Decreased In Situ Insulin Receptor Dephosphorylation in Hyperglycemia-Induced Insulin Resistance in Rat Adipocytes

Shangguo Tang, Hoang Le-Tien, Barry J. Goldstein, Phillip Shin, Robert Lai, and I. George Fantus

The regulation of insulin receptor (IR) tyrosine (tyr) phosphorylation is a key step in the control of insulin signaling. Augmented IR tyr dephosphorylation by protein tyrosine phosphatases (PTPs) may contribute to insulin resistance. To investigate this possibility in hyperglycemia-induced insulin resistance, primary cultured rat adipocytes were rendered insulin-resistant by chronic exposure (18 h) to 15 mmol/l glucose combined with 10^{-7} mol/l insulin. Insulin-resistant adipocytes showed a decrease in insulin sensitivity and a maximum response of 2-deoxyglucose uptake, which was associated with a decrease in maximum insulin-stimulated IR tyr phosphorylation in situ. To assess tyr dephosphorylation, IRs of insulin-stimulated permeabilized adipocytes were labeled with [γ-^{32}P]ATP and chased for 2 min with unlabeled ATP in the presence of EDTA. In a nonradioactive protocol, insulin-stimulated adipocytes were permeabilized and exposed to EDTA and erbstatin for 2 min, and IRs were immunoblotted with anti-phosphotyrosine (pY) antibodies. Both methods showed a similar diminished extent of IR tyr dephosphorylation in resistant cells. Immunoblotting of four candidate IR-PTPs demonstrated no change in PTP1B or the SH2 domain containing phosphatase-2 (SHP-2), whereas a significant decrease in leukocyte antigen-related phosphatase (LAR) (51 ± 3% of control) and an increase in PTP-α (165 ± 16%) were found. Activity of immunoprecipitated PTPs toward a triple tyr phosphorylated IR peptide revealed a correlation with protein content for PTP1B, SHP-2, and LAR but a decrease in apparent specific activity of PTP-α. The data indicate that decreased IR tyr phosphorylation in hyperglycemia-induced insulin resistance is not due to enhanced dephosphorylation. The diminished IR tyr dephosphorylation observed in this model is associated with decreased LAR protein content and activity. *Diabetes* 50:83–90, 2001

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2-DG, 2-deoxyglucose; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GST, glutathione S-transferase; high G/I, high glucose and high insulin; IR, insulin receptor; IRS, insulin receptor substrate; LAR, leukocyte antigen-related phosphatase; PKC, protein kinase C; PMSF, phenylmethylsulfonylfluoride; PTP, protein tyrosine phosphatase; pY, phosphotyrosine; SHP-2, SH2 domain containing phosphatase-2; TIU, trypsin inhibitor unit; tyr, tyrosine; WGA, wheat germ agglutinin.

Since the discovery of protein tyrosine (tyr) phosphatases (PTPs) it has become evident that these enzymes participate in cellular signaling in both a positive and negative manner (1–3). Growth factor receptors such as the insulin receptor (IR) undergo tyr autophosphorylation upon ligand binding. Subsequently, the activated IR phosphorylates a number of protein substrates on tyr such as insulin receptor substrate (IRS)-1/IRS-2 and Shc (4,5) which can then interact with multiple targets to mediate the pleiotropic actions of insulin (6–8). The importance of tyr phosphorylation in insulin signaling and the demonstration that the actions of insulin and other growth factors could be inhibited by PTPs (9,10) led to the hypothesis that resistance to insulin, a common feature of the obese and diabetic state in humans and rodent models, may in some instances be related to elevated PTP activity (11–14). To test this hypothesis in insulin-resistant states, PTP activity has been measured in cell extracts. Because the in vitro specificity of PTPs for a particular substrate such as the IR is not absolute (15–17) and it has been suggested that targeting or compartmentalization may be a critical determinant of substrate selectivity (1,2,18), it is not clear whether the in vitro assays reflect the in vivo state.

PTPs are classified into two major subfamilies: transmembrane and intracellular. Their activities may be regulated by cellular enzyme content, alternative splicing, cellular localization, cell–cell or cell–matrix interactions, and phosphorylation (1–3). Recent studies of insulin signaling have focused on the amount and activity of several candidate IR-PTPs, namely leukocyte antigen-related phosphatase (LAR), PTP1B, LRP/RPTP-α (LCA-related phosphatase/receptor-like PTP-α), and SH2 domain containing phosphatase-2 (SHP-2)/syp (19). A 37-kDa fragment of PTP1B, a single catalytic site PTP targeted to the endoplasmic reticulum (20), was first demonstrated to inhibit insulin/IGF-1 action in Xenopus oocytes (9). PTP1B has been demonstrated to dephosphorylate the IR and IRS-1 as well as associate with the tyr phosphorylated IR (21,22). An inhibitory anti-PTP1B antibody also enhanced insulin action (23). Recently, targeted disruption of the PTP1B gene resulted in mice that showed increased and prolonged IR tyr phosphorylation in liver and muscle (24). Similarly, the transmembrane double catalytic domain PTP LAR has been implicated in IR dephosphorylation. Reduction of cellular LAR content using an LAR antisense–containing vector augmented IR autophosphorylation and phosphatidylinositol 3-kinase activation in hepatoma cells, whereas overexpression blunted these responses (25,26). Insulin stimulation enhanced LAR-IR coimmunoprecipitation (27). There is less evidence at present

for significant dephosphorylation of the IR by PTP-α and SHP-2 (28,29), although PTP-α may suppress selective actions of insulin by another mechanism (30,31). SHP-2 may dephosphorylate IRS-1 (32,33); however, SHP-2 appears to be a positive mediator of insulin-stimulated ras activation (34,35). Transfection of a dominant-negative SHP-2 lacking the catalytic domain into rat adipocytes (36) and microinjection of either a glutathione S-transferase (GST)–SHP-2 SH2-domain fusion protein or anti-SHP2 antibodies into 3T3-L1 adipocytes (37) did not alter insulin-stimulated GLUT4 translocation.

