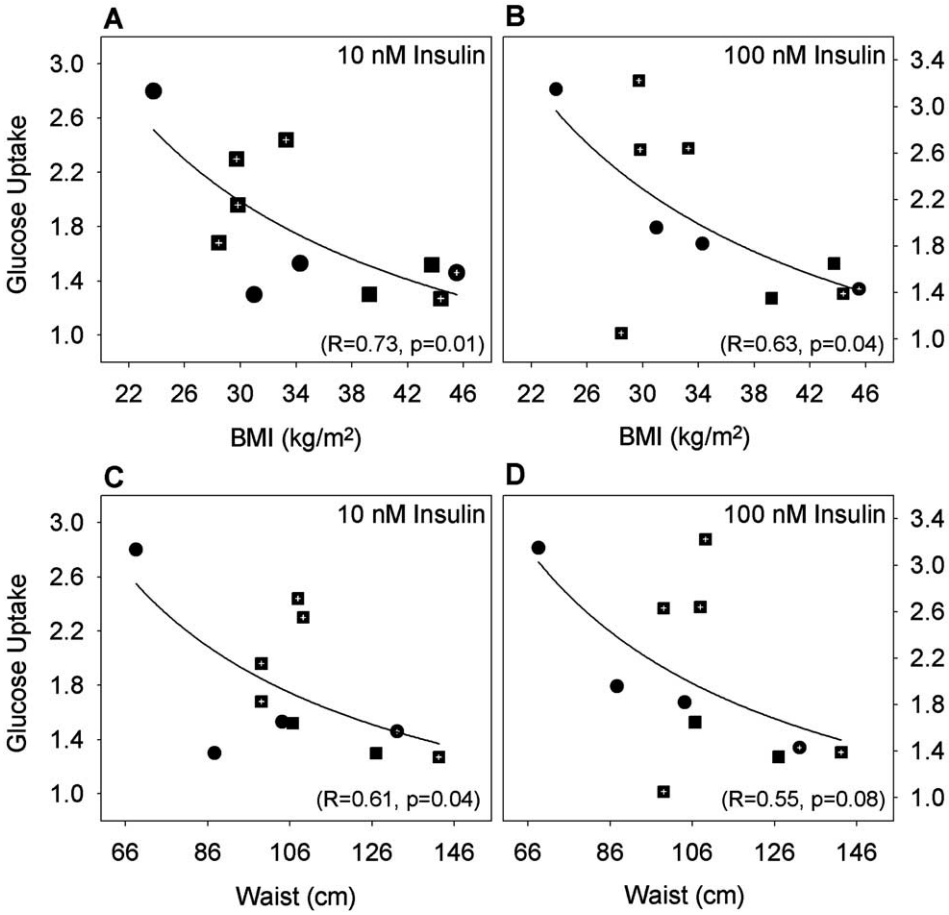

Fig 1. Correlation between insulin-stimulated glucose uptake with BMI and waist circumference. Isolated human subcutaneous adipocytes were stimulated with 10 nmol/L (A and C) or 100 nmol/L insulin (B and D) and the uptake of ^{14}C-D-glucose was measured as described in the Methods. Glucose uptake is presented as the ratio of CPM incorporated between insulin-stimulated and unstimulated (control) cells. Correlations with BMI and waist circumference are shown in A and B and in C and D, respectively. (■) Males; (●) females. Patients with type 2 diabetes are indicated by a "+" in the center of the symbol. The results of nonlinear regression analyses performed as described in the Methods are shown for each curve.

DTT in vitro, using the *p*NPP assay. The correlation between the subject's BMI and the specific activity of PTP1B either unreduced or following biochemical reduction was not significant ($P > .05$).

Association Between Insulin-Stimulated Adipocyte Glucose Uptake and Cellular PTPase Activity

To evaluate whether insulin-stimulated glucose uptake was associated with cellular PTPase content, we tested for a correlation between the PTPase activity in adipocyte cell lysates under anaerobic conditions with or without biochemical reduction with DTT and insulin-stimulated glucose uptake. The correlation between the endogenous, unreduced PTPase activity and insulin-stimulated glucose uptake was highly significant ($r = 0.89$, $P < .0001$) (Fig 2A). The correlation between glucose uptake and the total PTPase activity following reduction with DTT was also significant, but somewhat weaker ($r = 0.61$, $P = .034$) (Fig 2B).

The activity of PTP1B was also specifically measured by immunoprecipitation under anaerobic unreduced as well as reducing conditions. However, the activity of PTP1B, reduced or unreduced, was not significantly correlated to the level of glucose uptake stimulated by 10 nmol/L insulin or 100 nmol/L insulin ($P = 0.31$ to 0.73; data not shown).

