Tumor Necrosis Factor-α Induces Hepatic Insulin Resistance in Obese Zucker (*fa/fa*) Rats via Interaction of Leukocyte Antigen-Related Tyrosine Phosphatase With Focal Adhesion Kinase

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The molecular mechanism whereby tumor necrosis factor-α (TNF-α) induces insulin resistance in obesity is not well understood. Previously, we have shown that inhibition of TNF-α improved hepatic insulin sensitivity in obese Zucker rats without altering the tyrosine phosphorylation of liver insulin receptors (IRs), which indicates that the TNF-α and insulin-signaling cascades interact distally to the IR. To assess the effects of TNF-α on signaling molecules downstream from the IR, we analyzed the tyrosine phosphorylation patterns of liver homogenate proteins from TNF-α–neutralized *fa/fa* rats and showed that focal adhesion kinase (FAK) was consistently hyperphosphorylated (4.5-fold). Moreover, intravenous insulin increased hepatic FAK phosphorylation in a time-dependent manner in Sprague-Dawley rats, which suggests that TNF-α may induce hepatic insulin resistance by preventing FAK phosphorylation in response to insulin treatment. To explore the cellular mechanism whereby TNF-α regulates phosphorylation of FAK in the liver, we measured c-Src kinase activity and the abundance of 3 major protein tyrosine phosphatases (PTPs) (PTP-1B, leukocyte antigen-related tyrosine phosphatase [LAR], and src homology 2 domain–containing protein-tyrosine phosphatase [SHPTP-2]) in liver homogenates from obese Zucker rats after TNF-α blockade. Hepatic c-Src kinase activity was unaltered, but LAR protein was reduced by 75%. In addition, TNF-α blockade reduced hepatic PTP activity toward tyrosine phosphorylated FAK by 70%, and this was accounted for by immunodepletion of LAR. Incubation of HepG2 cells with TNF-α increased LAR protein levels in a dose-dependent manner. Additionally, pretreatment with TNF-α abolished insulin-stimulated tyrosine phosphorylation of FAK in HepG2 cells but had no effect on IR tyrosine phosphorylation or expression. These data suggest that TNF-α promotes LAR expression and thus prevents insulin-mediated tyrosine phosphorylation of FAK. This probably represents the interface between TNF-α and insulin signaling in the liver. *Diabetes* 49:810–819, 2000

Tumor necrosis factor-α (TNF-α) is a cytokine expressed by macrophages and other cell types whose expression is upregulated in the adipose tissue and/or skeletal muscle of both obese insulin-resistant human subjects and experimental animal models (1–4). Administration of TNF-α to experimental animals induces insulin resistance (5) and in vivo inhibition of TNF-α by blockade of TNF-α bioactivity (1,6–8), and genetic knockout of the TNF receptor (9,10) and/or the TNF-α ligand (11) has been shown in most (1,6,8,9,11) but not all (7,10) studies to improve insulin sensitivity and glucose metabolism. In addition, reversal of insulin resistance by weight reduction is associated with decreased adipose tissue expression of TNF-α (3,4,12). Thus, a potential role for TNF-α in the pathogenesis of insulin resistance in obesity and type 2 diabetes has been proposed (1,6,13).

Currently, however, the molecular mechanism whereby TNF-α attenuates insulin signaling is not well understood. Presumably, this entails interface between the TNF-α and the insulin signal transduction cascades at an undetermined site. Recent reports have suggested potential mechanisms whereby TNF-α impairs insulin signaling. These include decreased insulin receptor (IR) kinase activity (14–17) together with increased serine phosphorylation of insulin receptor substrate 1 (IRS-1) (6), decreased activity of protein tyrosine phosphatases (PTPs) (8), and reduced IR and IRS-1 expression (18) or reduced expression of the GLUT4 glucose transporter (18,19). Recently, we have shown that neutralization of TNF-α activity in obese Zucker rats (achieved through endogenous expression of a TNF-α inhibitor [TNFi] using an adenovirus vector) led to significant improvements in both peripheral and hepatic insulin sensitivity compared with obese Zucker rats infected with the same titer of adenovirus carrying the *lac-z* cDNA (8). However, in the liver, the improved insulin sensitivity was not associated with either

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Ad5, adenovirus 5; Ad5-β-gal, recombinant adenovirus 5 carrying *lac-Z* cDNA; Ad5-TNFi, recombinant adenovirus 5 carrying tumor necrosis factor-α inhibitor cDNA; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase; GAP, guanosine triphosphatase activating protein; GST, glutathione S-transferase; HGO, hepatic glucose output; HRP, horseradish peroxidase; IR, insulin receptor; IRS-1, insulin receptor substrate 1; LAR, leukocyte antigen-related tyrosine phosphatase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PTP, protein tyrosine phosphatase; SHPTP-2, src homology 2 domain–containing protein-tyrosine phosphatase; TBST, Tris-buffered saline with Tween; TNF-α, tumor necrosis factor-α; TNFi, tumor necrosis factor-α inhibitor.

altered IR number, insulin binding affinity, or increased insulin-stimulated tyrosine phosphorylation of IR, which indicates that, at least in this tissue, the TNF-α and insulin-signaling cascades interact at a level distal to the IR.

The present study was undertaken to determine potential molecular mechanisms of interaction between TNF-α and the insulin-signaling cascade in the liver. We observed consistent hyperphosphorylation of a 125-kDa protein after TNF inhibition and confirmed this to represent focal adhesion kinase (FAK). The role of FAK in insulin signal transduction has not been clearly established, although its phosphorylation state is altered by insulin stimulation in various cell types (20–23). We show herein that insulin stimulates hepatic FAK tyrosine phosphorylation both in rats and in HepG2 hepatoma cells and that insulin-stimulated phosphorylation of FAK in the liver of *fa*/*fa* rats is increased after TNF-α neutralization. We then searched for candidate PTPs that may play a role in TNF-α–mediated dephosphorylation of FAK. We found that inhibition of TNF-α in obese *fa*/*fa* rats led to decreased expression of leukocyte antigen-related tyrosine phosphatase (LAR), which is a putative negative regulator of insulin action. Furthermore, immunodepletion studies confirmed that reduced hepatic PTP activity toward FAK in TNF-α–neutralized animals was attributable to LAR.

RESEARCH DESIGN AND METHODS

Materials. Recombinant human TNF-α was purchased from Genzyme (Cambridge, MA). Antibodies to PTP1-B, SHPTP-2, and LAR were from Oncogene Science (Cambridge, MA), Upstate Biotechnology (Lake Placid, NY), and Transduction Labs (Lexington, KS), respectively. Glutathione S-transferase (GST)-guanosine triphosphatase activating protein (GAP)p^{62} fusion protein, antibodies against phosphotyrosine (pY99), FAK, c-Src, and IR were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LAR rabbit antisera for the immunodepletion experiment were kindly provided by Dr. Barry J. Goldstein (Thomas Jefferson School of Medicine, Philadelphia). The chemiluminescence kit, protein A/G–conjugated agarose beads, and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were all purchased from Pierce (Rockford, IL). Cell culture reagents were all from Gibco (Grand Island, NY). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Generation and propagation of recombinant adenovirus. The TNFi cDNA was subcloned into the pCCMV plasmid, which contains 1.3 map units of sequence taken from the left end of the adenovirus 5 (Ad5) genome, the cytomegalovirus early promoter, the pUC19 cloning site, followed by the SV40 splice, poly-A signal sequences, and map units 9–17 of the Ad5 genome. The recombinant pCCMV plasmid was cotransfected into the 293 packaging cell line with the pJM17 plasmid, which carries genomic Ad5 DNA. Mature recombinant Ad5 was generated after homologous recombination between the 2 plasmids had occurred in vivo (24). Ad5 was propagated and purified as previously described (25).