In clinical studies of obese insulin-resistant human subjects, a role for enhanced LAR PTP activity in both adipose (38) and muscle tissue (39) has been suggested as a cause of the resistance. However, in another report, skeletal muscle PTP1B content and IR tyr dephosphorylating activity correlated negatively with insulin resistance (40).

The purpose of this study was first, to determine in a defined model of insulin resistance in adipocytes whether enhanced IR tyr dephosphorylation was responsible for the resistance, and second, whether any change in a candidate IR-PTP could be used to support the identity of the physiologically relevant adipocyte IR-PTP.

RESEARCH DESIGN AND METHODS

Materials. Male Sprague-Dawley rats were from Charles River (St. Constant, Quebec, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY). Type 1 collagenase was from Worthington Biochemical (Freehold, NJ). 2-Deoxy-D-[^{3}H]glucose (10 Ci/mmol) and [γ-^{32}P]ATP (289 Ci/mmol) were from DuPont-New England Nuclear (Lachine, Quebec, Canada), and [γ-^{32}P]ATP (650 Ci/mmol) was from ICN (Costa Mesa, CA). Wheat germ agglutinin (WGA) coupled with agarose and protein A-Sepharose were obtained from Pharmacia (Uppsala, Sweden). The enhanced chemiluminescence (ECL) detection kit was from Amersham (Baie d'Urfe, Quebec, Canada). Aprotinin, bovine serum albumin (BSA), HEPES, *N*-acetyl-D-glucosamine, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (vanadate), and Triton X-100 were from Sigma (St. Louis, MO). Anti-phosphotyrosine (pY) antibody (PY20) was purchased from Transduction Laboratories (Lexington, Kentucky). Anti-PTP1B antibody was from Oncogene Research (Cambridge, MA). Anti-LAR antibodies were prepared as described (25), anti-SHP2/Syp was from Santa Cruz (Santa Cruz, CA), and anti-IR antibodies were from Dr. C. Yip (University of Toronto) or Santa Cruz. The anti–PTP-α antibodies used were raised in rabbits using a GST fusion protein of the PTP-α cytoplasmic domain (amino acids 175–275 of rat PTP-α) or from Dr. J. Sap (New York University). Human insulin was a gift from Eli Lilly (Indianapolis, IN).

Preparation of adipocytes and induction of insulin resistance. Adipocytes from epididymal fat pads of male Sprague-Dawley rats (160–220 g) were isolated by collagenase digestion as previously described (41). Cells were resuspended in DMEM supplemented with 1% BSA, 0.5% FBS, 1% penicillin/streptomycin, and 25 mmol/l HEPES (pH 7.4) and incubated at 37°C for 18 h in a humidified atmosphere of 5% CO_2 and air. To induce insulin resistance, 10^{-7} mol/l insulin and 15 mmol/l glucose (final concentrations) were added to the medium. The DMEM used in control cells contained 5.6 mmol/l glucose. At the end of the 18 h, cells were harvested followed by minor modifications of the washing procedure to remove insulin (42,43). In brief, this consisted of washing twice in 30 mmol/l insulin-free Krebs-Ringer HEPES buffer, pH 7.0 (137 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 1.25 mmol/l $CaCl_2$, 1 mmol/l Na pyruvate, 30 mmol/l HEPES, and 3% BSA), and incubating for an additional 30 min in this buffer at 37°C. Cells were then washed twice in Krebs-Ringer bicarbonate HEPES buffer (118 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 30 mmol/l HEPES, and 1 mmol/l Na pyruvate) with 3% BSA, pH 7.4, and resuspended in the same buffer with 1% BSA for measurement of 2-deoxyglucose (2-DG) uptake.

2-DG uptake. The 2-DG uptake assay was performed as previously described (44) with minor modifications. Adipocytes (8×10^5 cells/ml) were preincubated in the presence of 0–17.2 nmol/l insulin for 30 min. Labeled 2-[^{3}H]-DG (final concentration 50 μmol/l) was added and after 3 min at 37°C, the reaction was terminated by adding 500 μl of ice-cold 0.25 mmol/l phloretin. Nonspecific uptake mediated by simple diffusion, and trapping was determined by measuring 2-[^{3}H]-DG uptake in the presence of 0.25 mmol/l phloretin and subtracted from total uptake to yield carrier-mediated 2-DG uptake.

Anti-pY immunoblotting of insulin receptors. Adipocytes were incubated overnight, washed as described above, and then stimulated for 15 min at 37°C with insulin. The reaction was terminated by the addition of ice-cold solubilization buffer (1% Triton X-100, 4 mmol/l EDTA, 2 mmol/l NaF, 1 mmol/l PMSF, 1 trypsin inhibitor unit (TIU)/ml aprotinin, 2 mmol/l vanadate, and 30 mmol/l HEPES, pH 7.6) and immediate freezing to –70°C. IRs were partially purified by WGA-agarose chromatography and ^{125}I-insulin binding in the lectin-purified fraction determined (41). Aliquots of the WGA-purified fractions containing equal amounts of IR were submitted to SDS-PAGE (7.5%) under reducing conditions as described (45).

After electrophoretic transfer of proteins to nitrocellulose membranes, the membranes were washed, blocked with 10% FBS, and immunoblotted with a 1:1,000 dilution of anti-pY antibody as described (41). Labeled proteins were visualized by autoradiography, and intensities of the 95-kDa bands were determined by densitometry. Receptor content was confirmed by immunoblotting with antibodies against the β-subunit (Dr. B. Posner, McGill University) (41).