Evaluating each of the tested comparisons above by multiple linear regression analysis, we found that glucose transport stimulated by 100 nmol/L insulin as a dependent variable can be significantly predicted from the unreduced total PTPase activity in the cell lysate ($P < .05$), reflecting the endogenous PTPase activity level.

Effect of Insulin Stimulation on Cellular PTPase Activities

In recent studies, we have shown that insulin has a dynamic effect of the activity of endogenous PTPase enzymes, by a mechanism that involves oxidative inhibition of the PTPase catalytic thiol residue.[24] To determine whether this phenomenon also occurs in human adipocytes, we treated human adipocytes with 100 nmol/L insulin for 5 minutes and measured endogenous PTPase activity, preserving the oxidation state of the enzymes by working within the environment of the anaerobic chamber (Table 1). Prior to insulin treatment, DTT did not significantly affect the PTPase activity in the cell lysate. Insulin treatment dramatically reduced the PTPase activity in cell lysates to 37% of control ($P < .001$). Treatment of the cell lysates from the insulin-stimulated cells in vitro with DTT prior to enzyme assay tended to increase the PTPase activity, suggesting that the enzyme activity may be partially restored by biochemical reduction (Table 1).

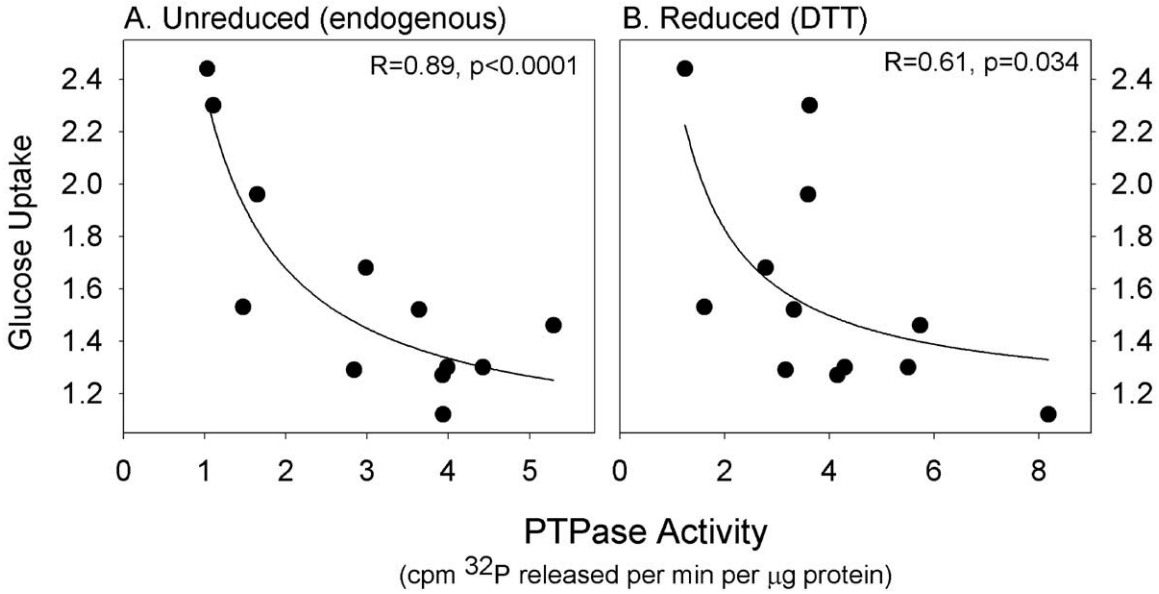

Fig 2. Correlation between insulin-stimulated glucose uptake and cellular PTPase activities assayed under anaerobic conditions with or without biochemical reduction in vitro prior to enzyme assay. Lysates of isolated subcutaneous adipocytes were prepared and assayed under an anaerobic environment (A) without or (B) with biochemical reduction by treatment with 1 mmol/L DTT for 10 minutes prior to assay using ^{32}P-RCM lysozyme as substrate as described in the Methods. Glucose transport was also measured in the isolated adipocytes from these same subjects using 10 nmol/L insulin, and presented as the ratio of CPM incorporated between insulin-stimulated and unstimulated (control) cells. The results of the nonlinear regression analyses performed as described in the methods section are shown for each curve.