Administration of adenovirus to Zucker rats and glucose clamp studies. The study was approved by the Animal Research and Use Committee of the University of Tennessee Memphis and the Subcommittee on Animal Studies of the Veterans Administration Medical Center. Seven-week-old obese *fa*/*fa* Zucker rats (Pennington Biomedical Research Center, Baton Rouge, LA) were either infected with 10^9 plaque-forming units of recombinant Ad5 carrying TNFi cDNA (Ad5-TNFi) or the *lac-z* gene, which encodes for β-galactosidase (Ad5-β-gal), as a control. Under brief isofluorane inhalational anesthesia, 10^9 plaque-forming units of virus in 30 µl phosphate-buffered saline (PBS) was administered via the tail vein. Infected rats were housed individually and weighed daily. Inhibition of plasma TNFi activity was assayed 4 days after Ad5 injection according to a previously described technique (24). The data have been reported previously and confirmed successful suppression of TNF bioactivity in animals receiving Ad5-TNFi (8). A total of 4 days after Ad5 injection, animals (who were fasted overnight) were anesthetized, and cannulas were placed in the carotid arteries and jugular veins. After a 20-min equilibration period, the animals underwent euglycemic-hyperinsulinemic clamp studies according to a previously described method (26). The insulin infusion rate was 70 pmol $\cdot$ kg^{-1} $\cdot$ min^{-1}, and plasma glucose was clamped at basal levels. A steady state was achieved after ~70–90 min. At the end of a 20-min steady-state period, liver and rectus muscles were exposed and snap-frozen in liquid N_2 and were stored at –70°C until subsequent in vitro experiments were performed. Animals were then euthanized in a CO_2 chamber. Mean steady-state serum insulin levels were 565 and 633 pmol/l in the Ad5-TNFi and Ad5-β-gal groups, respectively. The clamp data have been reported previously (8).

Preparation of liver homogenates. Frozen livers were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) and were solubilized in the presence of 1.5% Triton X-100, 5 mmol/l EDTA, 100 mmol/l NaF, 1 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 5 kallikrein inhibitory units/ml aprotinin, 2 mmol/l phenylmethylsulfonyl fluoride, and 1.5 mg/ml bacitracin. The insoluble particles were precipitated from the tissue homogenate by centrifugation at 100,000*g*. Protein concentration of homogenates was determined with the Bradford method (Bio-Rad, Hercules, CA).

Immunoblot analysis of PTP abundance. The 30-µg aliquots of liver homogenate protein were fractionated on 7.5% (for LAR) or 10% (for SHPTP-2 and PTP-1B) SDS-PAGE. Proteins were transferred by electroblotting to nitrocellulose membrane in 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol. After transfer, the membrane was blocked in Tris-buffered saline with Tween (TBST) containing 5% nonfat dry milk and then in TBST with 5% milk and primary antibody (1:500 dilution) followed by appropriate HRP-conjugated secondary antibody. Immunoreactive proteins were visualized with a Supersignal Chemiluminescence kit (Pierce) according to the manufacturer's instructions. Quantitation of immunoblots was made with a PDI densitometer and the accompanying Quantity One image analysis software (PDI, Huntington Way, NY).

Analysis of FAK tyrosine phosphorylation. A total of 2 mg protein extract from the liver was incubated with 1.5 µg anti-FAK antibody (Santa Cruz Biotechnology) and agarose-conjugated protein G (Pierce) at 4°C overnight. The immunoprecipitates were then washed extensively in a buffer containing 50 mmol/l HEPES, 100 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 10 mmol/l EDTA, 2 mmol/l Na_3VO_4, 2 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin boiled in 2× Laemmli buffer and separated by 7.5% SDS-PAGE. The proteins were then electroblotted onto nitrocellulose membranes, and the membrane was blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 20 immunoblotted with HRP-conjugated pY99 antibody. Results were visualized with chemiluminescence. The membrane was then stripped and reblotted with a monoclonal antibody specific for FAK (Transduction Labs) to determine the amount of FAK protein. Immunoreactive proteins were visualized and quantified by the Supersignal Chemiluminescence kit and densitometry, respectively, as described above.

Determination of the effect of insulin on hepatic FAK phosphorylation state and PTP activity toward FAK in vivo. Sprague-Dawley rats (6 weeks old, 200–250 g) were fasted overnight and were anesthetized with sodium pentobarbital. The internal jugular vein and carotid artery were then cannulated, and the abdominal cavity was opened. A 1-U bolus of insulin was administered via the jugular vein. Blood was sampled from the carotid artery at 2-min intervals for the determination of glucose concentrations, and 20% D-glucose was administered via the jugular vein as necessary to maintain blood glucose at basal fasting levels. At 0, 2, 4, 6, or 10 min after insulin administration, the liver was snap-frozen in liquid N_2. Liver homogenates were prepared as described above. FAK was then immunoprecipitated from the liver homogenate, and the levels of phosphorylation were analyzed by immunoblotting as described earlier. The membrane was then stripped and reblotted with anti-FAK antibody to confirm consistent precipitation of FAK protein.

For measurement of PTP activity toward FAK, 0.5 mg liver homogenate protein obtained 6 min after intravenous insulin bolus administration (at which time FAK was shown in control experiments to be maximally phosphorylated) was incubated with 2 µg anti-FAK antibody and immobilized protein A/G for 16 h at 4°C. The immunoprecipitate was then washed and resuspended in 100 µl dephosphorylation buffer (25 mmol/l imidazole, pH 7, 1 mmol/l EDTA, 0.1% 2-mercaptoethanol, 2 mmol/l $MgCl_2$, 2 mmol/l phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 0.1 mmol/l benzamidine, and 250 mmol/l sucrose). A total of 15 µg liver homogenate protein from TNF-α–neutralized *fa*/*fa* rats or controls was added to the immunoprecipitated FAK and was incubated at 30°C for 10 min. The reaction was stopped by adding 25 µl 6× Laemmli buffer and then boiling for 10 min. The supernatant was then separated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibody. The membrane was then stripped and reprobed with anti-FAK antibody to ensure constant substrate content. We have found that, under such conditions, the FAK dephosphorylation reaction stays linear up to 15 min (data not shown).

For measurement of FAK-specific PTP activity after immunodepletion of LAR, 100 µg liver homogenate protein from *fa*/*fa* rats treated with Ad5-TNFi or Ad5-β-gal was incubated with 5 µg rabbit polyclonal anti-LAR or a matching amount of rabbit IgG (Sigma) for 16 h at 4°C. The samples were then spun,

and aliquots of the supernatant (15 μg protein) were transferred to separate microcentrifuge tubes containing tyrosine phosphorylated FAK immunoprecipitated from liver homogenates of insulin-treated Sprague-Dawley rats. The dephosphorylation assay was carried out as described above.

Assay for Src kinase activity. Liver homogenate (0.5 mg) was incubated with 4 μg anti-Src antibody (Santa Cruz Biotechnology) and immobilized protein A/G at 4°C overnight. Immunoprecipitates were then washed twice with PBS containing various protease inhibitors and twice with kinase buffer (10 mmol/l Tris-Cl, pH 7.0, 5 mmol/l $MgCl_2$, 0.5 μmol/l ATP, 1 mmol/l dithiothreitol, 1 mg/ml leupeptin, 2 mmol/l phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin). A total of 5 μCi [^{32}P]ATP (Amersham, Amersham, U.K.) was added to the immunoprecipitate, and the phosphorylation reaction was then initiated by adding 10 μg substrate GST-GAPp[62]. The reaction was allowed to proceed for 20 min on ice. The reaction was stopped by boiling the sample in 2× Laemmli buffer, the supernatant was then separated by 12% SDS-PAGE, and substrate phosphorylation was quantified by direct phosphoimager analysis of the dried gel (Molecular Devices, Sunnyvale, CA).

Effect of TNF-α on insulin-induced FAK tyrosine phosphorylation in suspended HepG2 cells. HepG2, a human hepatoma cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 200 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Cells were grown to ~70% confluence and were incubated with or without 0.8 nmol/l human recombinant TNF-α for 72 h. After serum starvation overnight in medium containing the same concentration of TNF-α, the cells were gently scraped off the culture dish and were allowed to stabilize for 30 min in PBS at 37°C. The suspended cells were pelleted by centrifugation at 800*g* for 3 min, and the supernatant was resuspended in DMEM supplemented with or without 100 nmol/l insulin (Lilly, Indianapolis, IN) for the times indicated. After incubation, the cells were washed twice with PBS. After centrifugation, the cells were lysed in a buffer containing 50 mmol/l HEPES, pH 7.4, supplemented with 100 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 10 mmol/l EDTA, 2 mmol/l Na_3VO_4, 2 mmol/l phenylmethylsulfonyl fluoride, and 0.1 mg/ml aprotinin for 30 min on ice with a brief vortex every 5 min. The lysates were cleared by centrifugation at 4°C for 15 min at 15,000*g*. Lysate protein concentration was determined with the Bradford method (Bio-Rad). Cell lysate protein (0.5 mg) was incubated with 2 μg anti-FAK antibody and immobilized protein A/G agarose beads overnight at 4°C. The next morning, immunoprecipitates were washed 3 times with lysis buffer and were separated by 7.5% SDS-PAGE, and immunoblot analysis was performed as described above. To rule out the possibility that TNF-α may affect FAK tyrosine phosphorylation in suspended HepG2 cells through an effect on IR expression and/or tyrosine phosphorylation, 1 mg of the above cell lysate protein was incubated with 0.25 μg of c-19 anti–IR-β antibody (Santa Cruz Biotechnology) for 2 h, and then immobilized protein A/G agarose beads were added. After overnight incubation at 4°C, immunoprecipitates were washed 3 times with cold PBS and were separated by 7.5% SDS-PAGE, and immunoblot analysis was performed as described above with pY99 monoclonal antibody. The membrane was then stripped and reblotted with c-19 anti–IR-β antibody.