In situ IR phosphorylation and dephosphorylation: [γ-^{32}P]ATP labeling. To determine the extent of IR phosphorylation and dephosphorylation in the living adipocytes, the permeabilization protocol described by Mooney and Anderson (46) was adapted. After overnight incubation and washing as above, the adipocytes were resuspended in permeabilization medium (20 μg/ml digitonin, 20 mmol/l Tris, 125 mmol/l KCl, 5 mmol/l NaCl, 10 mmol/l $MgCl_2$, 11.1 mmol/l glucose, and 1% BSA, pH 7.4) for 15 min at 37°C. After an additional 15 min in the presence or absence of insulin, 75 μmol/l [γ-^{32}P]ATP (8 μCi/nmol) and 5 mmol/l $MnCl_2$ were added. After 5 min, the cells were rapidly separated by centrifugation through oil and immediately frozen in liquid N_2. Adipocytes were thawed and solubilized at 4°C in stopping solution (30 mmol/l Tris, 1% Triton X-100, 0.01% SDS, 10 mmol/l ATP, 30 mmol/l Na phosphate, 10 mmol/l Na pyrophosphate, 1 mmol/l p-nitrophenylphosphate, 10 mmol/l β-glycerophosphate, 2 mmol/l phosphotyrosine, 50 mmol/l NaF, 1 mg/ml benzamidine, 10 TIU/ml aprotinin, 1 mg/ml bacitracin, 1 mmol/l PMSF, and 2 mmol/l vanadate, pH 7.5). The fat cake was removed, and the solubilized extract was centrifuged at 100,000*g* for 1 h.

IRs were immunoprecipitated from aliquots of the cell extracts containing equal amounts of protein, subjected to SDS-PAGE on 5–15% resolving gels, and dried and exposed to film (Kodak X-Omat; Kodak). The intensities of the 95-kDa bands on the autoradiograms were quantified by densitometry.

To determine the extent of dephosphorylation, the [γ-^{32}P]ATP was chased with excess unlabeled ATP (8 mmol/l) and 11 mmol/l EDTA. EDTA was previously shown to markedly inhibit further tyr phosphorylation and not to alter the rate of IR tyr dephosphorylation (46). The reactions were terminated after 2 min of chase, and residual labeling of IRs was determined as described above for phosphorylation. To be certain that any difference in dephosphorylation was attributable to tyr dephosphorylation, representative gels were extensively washed with KOH to remove ^{32}P labeling of phosphoserine and phosphothreonine as previously described (45). The intensity of the 95-kDa β-subunit in each dephosphorylation experiment was expressed relative to the insulin-stimulated phosphorylation, which was designated as 100%.

In situ IR dephosphorylation: anti-pY immunoblotting. To confirm the results of the labeling studies and establish a nonradioactive protocol to assay in situ dephosphorylation, the above permeabilization procedure was modified. Briefly, the adipocytes were exposed to 10^{-7} mol/l insulin in the absence and presence of 1 mmol/l erbstatin for 15 min. The cells were centrifuged, and the medium was replaced with permeabilization medium without insulin, ATP, Mg^{2+} or Mn^{2+} and supplemented with 1 mmol/l erbstatin and 10 mmol/l EDTA. After 2 min at 37°C, the cells were harvested and rapidly frozen to –70°C. The adipocytes were solubilized, and IRs were partially purified by WGA chromatography. Equal amounts of IRs were subjected to SDS-PAGE, transferred to membranes, and immunoblotted with anti-pY and anti-IR antibodies as above. Detection was with ECL (Amersham) performed according to the manufacturer's protocol. The extent of pY dephosphorylation was calculated after subtraction of the basal level of IR pY detected in the absence of insulin from the maximum and dephosphorylated levels as indicated below:

$$\%\text{Dephosphorylation} = \frac{[(\text{max pY} - \text{basal}) - (\text{dephosphorylated pY} - \text{basal})]}{(\text{max pY} - \text{basal})} \times 100$$

Immunoblotting of PTPs. At the end of the overnight incubations, the adipocytes were washed and homogenized in lysis buffer (10 mmol/l Tris, 1% Triton X-100, 0.5% NP-40, 10% glycerol, 150 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l vanadate, 50 mmol/l NaF, 7.5 mmol/l Na phosphate, 10 mmol/l dithiothreitol (DTT), 1 mmol/l PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2.5 μmol/l pepstatin A, pH 7.5). Equal amounts of protein (100 μg) were separated by SDS-PAGE (7.5%), transferred to membranes, and immunoblotted with anti-PTP antibody—1:1,000 dilution for PTP-α and SHP-2 and 1:100 for PTP1B and LAR. Detection was performed by ECL, and intensity of