Effect of NADPH Oxidase Inhibition on Insulin-Stimulated Reduction in Cellular PTPase Activity and Glucose Uptake in Isolated Human Adipocytes

In recent work, we and others have provided evidence that the oxidative burst of H_2O_2 triggered by insulin stimulation of differentiated 3T3-L1 cells and hepatoma cells is mediated via NADPH oxidase.[7,25] This enzyme is the target of a number of growth factors and cytokines in the cell that induce the formation of intracellular H_2O_2, which is further coupled to various downstream signal transduction pathways.[26] To determine whether the insulin-stimulated burst of H_2O_2 in the human adipocytes leads to the oxidative inhibition of cellular PTPase activity, and to evaluate their potential effect of these changes on insulin-stimulated glucose uptake, we treated the isolated cells with DPI, an inhibitor of NADPH oxidase inhibitor that effectively blocks the insulin-stimulated production of H_2O_2 in a variety of cell types.[24] DPI had no effect on the basal level of endogenous PTPase activity, in the absence of insulin stimulation (Table 1). Following cellular stimulation with insulin, the level of endogenous PTPase activity in human subcutaneous adipocytes was reduced to 53% of control ($P < .001$) (Table 1). Treatment with DPI significantly blocked the insulin-induced reduction in cellular PTPase activity to 80% of the level observed in insulin-treated control adipocytes ($P = .037$).

Table 1. Effect of Insulin and DPI on the Endogenous PTPase Activity of Human Subcutaneous Adipocytes

Control	Control ± DTT	Insulin	Insulin ± DTT
6.26 ± 0.62	7.33 ± 0.25	2.29 ± 0.36†	3.36 ± 0.70
Control	**Insulin**	**Control + DPI**	**Insulin + DPI**
7.34 ± 0.81	3.89 ± 0.52†	7.45 ± 0.70	5.98 ± 0.79*

NOTE. After cell treatments (100 nmol/L insulin for 5 minutes, preceded by 10 μmol/L DPI for 30 minutes where indicated), lysates of isolated human subcutaneous adipocytes were prepared under an anaerobic environment. PTPase activity was determined in the cell lysates within the anaerobic chamber using ^{32}P-RCM lysozyme as substrate as described in the methods. Where indicated, lysates were also subjected to biochemical reduction in vitro by treatment with 1 mmol/L DTT for 10 minutes prior to assay.

*$P = .037$ *v* insulin alone.

†$P < .001$ *v* control

Insulin stimulation enhanced glucose uptake in the human subcutaneous adipocytes by 2-fold (Fig 3). Cellular treatment with DPI for 30 minutes prior to insulin stimulation decreased basal and insulin-stimulated glucose uptake to only 39% ($P < .001$) and 18% ($P < .001$) of the control values in the absence of DPI treatment, respectively (Fig 4). As in our previous work in other cell types,[7] these data suggest that cellular H_2O_2 has a permissive role in both basal and insulin-stimulated glucose transport in human adipocytes.

DISCUSSION

PTPases have received attention recently as potential targets for therapeutic intervention in insulin-resistant states, as it has been recognized from a variety of cellular and animal studies that this important class of enzymes plays a key role in the regulation of the insulin signaling pathway.[9] While PTP1B has emerged as one of the leading candidate PTPases that appears to regulate insulin action, studies in knock-out mice as well as

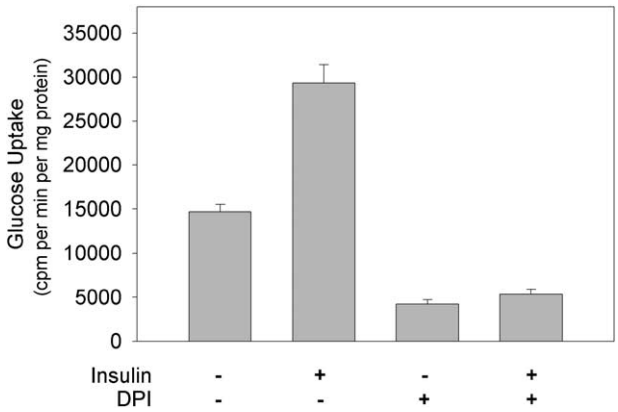

Fig 3. Effect of NADPH inhibition on insulin-stimulated glucose uptake in human subcutaneous adipocytes. Isolated human subcutaneous adipocytes were treated with the NADPH oxidase inhibitor DPI (10 μmol/L) for 30 minutes where indicated, prior to measurement of ^{14}C-D-glucose uptake as described in the Methods using stimulation with 100 nmol/L insulin for 5 minutes.

in cellular systems, as described above, have shown that PTP1B affects insulin signaling predominantly in skeletal muscle and liver, with little effect on distal signaling in adipose cells.[10-12] These studies raised awareness of the possibility that PTP1B may differentially influence the actions of insulin in the various tissues that are the major recognized targets for the metabolic actions of insulin.