Statistical analysis. All statistical comparisons were conducted with Student's 2-tailed *t* test for paired or unpaired samples as appropriate. Data are means ± SE. The immunodepletion study was evaluated with Student's unpaired 1-tailed *t* test.

RESULTS

Tyrosine phosphorylation of FAK in the liver of *fa/fa* rats is significantly increased after neutralization of TNF-α. To further identify the candidate-signaling proteins that may be responsible for the improvement in hepatic insulin sensitivity after TNF-α inhibition, liver was collected and snap-frozen during the steady state, and the phosphorylation pattern of liver homogenate proteins was analyzed by immunoblotting. We noted that a 125-kDa liver protein was consistently hyperphosphorylated after TNF-α neutralization in *fa/fa* rats (data not shown). Previous reports have indicated that FAK, a 125-kDa signaling protein downstream of the IR, is regulated by both insulin and TNF-α (23,27). To further confirm the identity of this protein and its phosphorylation state, we immunoprecipitated FAK from liver homogenates and analyzed its phosphorylation levels by immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 1, tyrosine phosphorylation levels of FAK from the liver of

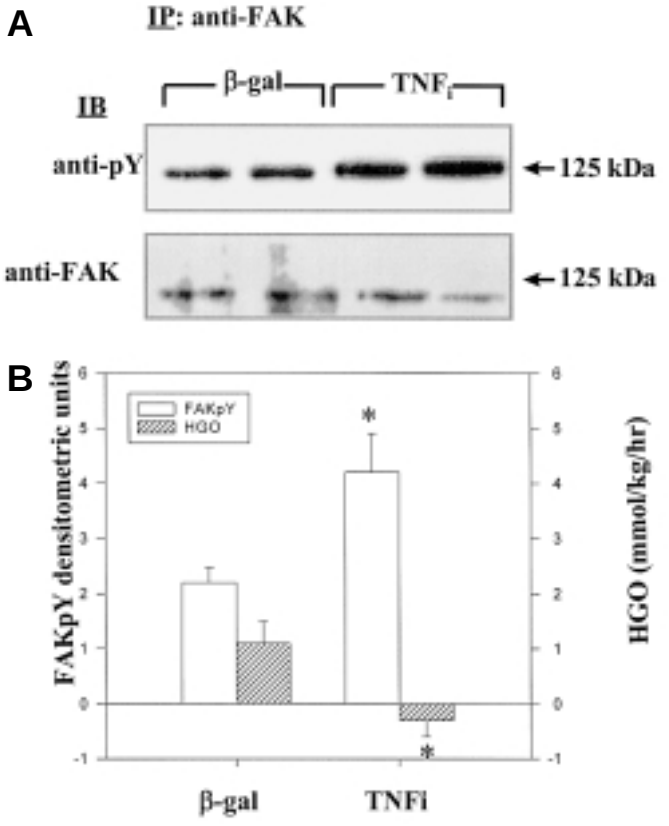

FIG. 1. Effects of TNF neutralization on HGO and liver FAK tyrosine phosphorylation stimulated by insulin in vivo during the euglycemic-hyperinsulinemic glucose clamp studies. *A*: Western blot analysis of tyrosine phosphorylation levels of liver FAK in representative samples from Ad5-TNFi (TNF_i)- and Ad5-β-gal (β-gal)–infected obese Zucker rats. FAK was immunoprecipitated from liver homogenates and was immunoblotted with anti-phosphotyrosine (anti-pY) and anti-FAK antibodies. *B*: Quantitation of phosphotyrosine levels of FAK from liver and corresponding mean HGO levels. FAK tyrosine phosphorylation levels were obtained from densitometric analysis of immunoblots and were normalized to the corresponding amount of FAK protein (*n* = 4 in each group). HGO was measured with the euglycemic-hyperinsulinemic clamp studies as described in RESEARCH DESIGN AND METHODS. Error bars represent SE. □, Phosphorylation levels of FAK; ▨, HGO. **P* < 0.05 vs. β-gal. IB, immunoblot antibody; IP, immunoprecipitation antibody.

TNF-α–neutralized *fa/fa* rats were about 4.5-fold greater than that of controls ($P < 0.05$; $n = 4$ in each group) (Fig. 1*A*). In addition, as shown in Fig. 1*B*, higher FAK phosphorylation levels in the liver of TNF-α–neutralized animals were associated with lower hepatic glucose output (HGO) during the steady state in the euglycemic-hyperinsulinemic clamp studies.

Insulin regulates FAK tyrosine phosphorylation in vivo. Because the effect of insulin on FAK phosphorylation is still controversial (20–23), and because no data exist regarding insulin's effect on FAK phosphorylation in intact animals, we next examined the effects of insulin on FAK phosphorylation in the liver of normal Sprague-Dawley rats. As shown in Fig. 2, in rat liver, insulin induced a rapid phosphorylation of FAK. Maximal stimulation of FAK phosphorylation occurred at 6 min with phosphorylation levels more than 3-fold higher than in the basal state. Because blood glucose was maintained at basal levels, the effect of insulin on FAK phosphorylation resulted neither from hypoglycemia nor indirectly from a metabolic response to hypoglycemia.

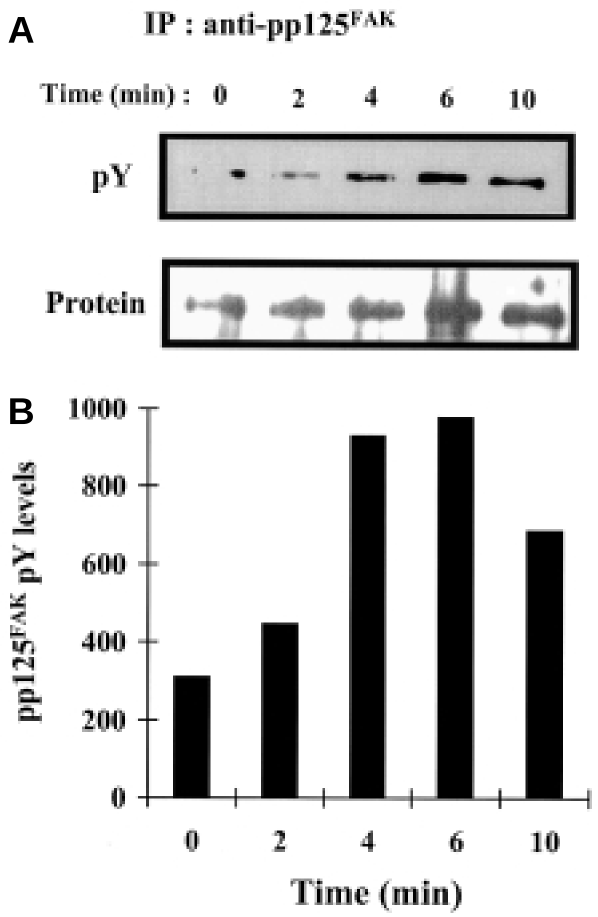

FIG. 2. Effects of intravenous insulin on liver FAK tyrosine phosphorylation (pY) in Sprague-Dawley rats. *A*: Rats were given a bolus of 1 U of intravenous insulin for the periods indicated. Livers were then snap-frozen and homogenized, and the phosphorylation level was analyzed by Western blot. The membrane was then stripped and reprobed with anti-FAK antibody. *B*: Quantitation of FAK phosphorylation levels with densitometry. Values were normalized to FAK protein levels. Each time point represents an average value from 2 animals.

Thus, insulin effectively promotes FAK phosphorylation in the liver of Sprague-Dawley rats in vivo.