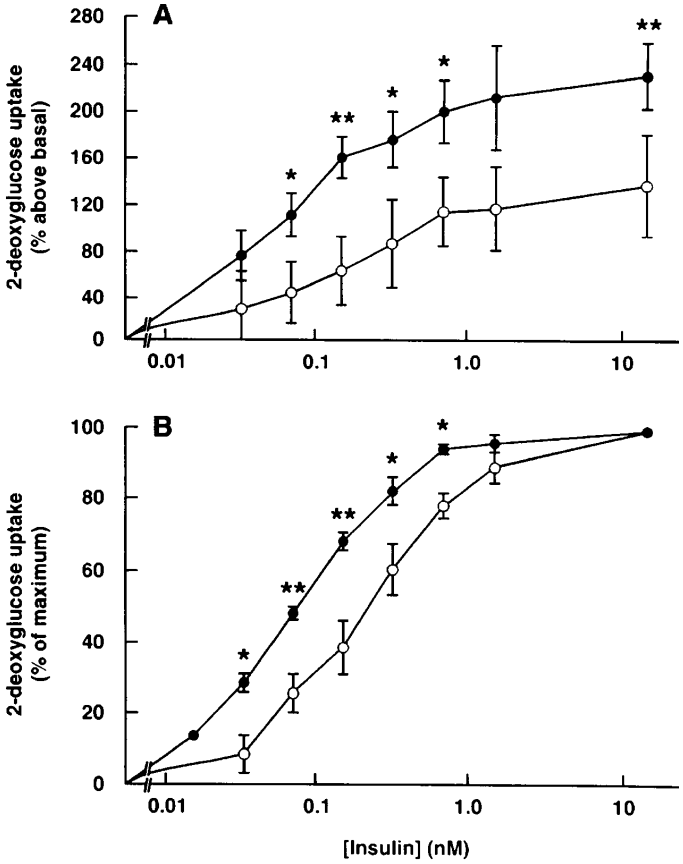

FIG. 1. Insulin-stimulated glucose uptake in control and insulin-resistant adipocytes. Rat adipocytes were incubated in DMEM supplemented with 1% BSA, 0.5% FBS, and 25 mmol/l HEPES in 5.6 mmol/l (●, control) or 15 mmol/l glucose and 10^{-7} mol/l insulin (○, resistant). After 18 h at 37°C, the cells were washed, and 2-DG was uptake assayed. *A*: Results are means ± SE of five to six separate experiments and plotted as the percent above basal. *B*: The data are plotted as the percent of maximum to illustrate the difference in insulin sensitivity. Absolute glucose uptake values in the basal state were 95 ± 6.8 and 83 ± 17.3 pmol/ 3 min/ 8 × 10^5 cells in control and resistant cells, respectively (NS). **P* < 0.05; ***P* < 0.01.

the bands was determined by densitometry. The linear range of detection was confirmed by loading and immunoblotting half and twice the initial protein concentrations (data not shown). β-Actin was immunoblotted as a control.

In vitro PTP assay. One milligram of total protein (500 µg for PTP1B) from control and resistant cell lysates was precleared with protein A-Sepharose. Immunoprecipitated PTPs were washed with phosphatase assay buffer (100 mmol/l HEPES, pH 7.6, 1 mmol/l DTT, 2 mmol/l EDTA, 150 mmol/l NaCl, and 1 mg/ml BSA) and incubated in a final volume of 60 µl with 100 µmol/l of IR peptide TRDIpYETDpYpYRK (Biomol, Plymouth Meeting, PA). After 2 h at 22°C, phosphatase reactions were terminated by adding 40 µl aliquots to 100 µl of Biomol Green reagent, and the release of inorganic phosphate (P_i) was determined by the absorbance measured at 630 nm using the Titertek Plus 96-well plate reader (47). Measured PTP activity was linear from 0.5 to 2 times the total lysate protein concentrations used for immunoprecipitation (data not shown).

Data analysis. Results are presented as means ± SE. The significance of the differences between groups was determined using paired or unpaired Student's *t* test (two-tailed) and analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

2-DG uptake. The 18-h culture of isolated rat adipocytes in a high glucose and high insulin (high G/I)-containing medium resulted in insulin resistance of glucose uptake. Basal glucose uptake was not significantly altered (control, 95 ± 6.8 pmol/3 min/8 × 10^5 cells; resistant, 83 ± 17.3 pmol/3 min/8 × 10^5 cells; NS). However, maximum insulin-stimulated 2-DG uptake was significantly decreased in resistant cells (control, 306 ± 20.4 pmol/3 min/8 × 10^5 cells; resistant, 189 ± 24.4 pmol/3 min/8 × 10^5 cells; $P < 0.01$). The insulin dose-response curves are depicted as percent above basal in Fig. 1*A*. When normalized and plotted as a percent of maximum, the data demonstrate a decrease in insulin sensitivity. Thus, the concentration of insulin required to stimulate glucose uptake to 50% of maximum was significantly increased in resistant cells (control, 0.12 ± 0.01 nmol/l; resistant, 0.34 ± 0.10 nmol/l; $P < 0.05$) (Fig. 1*B*). These results are similar to those previously reported in this model (42).

Insulin receptor autophosphorylation. To determine whether resistance to insulin is present at the level of the IR, the washed adipocytes were stimulated for 15 min with various concentrations of insulin, and the pY content of WGA-purified IRs was determined by immunoblotting. Basal IR pY content corrected for the amount of IR was not different (arbitrary densitometric units: control, 0.42 ± 0.165; resistant, 0.49 ± 0.176; $n = 4$). However, the stimulation by maximal insulin was decreased in the high G/I exposed cells (control, 7.3 ± 1.56; resistant, 3.35 ± 0.46; $P < 0.05$) (Fig. 2). Two insulin

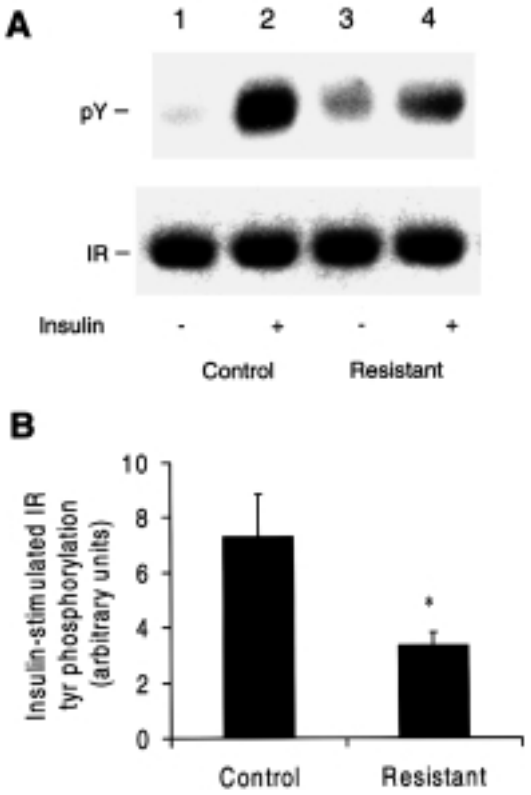

FIG. 2. Insulin-stimulated insulin receptor tyr phosphorylation in control and insulin-resistant adipocytes. Rat adipocytes were rendered insulin resistant over 18 h, washed, and stimulated for 15 min with and without 10^{-7} mol/l insulin. Equal amounts of WGA-purified IRs were separated by SDS-PAGE and immunoblotted with anti-pY antibodies. *A*: Representative immunoblot showing tyr phosphorylation of a 95-kDa IR β-subunit in control and resistant adipocytes. Membranes were reprobed to determine the total IR β-subunit. *B*: Intensities (arbitrary units) of anti-pY immunoblots were corrected for total IR. Results depicted are the means ± SE increases above basal of four independent experiments. There was no difference in basal pY/IR values between control and resistant adipocytes (see text), whereas after insulin stimulation, pY/IR was 54% lower in resistant cells. **P* < 0.05.