In the present work, we demonstrate in isolated subcutaneous human adipocytes that the level of overall cellular PTPase activity correlates strongly with insulin-stimulated glucose uptake, when measured under an anaerobic environment, reflecting the endogenous level of PTPase activity. This was also correlated with BMI, again reflecting an important influence of body mass on the level of cellular PTPase activity, as we have reported previously.[5] As it has been recognized for years that insulin-stimulated glucose transport is diminished in adipocytes obtained from obese, nondiabetic subjects (for example, see Bogardus et al[27]), our data provide evidence that increased cellular PTPase activity may play a role in the regulation of insulin action on glucose transport in human adipocytes, in addition to other processes, such as changes in GLUT4 protein abundance or gene transcription.[28]

Consistent with the suggestion from available literature that PTP1B itself may not have a critical influence on insulin signaling in adipose tissue, especially involving glucose transport, we also found that the level of PTP1B, either in the endogenous state, or following biochemical reduction, was not correlated with insulin-stimulated glucose transport. Thus, while the weight of evidence suggests that PTP1B regulates insulin action in skeletal muscle and liver, its effects on adipose tissue glucose uptake, including in human adipocytes, may not be of critical importance. Since the overall level of cellular PTPase activity correlates with the rate of insulin-stimulated glucose uptake, one or more additional PTPases, besides PTP1B, may be important in the regulation of insulin signaling.

In the present work, we have been able to link the increase in cellular H_2O_2 induced by insulin stimulation via a cellular NADPH oxidase activity, as has been reported for human adipose cells,[25,29] to the inhibition of cellular PTPase activity. The family of homologous PTPase enzymes have in common a conserved approximately 230–amino acid domain that contains the cysteine residue that catalyzes the hydrolysis of protein phosphotyrosine residues by the formation of a cysteinyl-phosphate intermediate.[30,31] Several laboratories have recently provided evidence that reactive oxygen species, including H_2O_2, can oxidize and inactivate PTPases in vivo.[32,33] Since only the reduced form of the catalytic site is enzymatically active, stepwise and progressively irreversible oxidative inhibition is emerging as an important means by which PTPase activity can be suppressed in specific signal transduction pathways.[34-36] Insulin stimulation of cultured hepatocytes and adipose cell models generates a burst of H_2O_2 that rapidly inhibits cellular PTPases and that this oxidant signal is involved in potentiating both the early initial phases of insulin signal transduction as well as the late, integrated responses to insulin.[7,24] Here, we also show that cell treatment with the NADPH oxidase inhibitor DPI, which prevents the rise in cellular H_2O_2 content in response to insulin, blocked the insulin-induced reduction in cellular PTPase activity and also resulted in sharply diminished glucose uptake in the adipocytes (Table 1). This effect is similar to our data reported for glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes,[7] and suggests a potentially important role in the oxidative modification of PTPases or other cellular targets involved in the glucose uptake machinery stimulated by insulin.

In parallel studies, we also observed identical changes in cellular PTPase activity in response to insulin using isolated human omental, as opposed to subcutaneous, adipocytes (data not shown). Similar to our previous report,[37] we found an approximately 35% increase in PTPase activity in omental adipose tissue lysates in the presence or absence of DPI without insulin stimulation. As we found with the subcutaneous samples reported here, insulin reduced the PTPase activity in omental adipose tissue to 53% of control and DPI treatment diminished this effect of insulin to 77% of control (data not shown). Thus, mechanisms for the regulation of cellular PTPase activity are evident to a similar magnitude in both subcutaneous and omental human adipocytes.

We also found in the present work that cellular PTPase activity in the subcutaneous adipocytes from subjects with type 2 diabetes was not correlated with the presence or absence of clinical diabetes. This differs from our previously reported data for skeletal muscle tissue from human subjects where PTPase activity was significantly lower in subjects with overt diabetes.[23] This finding suggests that PTPase activity in skeletal muscle is regulated differently in simple obesity compared to obese subjects with type 2 diabetes, but that this regulation does not seem to occur in adipose tissue, at least not in the subcutaneous adipose depot.

In summary, our findings suggest that total cellular PTPase activity, but not the endogenous activity level of PTP1B itself, is higher in more obese subjects and is negatively associated with insulin-stimulated glucose transport. Therefore, another PTPase homolog may be more critical to the regulation of insulin signaling in adipose cells but does not play as pivotal a role in skeletal muscle and liver, as PTP1B. The insulin-

stimulated oxidative inhibition of PTPases may also have an important permissive role in the transmission of the insulin signal to glucose transport in human adipocytes. Given the important role of adipose tissue in the pathogenesis of the metabolic syndrome and type 2 diabetes mellitus, further insight into the mechanisms regulating insulin signaling in this tissue may help in developing new strategies to combat obesity and the devastating health implications of its associated disorders.

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