TNF-α neutralization downregulates LAR expression without altering c-Src kinase activity in *fa*/*fa* rat liver. To explore the putative mechanism whereby inhibition of TNF-α leads to increased phosphorylation of FAK in the liver, we examined the expression of various candidate PTPs and c-Src kinase activity in liver homogenates from *fa*/*fa* rats treated with either adenovirus expressing the TNFi or β-galactosidase gene products. Numerous reports have shown that the phosphorylation state of FAK is regulated by PTPs (28–30) and Src kinase (21,31,32). The *fa*/*fa* rats infected with Ad5-TNFi and Ad5-β-gal for 4 days were used. Liver was removed during the steady state in the euglycemic clamp studies and was snap-frozen in liquid N_2. Homogenates were prepared, and the relative abundance of 3 candidate PTPs (PTP-1B, LAR, and SHPTP-2), which have been proposed previously to be mediators of insulin action (33–36), was determined by immunoblotting. As shown in Fig. 3, LAR protein levels were dramatically reduced in TNF-α–neutralized animals compared with animals treated with Ad5-β-gal, whereas protein levels of PTP-1B and SHPTP-2 were unchanged in both

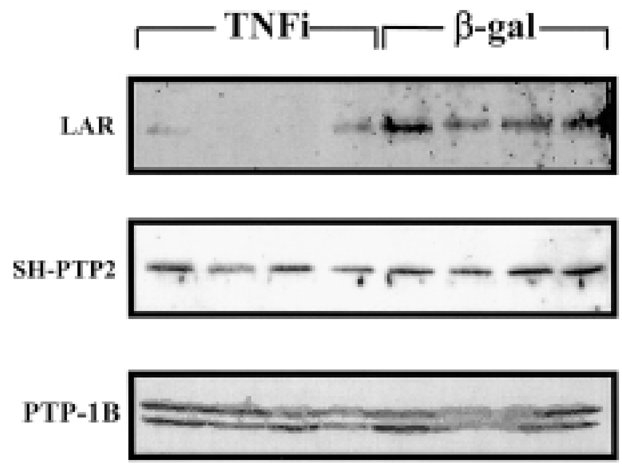

FIG. 3. Abundance of LAR, SHPTP-2, and PTP-1B in liver homogenates from obese Zucker rats treated with either Ad5-TNFi or Ad5-β-gal. Liver tissues were snap-frozen in liquid N_2 during the steady state in the euglycemic-hyperinsulinemic glucose clamps and stored at –70°C until processed. Tissue homogenates were prepared. A total of 20 µg liver homogenate protein was loaded by each lane, separated in 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with specific antibodies. LAR and SHPTP-2 migrate as a single protein band at ~80 and ~65 kDa, respectively. PTP-1B is identified as 2 protein bands consisting of the full-length form (~50 kDa) and the truncated form (40 kDa). Each lane corresponds to 1 rat (*n*= 4 per group).

groups. Densitometric analysis of LAR expression revealed a highly significant mean reduction of 75% in the liver of TNF-α–neutralized *fa*/*fa* rats (TNFi 4,721 ± 746 vs. β-gal 21,523 ± 4,078 arbitrary units [$n = 4$ in each group]; $P < 0.05$).

Src kinase plays an important role in regulating FAK phosphorylation levels and is critical in the assembly of focal adhesion complexes (31,37,38). In addition, reports have shown that insulin's effects on FAK may be mediated through the Src kinase pathway (21). We postulated that, if the elevation in phosphorylation levels of FAK in TNF-α–neutralized animals is mediated through the Src kinase pathway, then Src kinase activity in the liver of TNF-α–neutralized animals would be higher than that in controls. The kinase assay was performed as described in RESEARCH DESIGN AND METHODS. Again, liver tissue was collected during the steady state in the euglycemic-hyperinsulinemic clamp studies. Mean Src activity in liver homogenates of *fa*/*fa* rats infected with Ad5-TNFi was not significantly different from rats infected with Ad5-β-gal (90.9 ± 14.5 vs. 74.2 ± 8.5 arbitrary units [$n = 4$ per group]; NS), which indicates that upregulation of Src activity is not likely to be responsible for the improvement of insulin-induced FAK phosphorylation after TNF-α inhibition (data not shown).

Immunodepletion of LAR from liver homogenates restores the phosphorylation state of FAK. Although lowered LAR abundance in TNF-α–neutralized animals corresponded with higher FAK phosphorylation in the liver, we had to establish that increased LAR abundance was specifically responsible for the reduction of insulin-stimulated FAK phosphorylation levels in *fa*/*fa* rats. To address this issue, we compared the levels of FAK-specific PTP activity in liver homogenates of Ad5-TNFi– and Ad5-β-gal–treated *fa*/*fa* rats collected during the steady state in the glucose clamp studies after immunodepletion of LAR with excess anti-LAR antibody. Maximally phosphorylated FAK isolated from the liver

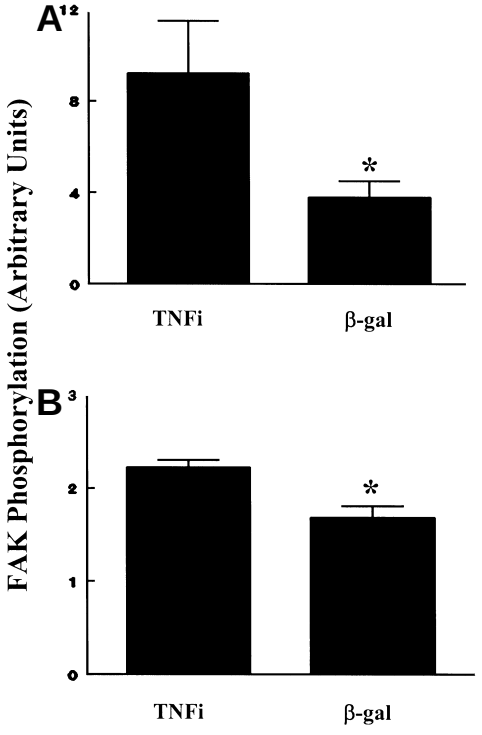

FIG. 4. Effect of immunodepletion of LAR from the particulate fraction of liver homogenates on PTP activity toward tyrosine phosphorylated FAK. Particulate fractions of tissue homogenates from liver removed from obese Zucker rats during the steady state in the glucose clamp studies were incubated with excess anti-LAR antibody or matching amounts of normal rabbit IgG. The resulting supernatants were then incubated with prephosphorylated immunoprecipitated FAK isolated from Sprague-Dawley rat liver as described in RESEARCH DESIGN AND METHODS, and residual FAK phosphotyrosine content was determined densitometrically (n = 4 in each group). Results are shown for rabbit IgG as control (*A*; 9.0 ± 2.4 for TNFi vs. 3.8 ± 0.7 arbitrary units for β-galactosidase [β-gal]) and anti-LAR antibody (*B*; 2.2 ± 0.1 for TNFi vs. 1.7 ± 0.1 arbitrary units for β-galactosidase). Error bars represent SE. *P < 0.05.

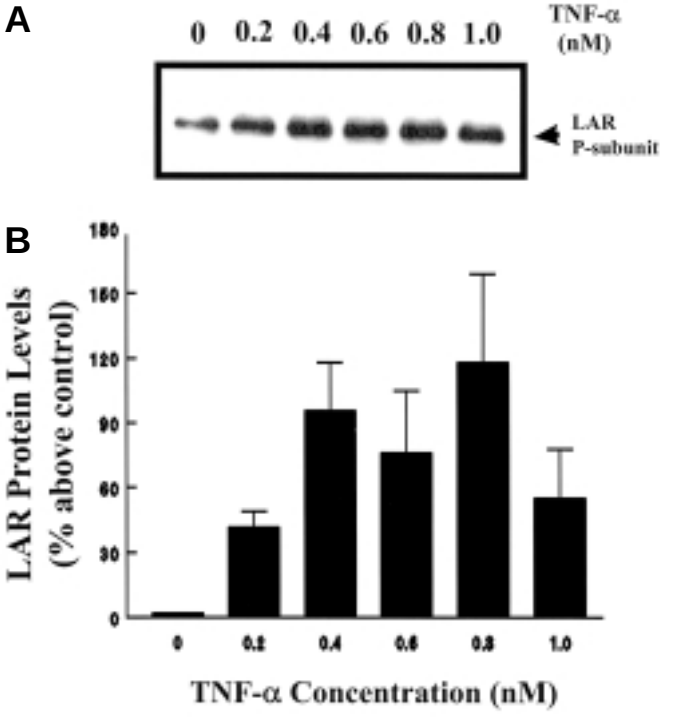

FIG. 5. Effects of TNF-α on the expression of LAR in HepG2 cells. The upper panel shows a representative immunoblot of HepG2 extracts after treatment with the indicated concentrations of recombinant human TNF-α for 72 h (n = 4 for each group). The anti-LAR antibody used recognizes the P-subunit of LAR. The lower panel shows quantification of LAR protein levels with densitometric analysis. Data are means from 3 separate experiments, and the error bars represent SE. anti-pY, anti-phosphotyrosine; IB, immunoblot antibody; IP, immunoprecipitation antibody.