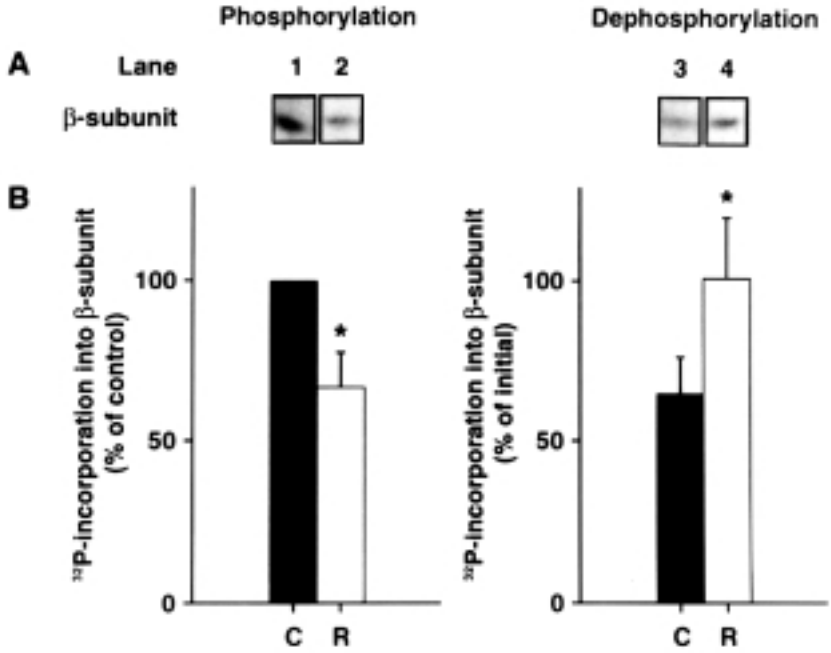

FIG. 3. Insulin receptor phosphorylation and dephosphorylation in permeabilized adipocytes. Adipocytes were incubated for 18 h, washed, permeabilized with digitonin, and stimulated with and without 10^{-7} mol/l insulin in the presence of [γ-^{32}P]ATP. Labeled ATP was chased with excess unlabeled ATP and EDTA, and dephosphorylation was allowed to proceed for 2 min. IRs were immunoprecipitated and subjected to SDS-PAGE, and the extent of ^{32}P labeling of the 95-kDa β-subunit was determined by densitometry of autoradiograms. *A*: Representative experiment showing insulin-stimulated ^{32}P labeling of an IR from control (*lane 1* and *2*) and resistant (*lane 3* and *4*) adipocytes. Phosphorylation (*lane 1* and *3*) and 2-min dephosphorylation (*lane 2* and *4*) are shown. *B:* Left panel: Phosphorylation stimulated by insulin, designated as 100% in control cells (C), was decreased in resistant cells (R) (n = 5) (*P < 0.05, C vs. R). Right panel: The extent of ^{32}P label remaining after 2 min of dephosphorylation is shown as the percent of maximum insulin-stimulated phosphorylation in each condition. There was ~35% dephosphorylation in control cells but no significant dephosphorylation observed in resistant cells (n = 5; *P < 0.05, C vs. R) (see text for details).

dose-response curves did not show any shift in sensitivity to insulin of IR tyr phosphorylation (data not shown).

This technique assessed the extent of IR tyr autophosphorylation in the intact cell. In a cell-free system, IR tyr kinase activity of the WGA-purified IRs was assessed using poly-[glu:tyr] (4:1) as substrate. There was no significant change in either the insulin dose-response curves (sensitivity) or in maximum response (data not shown). These data indicate that there is no apparent defect in enzymatic activity if the receptor is removed from its cellular environment. It should be noted that ^{125}I-insulin binding to intact adipocytes was minimally decreased (10 ± 5%) by the 18-h incubation in high G/I (data not shown). This is consistent with previous studies showing an efficient IR recycling mechanism in adipocytes and an inability of IR downregulation to account for the insulin resistance in this model (42).

In situ ^{32}P labeling of the IR. One possible explanation for the observed defect in insulin-stimulated IR autophosphorylation was an increased activity of an IR-PTP. As a means to study IR phosphorylation and dephosphorylation in situ, the digitonin-permeabilized adipocyte model was used. Using this technique, IR autophosphorylation remained responsive to insulin, and the IR was rapidly labeled using [γ-^{32}P]ATP in situ. In preliminary experiments carried out with unlabeled ATP, it was demonstrated that equal amounts of IRs were immunoprecipitated from control and resistant cells (data not shown). The extent of ^{32}P labeling of the IR in the unstimulated state was not different in insulin-resistant adipocytes (94.9 ± 7.04% of control, n = 3). However, after insulin stimulation, labeling was significantly decreased to 66.5 ± 10.7% of the control (P < 0.05, n = 5) (Fig. 3). This decrease was consistent with the results obtained by immunoblotting with anti-pY antibody.