of Sprague-Dawley rats treated with 1 U of intravenous insulin was used as a substrate in this assay. Equal amounts of particulate protein from the liver of experimental animals were incubated with excess anti-LAR antiserum (10 μg) and protein A/G–conjugated agarose. The particulate fraction of liver homogenate was used because LAR is localized in this tissue fraction (39). Equal aliquots of the resulting LAR-depleted supernatant (~15 μg protein) were then transferred to separate microcentrifuge tubes containing equal amounts of substrate (immobilized FAK from insulin-treated Sprague-Dawley rat liver). In preliminary experiments, the amount of anti-LAR antibody used specifically depleted 80–90% of the LAR protein from the particulate fraction of liver homogenates (data not shown). The reaction was allowed to proceed for 6 min at room temperature. Control samples were incubated with normal rabbit IgG. As shown in Fig. 4, FAK-specific PTP activity was 58% higher in Ad5-β-gal–treated control animals (as indicated by lower FAK phosphorylation levels) compared with Ad5-TNFi–treated animals (P < 0.05; n = 4 in each group). The higher hepatic PTP activity in Ad5-β-gal–treated animals correlated well with their higher LAR abundance (Fig. 3). Moreover, after depletion of the LAR enzyme, the difference in FAK-specific PTP activity in the same tissue fraction between the 2 groups, although still statistically significant, was greatly minimized to 23% (Fig. 4*B*). These results directly link higher FAK phosphorylation levels in the liver from TNF-α–neutralized *fa*/*fa* rats to reduced LAR expression.

TNF-α induces LAR expression in HepG2 cells. Neutralization of TNF-α led to reduced LAR abundance in the liver of *fa*/*fa* rats, which indicates that elevated TNF-α levels in obesity may have led to higher-than-normal levels of LAR expression in these obese rodents. To ascertain whether similar regulation of LAR expression by TNF-α also occurs in cultured liver cells, LAR abundance in HepG2 cells was quantified with Western blot after a 72-h exposure to varying concentrations of TNF-α. Figure 5 shows the dose response of LAR expression in HepG2 cells to this TNF-α treatment. Stimulation of LAR expression occurred at 0.2 nmol/l TNF-α and reached maximal levels at 0.8 nmol/l. No TNF-α concentration had any adverse effect on cell viability as determined by trypan blue exclusion (data not shown).

TNF-α blunts insulin-stimulated tyrosine phosphorylation of FAK without altered IR tyrosine phosphorylation in HepG2 cells. As shown before, in *fa*/*fa* rats, inhibition of TNF-α activity was associated with increased tyrosine phosphorylation of FAK in the liver during the steady state in the glucose clamp studies, and insulin can stimulate tyrosine phosphorylation of FAK in vivo. These findings raise the possibility that TNF-α itself may interfere with insulin-induced tyrosine phosphorylation of FAK, which

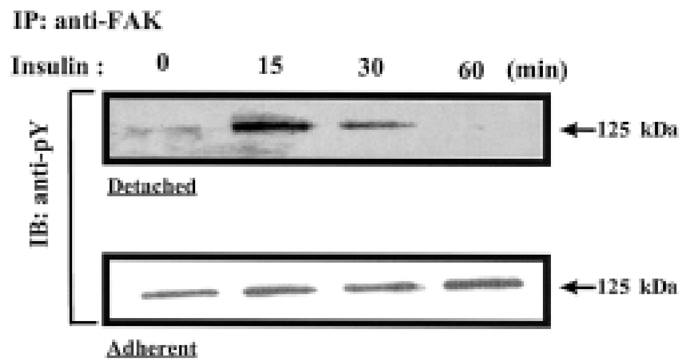

FIG. 6. The effect of insulin on FAK phosphorylation in detached and adherent HepG2 cells. Detached cells were obtained by gentle scraping from the culture flasks and were maintained in suspension for 30 min in PBS before addition of 100 nmol/l insulin for indicated periods. Cell lysates were collected and subjected to immunoprecipitation with anti-FAK antibody. FAK phosphorylation was visualized by antiphosphotyrosine immunoblotting. Anti-pY, anti-phosphotyrosine; IB, immunoblot antibody; Ins, insulin; IP, immunoprecipitation antibody.

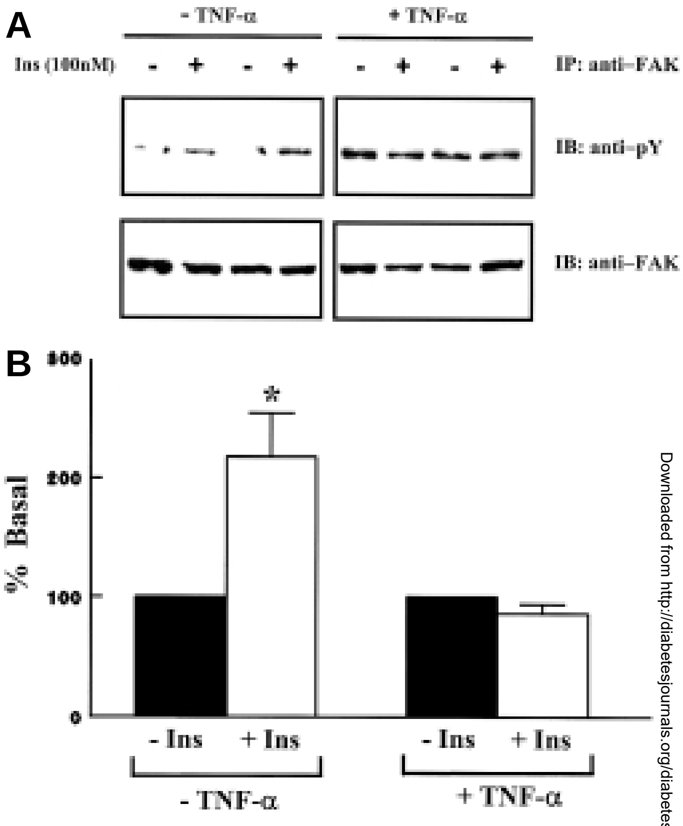

FIG. 7. Effect of TNF-α on insulin-stimulated FAK tyrosine phosphorylation. HepG2 cells were incubated with or without TNF-α (0.8 nmol/l) for 72 h. Cells were then detached and treated with or without 100 nmol/l insulin for 15 min. Cells were placed on ice and washed twice with ice-cold PBS before solubilization. Cell lysates were incubated with anti-FAK antibody, and tyrosine-phosphorylated proteins were revealed by immunoblotting. *A*: Representative immunoblots. The levels of FAK phosphorylation were quantified densitometrically (*B*). Data are means, and the error bars represent SE. Insulin increased FAK phosphorylation to 217 ± 34% (n = 6; *P < 0.03), and TNF-α treatment reduced insulin stimulation of FAK phosphorylation to 86 ± 16% of control (n = 4; NS).

could be one of the mechanisms whereby TNF-α affects insulin signaling. We tested this hypothesis in suspended HepG2 cells because we believe that suspended HepG2 cells best simulate the in vivo tyrosine phosphorylation response of FAK to insulin in livers of intact animals, as shown earlier in Fig. 2. Similar to the findings of Baron et al. (20), we also demonstrated that insulin induces tyrosine phosphorylation of FAK. HepG2 cells in suspension were treated with 100 nmol/l insulin, and the results are shown in Fig. 6. Tyrosine phosphorylation peaked 15 min after exposure to insulin. Next, the cells were pretreated with or without 0.8 nmol/l TNF-α for 72 h. As shown in Fig. 7*A*, TNF-α treatment completely abolished insulin-induced tyrosine phosphorylation of FAK (relative phosphotyrosine content vs. time 0: 86 ± 16% [n = 4]; NS), whereas FAK phosphorylation increased significantly after insulin treatment in cells without TNF-α preexposure (relative phosphotyrosine content vs. time 0: 217 ± 34% [n = 6]; P < 0.03). However, when immunoblots for IR content and insulin-stimulated tyrosine phosphorylation of IR were performed, no differences were evident between TNF-α pretreated and control cells (Fig. 8). These data indicate that TNF-α prevents insulin from stimulating FAK phosphorylation in cultured liver cells by a mechanism independent of inhibition of an early step of the insulin-signaling pathway.