To assess dephosphorylation, a chase with unlabeled ATP for 2 min was used. In control adipocytes, the ^{32}P labeling of the IR was decreased after 2 min to 64.7 ± 11.5% of that observed after insulin stimulation, whereas in the resistant cells, there was no apparent dephosphorylation (100.9 ± 18.9%) (P < 0.05 compared with control, n = 5) (Fig. 3). We noted that the extent of dephosphorylation over 2 min was less in these experiments in which adipocytes were cultured for 18 h compared with the results previously reported by Mooney and Anderson (46) in freshly isolated adipocytes. To determine whether any of the differences between fresh and cultured cells or between control and resistant cells could be accounted for by ser/thr phosphorylation, the gels from two experiments were washed with KOH. The alkali-resistant ^{32}P incorporation, i.e., ^{32}P-tyr, could then be compared. This comparison demonstrated a greater degree of dephosphorylation after 2 min of chase in both control (26% of maximum insulin-stimulated ^{32}P label remaining) and resistant (46% of maximum insulin-stimulated ^{32}P label remaining) adipocytes (data not shown). These results indicated that there was a substantial amount of ser/thr phosphorylation present and that the resistant cells showed a lesser extent of IR tyr dephosphorylation.

In situ IR tyr dephosphorylation: anti-pY immunoblotting. To confirm the findings of the alkali wash of the ^{32}P-labeled receptors, a nonradioactive modification of the permeabilized adipocyte method was developed. The intact adipocytes were stimulated with insulin for 15 min, followed by the permeabilization procedure. In the modified protocol, the tyr

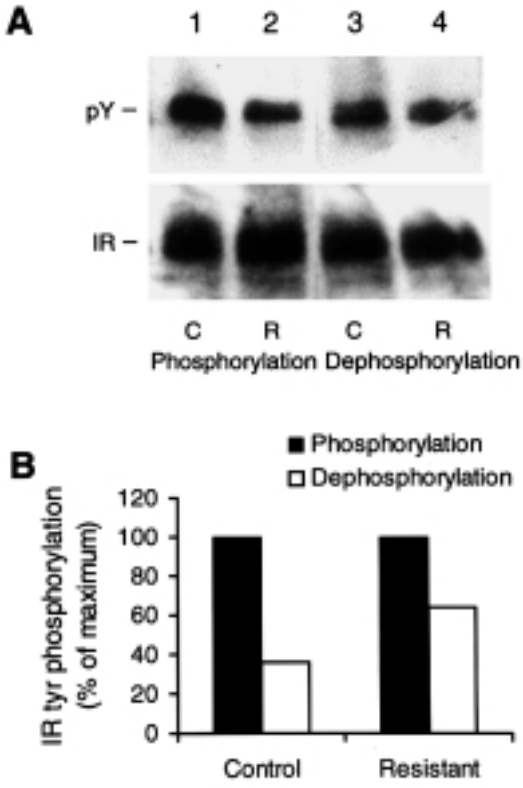

FIG. 4. In situ insulin receptor tyr phosphorylation and dephosphorylation. Adipocytes were incubated and washed as described in Fig. 1. Cells were stimulated with 10^{-7} mol/l insulin for 15 min at 37°C, then permeabilized with digitonin in medium supplemented with erbstatin and EDTA, and dephosphorylation was allowed to proceed for 2 min. Equal amounts of WGA-purified IRs were separated by SDS-PAGE and immunoblotted with anti-pY antibodies and anti–IR-β-subunit antibodies. *A*: Representative immunoblots are shown for IRs from control (C) and resistant (R) adipocytes. Maximum tyr phosphorylation (*lane 1* and *2*) and remaining pY after 2-min dephosphorylation (*lane 3* and *4*) are shown for control and resistant adipocytes. *B*: Densitometry (arbitrary units) of pY/IR was determined, and the extent of tyr dephosphorylation was calculated after designating maximum pY/IR as 100% in each condition (left panel, control; right panel, resistant). Similar results were obtained in three separate experiments.

kinase inhibitor erbstatin was added, which completely blocked insulin stimulation of IR tyr phosphorylation (data not shown). Equal amounts of WGA-purified IRs, determined by ^{125}I-insulin binding and confirmed by immunoblotting with anti-IR antibody, were immunoblotted with anti-pY. Similar to the results shown in Fig. 2, insulin-stimulated IR tyr phosphorylation was decreased in resistant cells. Furthermore, the extent of tyr dephosphorylation was greater in control adipocytes than in resistant adipocytes (Fig. 4). In three separate experiments, dephosphorylation ranged from 64 to 75% in control cells and 37 to 46% in resistant cells. These results showed the same difference as observed in the above in situ ^{32}P-labeling protocol in which the gels were treated with KOH (extent of dephosphorylation: control 74%, resistant 54%) and indicate decreased IR dephosphorylation in adipocytes exposed to high G/I.

PTP immunoblots. Previous studies have implicated several PTPs as candidate enzymes with activity toward the IR—namely LAR, PTP1B, PTP-α, and SHP-2. To determine whether the cellular protein content of any of these was altered in this model of insulin resistance and correlated with the apparent decrease in IR tyr dephosphorylation, total solubilized cell lysates were subjected to SDS-PAGE followed by immunoblotting with specific anti-PTP antibodies. There were no changes in SHP-2 or PTP1B. However, there was a significant increase in total cellular PTP-α (165 ± 16% of control, $P < 0.01$) and a significant decrease in LAR (51 ± 3% of control, $P < 0.01$) in the insulin-resistant adipocytes (Fig. 5). Immunoblotting of β-actin as a control showed no difference between control and resistant adipocytes (data not shown).

PTP activities. To investigate whether alterations in PTP protein level reflected changes in the activity of individual PTPs, the candidate PTPs immunoblotted above were assayed for activity after immunoprecipitation. A triphosphopeptide representing the catalytic regulatory domain of the IR was used as substrate. Correlating with protein levels, the activities of SHP-2 and PTP1B were unchanged, and LAR activity was decreased (59 ± 6%, $P < 0.01$) compared with control (Fig. 6*A*). PTP-α activity in the assay was much lower than expected from the protein level (insulin-resistant adipocytes, 60 ± 5% of control, $P < 0.01$). The specific activity of each PTP (calculated as the ratio of activity/protein content of resistant cells relative to control; designated as 100%) was not different for SHP-2, PTP1B, or LAR, whereas PTP-α specific activity was decreased (36 ± 7%) in the resistant cells (Fig. 6*B*). It should be noted that there were no differences in the extent of immunoprecipitation of the PTPs from control and resistant cell lysates (70 ± 5% for LAR and PTP-α and 90 ± 5% for PTP1B and SHP-2).

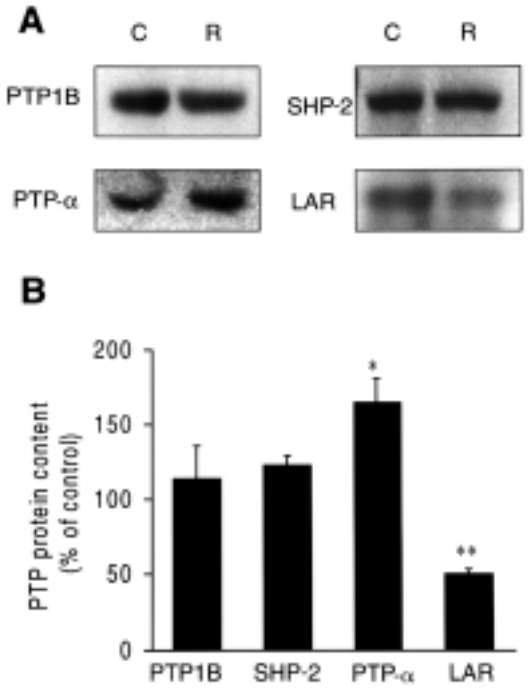

FIG. 5. Protein tyrosine phosphatase content in control and insulin-resistant adipocytes. Adipocytes were incubated as described in Fig. 1. Cells were solubilized, and equal amounts of protein were separated by SDS-PAGE, transferred to membranes, and immunoblotted with anti-PTP antibodies. *A*: Representative immunoblots. C, control adipocytes; R, resistant adipocytes. *B*: The intensities (arbitrary units) of bands from resistant adipocytes are shown as the percent of control. Total amount of SHP-2 and PTP1B were unchanged, whereas PTP-α was increased and LAR was decreased. **P* < 0.01; ***P* < 0.001, C vs. R. *n* = 4–6.

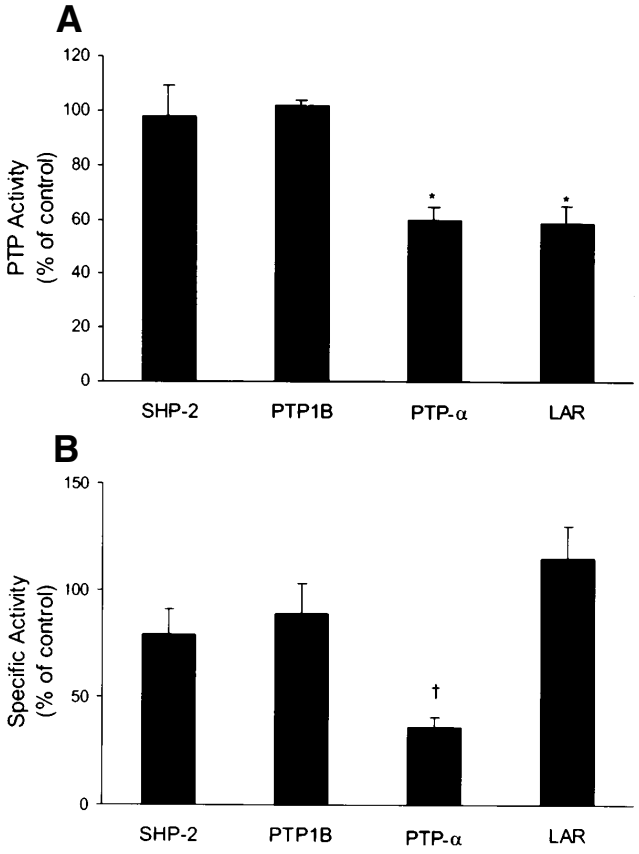

FIG. 6. PTP activities of SHP-2, PTP1B, PTP-α, and LAR from control and insulin-resistant adipocytes. Adipocytes were incubated as described in Fig. 1. Cell lysates were prepared, and PTPs were immunoprecipitated from equal amounts of protein. PTP activity was assessed in the immunocomplexes using the malachite green assay. *A*: Total phosphatase activities of immunocomplexes. There was no change in PTP1B and SHP-2, but activities of PTP-α and LAR ($P < 0.01$ for both) were significantly decreased in resistant adipocytes ($n = 4–6$). *B*: Specific PTP activities. PTP activities determined in *A* were corrected for PTP content, and the ratios from resistant cells were compared with control cells (designated as 1.0). Only PTP-α showed a decrease in specific activity in resistant cells ($P < 0.05$).

DISCUSSION

The cause of the insulin resistance found in type 2 diabetes is multifactorial and, at least in part, acquired (48). Some of the defects such as decreased IR autophosphorylation activity are reversible with improvement in metabolic control (49). Insulin resistance can be induced in vitro by exposure to a diabetic milieu. Primary rat adipocytes cultured in the presence of high G/I develop diminished insulin sensitivity and maximum response of glucose transport (42), defects similar to those observed in human adipocytes from subjects with type 2 diabetes (50). Diminished IR autophosphorylation has also been reported after exposure of adipocytes to high glucose concentrations (51). In this study, we found a decrease in insulin stimulation of glucose uptake and of IR autophosphorylation in situ in rat adipocytes exposed to high G/I.