DISCUSSION

We have previously reported that neutralization of circulating TNF-α through adenovirus-mediated expression of a TNFi in obese Zucker rats leads to significant improvement in hepatic insulin sensitivity (8). However, the improvement in hepatic insulin sensitivity was achieved without altering the liver IR number, insulin binding affinity, or IR kinase activation state, which signifies that the enhancement of insulin's molecular action in the liver was likely to be downstream of the IR (8). In contrast, Hotamisligil et al. (1) have reported that TNF-α neutralization with intravenous administration of a recombinant TNFi protein in obese Zucker rats improved peripheral insulin sensitivity, whereas hepatic insulin sensitivity remained unchanged. Such discrepancies may be due to differences in the concentrations of TNFi in the liver or due to clearance of TNFi proteins by the host immune response when inhibitor proteins were administered exogenously. We have previously shown that Ad5-TNFi gene expression provided a sustained high level of soluble circulating TNFi protein in mice (40) and was high at the time of the glucose clamp studies in the current study (8). To our knowledge, in other neutralization studies, circulating TNFi activity has not been measured at the time of clamp study. Kolls et al. (40) previously showed that the half-life of the TNFi proteins in mouse plasma is ~30 h (40). With such clearance kinetics, constant inhibition of TNF-α could not likely be achieved in between once-daily injections of TNFi proteins. Although sustained high levels of TNFi could be achieved by constant infusion or an implantable slow-release preparation, we are not aware that these methods have been used in reported studies. With our model, however, plasma levels of the inhibitor peak on the second day after adenovirus administration and maintain that level for up to 20 days (40). Additionally, our findings regarding the effects of TNF-α on the liver agree with previous reports showing that the administration of TNF-α to normal Sprague-Dawley rats impairs insulin's ability to suppress HGO (5) and that

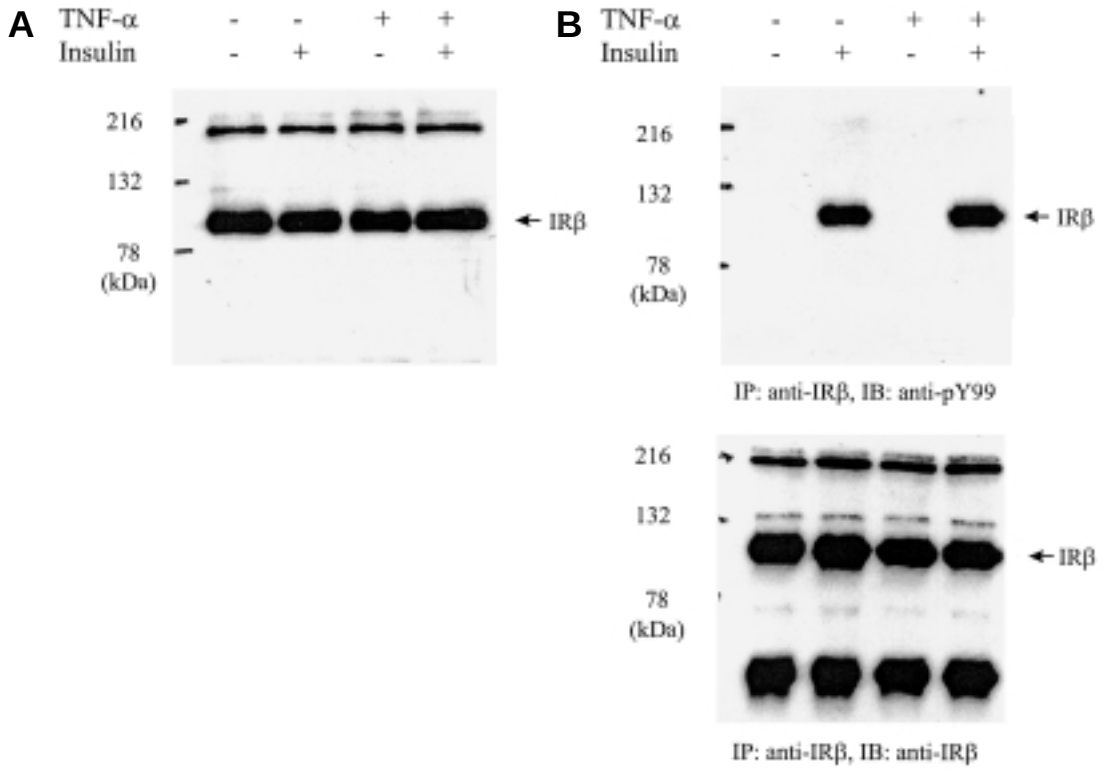

FIG. 8. Effect of TNF-α on IR-β subunit (IRβ) protein levels and insulin-stimulated IR-β subunit tyrosine phosphorylation. HepG2 cells were incubated with or without TNF-α (0.8 nmol/l) for 72 h. Cells were then detached and treated with or without 100 nmol/l insulin for 15 min. *A*: Whole-cell lysates were separated on 7.5% SDS-PAGE, and IR-β subunit protein levels were detected with a polyclonal anti–IR-β antibody. *B*: Whole-cell lysates were immunoprecipitated with anti–IR-β antibody, and tyrosine phosphorylation of IR-β was revealed by immunoblotting with an anti-phosphotyrosine antibody. The membrane was then stripped and reblotted with anti–IR-β antibody to confirm the presence of equal amounts of protein. The data shown resulted from a single experiment, which was repeated twice with similar results. IB, immunoblot antibody; IP, immunoprecipitation antibody.

TNF-α treatment of cultured liver cells induces insulin resistance (41). Recent reports have shown that removal of visceral fat leads to significant improvement in hepatic insulin sensitivity with concomitant marked reductions in TNF-α gene expression in subcutaneous fat, whereas plasma free fatty acids were unchanged (42). These results suggest that TNF-α may be an important mediator of hepatic insulin sensitivity. Also, although genetic knockout of the TNF-α ligand is known to lead to increased peripheral insulin sensitivity, hepatic insulin sensitivity has not been reported in these animal models (9,11).

Our data suggest that TNF-α upregulates LAR expression and that the resultant increased LAR activity may subsequently dephosphorylate FAK, thus preventing its activation by insulin. Although the exact role of FAK in insulin signal transduction remains unclear, recent studies have suggested that FAK is involved in many growth factor–signaling cascades (43). If our hypothesis was correct (i.e., that TNF-α attenuation of insulin action in the liver is mediated through downregulation of insulin-induced FAK activation), then we anticipated that insulin would promote FAK tyrosine phosphorylation and activation. However, in contrast with other receptor tyrosine kinases that induce tyrosine phosphorylation of FAK (43,44), earlier reports have shown that insulin induces dephosphorylation of FAK and reduces actin filament content in Chinese hamster ovary (CHO) cells and fibroblasts overexpressing IR (23,28). Because FAK plays a positive regulatory role in integrin signaling (45), insulin-mediated FAK dephosphorylation should exert an antagonistic effect on integrin signaling. However, synergy between the IR and integrin signaling has been reported (46,47). For example, insulin markedly promotes CHO–T-cell adhesion to the fibronectin matrix (a mechanism mediated by $\alpha_v\beta_1$ integrin), and activation of this integrin in turn enhances IR and IRS-1 phosphorylation and the association of phosphatidylinositol 3-kinase with IRS-1 in response to insulin (47). Furthermore, the mitogenic effects of insulin have been shown to be enhanced by $\alpha_v\beta_1$ integrins, which also associate with IRS-1 in an insulin-dependent manner (48).