One possible explanation for such a defect is elevated IR tyr dephosphorylation. Previous studies of human subjects with insulin resistance suggested that increased PTP activity is present in some cases (11,38,39). Elevated PTP activity has also been reported in several rodent models of insulin resistance (12–14). However, in these studies, PTP activity was only assessed in vitro, and it has been difficult to prove that augmented IR tyr dephosphorylation is responsible for a decrease in IR tyr phosphorylation.

Our approach to test this possibility in the rat adipocyte model was to assess IR dephosphorylation in situ using two permeabilization protocols. Labeling of the IR with [γ-^{32}P]ATP in permeabilized adipocytes revealed that insulin-stimulated ^{32}P labeling was diminished in the insulin-resistant cells to ~66% of that in control cells, consistent with a decrease in IR phosphorylation. Limiting further phosphorylation by the addition of EDTA and chasing with excess unlabeled ATP revealed that the extent of dephosphorylation after 2 min was significantly greater in control cells than resistant cells. Enriching for phosphotyrosine by removal of phosphate from ser and thr residues using alkali treatment showed that the extent of tyr dephosphorylation was greater in control cells (~74%) than in resistant cells (~54%). These results were confirmed using a modified protocol in which adipocytes were stimulated with insulin and then permeabilized with medium supplemented with EDTA and erbstatin to inhibit further IR tyr autophosphorylation. The extent of IR tyr dephosphorylation determined by immunoblotting with anti-pY was decreased in the insulin-resistant cells.

The difference in IR ^{32}P labeling before and after the KOH wash indicated that a substantial amount of ser/thr phosphorylation was present. It has been well documented that insulin stimulates ser/thr phosphorylation of its receptor (5,8). Furthermore, chronic exposure to high insulin and/or high glucose concentrations has been associated with enhanced IR ser/thr phosphorylation (52,53). Assuming that only and most ser/thr phosphate was removed by the alkali wash, it was estimated that ~39% of the ^{32}P labeling of the insulin-stimulated IR was associated with ^{32}P-ser and/or ^{32}P-thr in control cells and that this was increased to 55% in resistant cells. Several studies have implicated protein kinase C (PKC) as a mediator of IR ser/thr phosphorylation and suggested that this phosphorylation was the cause of the associated defect in insulin-stimulated IR tyr autophosphorylation (51,54–56). However, this has not been a universal finding (57). Furthermore, IRs with a truncated COOH-terminal domain in which a number of the ser/thr phosphorylation sites are absent remain sensitive to the effects of glucose and PMA (phorbol myristate acetate) (53). Thus, the role of the apparent increase in IR ser/thr phosphorylation in the insulin-resistant adipocytes is not clear.

The data in this study indicate that in this model, augmented IR tyr dephosphorylation does not account for the insulin resistance, and the decrease would in fact predict increased insulin signaling. There are a number of possible explanations for this apparent discrepancy. First, the defect in IR autophosphorylation may be related to increased ser/thr phosphorylation in the juxtamembrane region. Alternatively, the decrease may be caused by an endogenous IR kinase inhibitor. For example, in the case of tumor necrosis factor-α–induced insulin resistance, ser phosphorylated IRS-1 acts as such an inhibitor (58). A similar phenomenon has recently been described for the inhibition of IR autophosphorylation by a number of diacylglycerol-sensitive PKC isoforms (59). It has also been suggested that efficient IR tyr dephosphorylation is necessary to maintain insulin sensitivity (60). If this hypothesis is correct, the influence of an IR-PTP may be biphasic, with insulin resistance resulting from increased or decreased activity. Finally, it should be noted that the specific tyr residues that

are dephosphorylated more slowly in the resistant cells remain to be identified and may not be those that positively influence kinase activity in the regulatory domain.

At this time, it is not clear whether one or more than one PTP is responsible for the physiological regulation of IR tyr dephosphorylation. The major candidate enzymes are PTP1B, LAR, and PTP-α (19). Immunoblotting of whole-cell lysates showed that the only candidate enzyme that was decreased in resistant adipocytes was LAR. In addition, total-cell LAR-related PTP activity was decreased in proportion to its amount. There are previous data that support the notion that LAR plays a major role as an IR-PTP (25–27). In obese human subjects with insulin resistance, an increased cellular content of LAR was found in adipocytes (38) and, more recently, in muscle (39). This finding appeared to account for the increased PTP activity measured in cell lysates. However, it is noteworthy that these subjects did not manifest overt diabetes and that, in contrast, studies of PTP activity in subjects with type 2 diabetes showed a decrease in PTP activity toward the IR (39,40,61). The in vitro model used here, which combines the metabolic perturbations of both hyperinsulinemia and hyperglycemia, may be more representative of the latter subjects.

Although our data show a correlation between decreased IR dephosphorylation and diminished LAR protein content and activity, the results do not prove that the two are causally related. It is of interest that in the mouse lacking PTP1B, IR tyr phosphorylation was increased and prolonged in liver and muscle but not in adipose tissue (24). PTP1B has also recently been suggested to participate in IRS-1 dephosphorylation (62). On the other hand, in the $LAR^{-/-}$ mouse, in vivo peripheral insulin resistance was observed (63), but isolated adipose tissue was not studied. The possibility of tissue specificity in the relative contribution of different PTPs to IR dephosphorylation is suggested but remains to be clearly demonstrated.

Although PTP-α content was increased in the resistant cells, its specific activity was decreased. Whereas this may also provide an explanation for decreased IR tyr dephosphorylation (28,31), recent studies do not support the concept that the IR is an in vivo substrate of this PTP (30,64).

In summary, this study is the first in situ assessment of IR dephosphorylation in intact insulin-resistant cells. Our results demonstrate a diminished IR tyr dephosphorylation in adipose tissue rendered insulin-resistant by exposure to high G/I and indicate that the defect in insulin-stimulated IR autophosphorylation is not accounted for by excess IR dephosphorylation. The data also support, but do not prove, a role for LAR as an IR-PTP in adipose tissue.

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