Previous reports have also indicated that insulin's effects on FAK phosphorylation depend on cell type, cell adhesion status, and IR content (20,22). Baron et al. (20) reported that insulin promotes FAK phosphorylation in nonattached HepG2 cells and that FAK is a direct substrate of the IR tyrosine kinase. In CHO and rat hepatoma cells overexpressing the IR, insulin stimulates dephosphorylation of FAK and rearrangement of actin filaments and of focal adhesion complexes (22). In contrast, a completely opposite response was observed in the parental cell lines expressing physiological amounts of IR. In these insulin-responsive parental cells, rapid phosphorylation of FAK and polymerization of actin filaments were observed during insulin challenge (22). We have shown that insulin stimulates phosphorylation of FAK in Sprague-Dawley rat liver in vivo and that neutralization of TNF-α in obese *fa*/*fa* Zucker rats leads to increased insulin-stimulated FAK phosphorylation in the liver. We have addi-

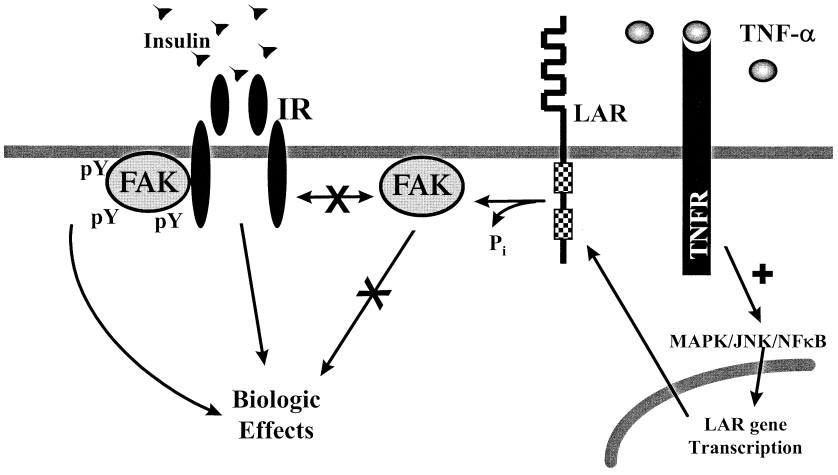

FIG. 9. Proposed schema for the interaction between the TNF-α and insulin-signaling cascades in the induction of insulin resistance in liver. TNF-α activates LAR gene transcription. LAR dephosphorylates FAK, which is normally phosphorylated in vivo in response to insulin binding. In the absence of FAK phosphorylation, IR and IRS proteins do not physically interact with FAK to form the focal adhesion complex, thus inhibiting IR signal transduction. JNK, Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; P_i, inorganic phosphate; pY, phosphotyrosine; TNFR, TNF receptor.

tionally demonstrated that TNF-α treatment of HepG2 cells in suspension abolishes insulin-mediated stimulation of FAK phosphorylation. This demonstration suggests that, under physiological conditions, FAK in the liver of intact animals is tyrosine phosphorylated during insulin challenge and that the increased TNF-α levels in obese animals may prevent this tyrosine phosphorylation and attenuate certain components of hepatic insulin signal transduction.

The activation state of FAK is tightly regulated by Src kinase (31,49) and PTPs (29,50). Tobe et al. (21) reported that insulin promotes the association of phosphorylated IRS-1 with Csk, whereas overexpression of Csk decreases basal FAK phosphorylation levels and enhances insulin's effect to dephosphorylate FAK in CHO cells overexpressing the IR. Because Csk inhibits Src activity by phosphorylating tyrosine residues on the COOH-terminal tail of Src kinase, these observations led the researchers to propose that insulin's effects on FAK are mediated through the Src kinase pathway (21). However, we did not find any alterations in hepatic Src activity after neutralization of TNF-α in *fa*/*fa* rats, which indicates that the increase in insulin-stimulated FAK phosphorylation in our model did not likely result from upregulation of Src activity.

Many reports have also shown that FAK phosphorylation is regulated by PTPs (27,29,30,51). Although the precise role of PTPs in the pathogenesis of insulin resistance is still controversial (52), it is generally believed that PTPs are potential regulators of insulin action (34,53) and that abundance and activity of specific PTPs are altered in insulin target tissues from obese insulin-resistant human subjects (33,54–56). LAR is a receptor-like PTP that is recognized as a putative negative regulator of insulin action (35,55,57,58). Ahmad et al. (33,55) reported that LAR expression and activity in adipose tissue and skeletal muscle from obese insulin-resistant human subjects are significantly higher than in healthy nonobese subjects. Reports have also shown that inhibition of LAR expression by antisense methodology augments insulin signaling (35). Recently, a transgenic LAR knockout mouse model was reported to exhibit reduced fasting glucose and insulin levels, which suggest improved hepatic insulin sensitivity (59). Our data indicate that TNF-α–induced hepatic insulin resistance may be mediated through LAR, which supports the established negative regulatory role of LAR in insulin signaling and its expression pattern in insulin-sensitive tissues from obese type 2 diabetic subjects.

Reports have shown that incubation with TNF-α may reduce insulin-stimulated IR tyrosine phosphorylation (6,14–17) or downregulate IR expression (18). These upstream effects could potentially influence downstream FAK activation independent of its proposed interaction with LAR. However, our data show that, in HepG2 cells, preincubation with TNF-α resulted neither in insulin-independent IR tyrosine phosphorylation nor in altered insulin-mediated IR tyrosine phosphorylation. Furthermore, expression of IR was unchanged. Thus, our findings cannot be accounted for by upstream impairment in activation of the insulin-signaling cascade by TNF-α in this model.

The data we present herein also show that TNF-α is a potent stimulator of LAR expression in human hepatoma cells and that neutralization of TNF-α in *fa*/*fa* rats reduces LAR expression in the liver. Furthermore, we show that immunodepletion of LAR normalizes most of the difference in FAK-specific PTP activity in liver homogenates of TNF-α–neutralized and control *fa*/*fa* rats. Our findings suggest that TNF-α may attenuate insulin action through stimulation of LAR expression and activity, which agrees with the findings of Kroder et al. (27), who reported that the TNF-α inhibitory effect on insulin action in NIH-3T3 cells was completely prevented by PTP inhibitors.

Taken together, our results indicate that TNF-α induces LAR expression and hence activity in the liver, which leads to impaired insulin-stimulated FAK phosphorylation and activation. LAR colocalizes with FAK at focal adhesions in vivo (60), and several reports have shown that PTPs are positive regulators of TNF-α action (27,61,62). First, it has been shown that PTP inhibitors block TNF-α–induced insulin resistance in NIH-3T3 cells (27). In addition, Aggarwal and colleagues (61,62) have shown that PTP inhibitors block TNF-α–induced growth modulation and nuclear factor (NF)-κB activation. They have further shown that PTPs play an essential role in phosphorylation of the cytoplasmic domain (residue Y_{331} localized in the death domain) of the TNF receptor (61,62), which in turn regulates the receptor-associated kinase and NF-κB activation.

Our results can be summarized in the model of TNF-α–induced hepatic insulin resistance illustrated in Fig. 9. Under normal conditions, insulin activates FAK in the liver by promoting its tyrosine phosphorylation. However, the increased TNF-α levels in obesity trigger the expression of LAR in the

liver via an undetermined mechanism. The increased LAR abundance may simultaneously dephosphorylate FAK, thus antagonizing signal transduction via the IR. Clearly, additional studies are required to understand the physiological role of FAK in hepatic insulin action, the molecular interaction between LAR and FAK, and the precise mechanism whereby TNF-α promotes increased LAR expression.

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REFERENCES

1. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993
2. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
3. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB: The expression of tumor necrosis factor in human adipose tissue: regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* 95:2111–2119, 1995
4. Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA: The expression of TNF alpha by human muscle: relationship to insulin resistance. *J Clin Invest* 97:1111–1116, 1996
5. Lang CH, Dobrescu C, Bagby GJ: Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology* 130:43–52, 1992
6. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM: Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes: central role of tumor necrosis factor-alpha. *J Clin Invest* 94:1543–1549, 1994
7. Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R: Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45:881–885, 1996
8. Cheung AT, Ree D, Kolls JK, Fuselier J, Coy DH, Bryer-Ash M: An in vivo model for elucidation of the mechanism of tumor necrosis factor-alpha (TNF-alpha)-induced insulin resistance: evidence for differential regulation of insulin signaling by TNF-alpha. *Endocrinology* 139:4928–4935, 1998
9. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610–614, 1997
10. Schreyer SA, Chua SC Jr, LeBoeuf RC: Obesity and diabetes in TNF-alpha receptor-deficient mice. *J Clin Invest* 102:402–411, 1998
11. Ventre J, Doebber T, Wu M, MacNaul K, Stevens K, Pasparakis M, Kollias G, Moller DE: Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. *Diabetes* 46:1526–1531, 1997
12. Hofmann C, Lorenz K, Braithwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS, Spiegelman BM: Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* 134:264–270, 1994
13. Hotamisligil GS, Spiegelman BM: Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 43:1271–1278, 1994
14. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM: Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci U S A* 91:4854–4858, 1994
15. Begum N, Ragolia L, Srinivasan M: Effect of tumor necrosis factor-alpha on insulin-stimulated mitogen-activated protein kinase cascade in cultured rat skeletal muscle cells. *Eur J Biochem* 238:214–220, 1996
16. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A: Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem* 268:26055–26058, 1993
17. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665–668, 1996
18. Stephens JM, Lee J, Pilch PF: Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. *J Biol Chem* 272:971–976, 1997
19. Garvey WT, Maianu L, Hancock JA, Golichowski AM, Baron A: Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. *Diabetes* 41:465–475, 1992
20. Baron V, Calleja V, Ferrari P, Alengrin F, Van Obberghen E: p125Fak focal adhesion kinase is a substrate for the insulin and insulin-like growth factor-I tyrosine kinase receptors. *J Biol Chem* 273:7162–7168, 1998
21. Tobe K, Sabe H, Yamamoto T, Yamauchi T, Asai S, Kaburagi Y, Tamemoto H, Ueki K, Kimura H, Akanuma Y, Yazaki Y, Hanafusa H, Kadowaki T: Csk enhances insulin-stimulated dephosphorylation of focal adhesion proteins. *Mol Cell Biol* 16:4765–4772, 1996
22. Wang Q, Bilan PJ, Klip A: Opposite effects of insulin on focal adhesion proteins in 3T3-L1 adipocytes and in cells overexpressing the insulin receptor. *Mol Biol Cell* 9:3057–3069, 1998
23. Knight JB, Yamauchi K, Pessin JE: Divergent insulin and platelet-derived growth factor regulation of focal adhesion kinase (pp125FAK) tyrosine phosphorylation, and rearrangement of actin stress fibers. *J Biol Chem* 270:10199–10203, 1995
24. Peppel K, Crawford D, Beutler B: A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. *J Exp Med* 174:1483–1489, 1991
25. Graham FL, Provec L: In *Methods in Molecular Biology*. Murray EJ, Ed. Clifton, NJ, Humana, 1990, p. 109–128
26. Bryer-Ash M: Regulation of rat insulin-receptor kinase by glucose in vivo. *Diabetes* 40:633–640, 1991
27. Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Muhlhofer A, Berti L, Horikoshi H, Ullrich A, Haring H: Tumor necrosis factor-alpha- and hyperglycemia-induced insulin resistance: evidence for different mechanisms and different effects on insulin signaling. *J Clin Invest* 97:1471–1477, 1996
28. Pillay TS, Sasaoka T, Olefsky JM: Insulin stimulates the tyrosine dephosphorylation of pp125 focal adhesion kinase. *J Biol Chem* 270:991–994, 1995
29. Shen Y, Schneider G, Cloutier JF, Veillette A, Schaller MD: Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. *J Biol Chem* 273: 6474–6481, 1998
30. Yamauchi K, Milarski KL, Saltiel AR, Pessin JE: Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. *Proc Natl Acad Sci U S A* 92:664–668, 1995
31. Schlaepfer DD, Broome MA, Hunter T: Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adapter proteins. *Mol Cell Biol* 17:1702–1713, 1997
32. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT: Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14:1680–1688, 1994
33. Ahmad F, Azevedo JL, Cortright R, Dohm GL, Goldstein BJ: Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J Clin Invest* 100:449–458, 1997
34. Byon JC, Kusari AB, Kusari J: Protein-tyrosine phosphatase-1B acts as a negative regulator of insulin signal transduction. *Mol Cell Biochem* 182:101–108, 1998
35. Kulas DT, Zhang WR, Goldstein BJ, Furlanetto RW, Mooney RA: Insulin receptor signaling is augmented by antisense inhibition of the protein tyrosine phosphatase LAR. *J Biol Chem* 270:2435–2438, 1995
36. Kenner KA, Anyanwu E, Olefsky JM, Kusari J: Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. *J Biol Chem* 271:19810–19816, 1996
37. Vuori K, Hirai H, Aizawa S, Ruoslahti E: Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol Cell Biol* 16:2606–2613, 1996
38. Schaller MD, Parsons JT: pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol Cell Biol* 15:2635–2645,

1995
39. Zhang WR, Hashimoto N, Ahmad F, Ding W, Goldstein BJ: Molecular cloning and expression of a unique receptor-like protein-tyrosine-phosphatase in the leucocyte-common-antigen-related phosphate family. *Biochem J* 302:39–47, 1994
40. Kolls J, Peppel K, Silva M, Beutler B: Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer. *Proc Natl Acad Sci U S A* 91:215–219, 1994
41. Solomon SS, Mishra SK, Cwik C, Rajanna B, Postlethwaite AE: Pioglitazone and metformin reverse insulin resistance induced by tumor necrosis factor-alpha in liver cells. *Horm Metab Res* 29:379–382, 1997
42. Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J, Rossetti L: Surgical removal of visceral fat reverses hepatic insulin resistance. *Diabetes* 48:94–98, 1999
43. Meredith JE Jr, Winitz S, Lewis JM, Hess S, Ren XD, Renshaw MW, Schwartz MA: The regulation of growth and intracellular signaling by integrins. *Endocrinol Rev* 17:207–220, 1996
44. Valentinis B, Reiss K, Baserga R: Insulin-like growth factor-I-mediated survival from anoikis: role of cell aggregation and focal adhesion kinase. *J Cell Physiol* 176:648–657, 1998
45. Schaller MD: The focal adhesion kinase. *J Endocrinol* 150:1–7, 1996
46. Guilherme A, Czech MP: Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by beta1-integrin signaling in rat adipocytes. *J Biol Chem* 273:33119–33122, 1998
47. Guilherme A, Torres K, Czech MP: Cross-talk between insulin receptor and integrin alpha5 beta1 signaling pathways. *J Biol Chem* 273:22899–22903, 1998
48. Vuori K, Ruoslahti E: Association of insulin receptor substrate-1 with integrins. *Science* 266:1576–1578, 1994
49. Calalb MB, Polte TR, Hanks SK: Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol* 15:954–963, 1995
50. Persson C, Carballeira N, Wolf-Watz H, Fallman M: The PTPase YopH inhibits uptake of yersinia, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. *EMBO J* 16:2307–2318, 1997
51. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT: pp125FAK: a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* 89:5192–5196, 1992
52. Goldstein BJ, Li PM, Ding W, Ahmad F, Zhang WR: Regulation of insulin action by protein tyrosine phosphatases. *Vitam Horm* 54:67–96, 1998
53. Goldstein BJ, Ahmad F, Ding W, Li PM, Zhang WR: Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. *Mol Cell Biochem* 182:91–99, 1998
54. Kusari J, Kenner KA, Suh KI, Hill DE, Henry RR: Skeletal muscle protein tyrosine phosphatase activity and tyrosine phosphatase 1B protein content are associated with insulin action and resistance. *J Clin Invest* 93:1156–1162, 1994
55. Ahmad F, Considine RV, Goldstein BJ: Increased abundance of the receptor-type protein-tyrosine phosphatase LAR accounts for the elevated insulin receptor dephosphorylating activity in adipose tissue of obese human subjects. *J Clin Invest* 95:2806–2812, 1995
56. Cheung A, Kusari J, Jansen D, Bandyopadhyay D, Kusari A, Bryer-Ash M: Marked impairment of protein tyrosine phosphatase 1B activity in adipose tissue of obese subjects with and without type 2 diabetes mellitus. *J Lab Clin Med* 134:115–123, 1999
57. Ahmad F, Goldstein BJ: Functional association between the insulin receptor and the transmembrane protein-tyrosine phosphatase LAR in intact cells. *J Biol Chem* 272:448–457, 1997
58. Zhang WR, Li PM, Oswald MA, Goldstein BJ: Modulation of insulin signal transduction by eutopic overexpression of the receptor-type protein-tyrosine phosphatase LAR. *Mol Endocrinol* 10:575–584, 1996
59. Ren JM, Li PM, Zhang WR, Sweet LJ, Cline G, Shulman GI, Livingston JN, Goldstein BJ: Transgenic mice deficient in the LAR protein-tyrosine phosphatase exhibit profound defects in glucose homeostasis. *Diabetes* 47:493–497, 1998
60. Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A, Streuli M: The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. *EMBO J* 14:2827–2838, 1995
61. Dhawan S, Singh S, Aggarwal BB: Induction of endothelial cell surface adhesion molecules by tumor necrosis factor is blocked by protein tyrosine phosphatase inhibitors: role of the nuclear transcription factor NF-kappa B. *Eur J Immunol* 27:2172–2179, 1997
62. Darnay BG, Aggarwal BB: Inhibition of protein tyrosine phosphatases causes phosphorylation of tyrosine-331 in the p60 TNF receptor and inactivates the receptor-associated kinase. *FEBS Lett* 410:361–367, 1997