Tumor Necrosis Factor-α– and Hyperglycemia-induced Insulin Resistance

Evidence for Different Mechanisms and Different Effects on Insulin Signaling

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Abstract

Inhibition of insulin receptor signaling by high glucose levels and by TNF-α was recently observed in different cell systems. The aim of the present study was to characterize the mechanism of TNF-α–induced insulin receptor inhibition and to compare the consequences of TNF-α– and hyperglycemia-induced insulin receptor inhibition for signal transduction downstream from the IR. TNF-α (0.5–10 nM) and high glucose (25 mM) showed similar rapid kinetics of inhibition (5–10 min, > 50%) of insulin receptor autophosphorylation in NIH3T3 cells overexpressing the human insulin receptor. TNF-α effects were completely prevented by the phosphotyrosine phosphatase (PTPase) inhibitors orthovanadate (40 μM) and phenylarsenoxide (35 μM), but they were unaffected by the protein kinase C (PKC) inhibitor H7 (0.1 mM), the phosphatidylinositol-3 kinase inhibitor wortmannin (5 μM), and the thiazolidindione troglitazone (CS045) (2 μg/ml). In contrast, glucose effects were prevented by PKC inhibitors and CS045 but unaffected by PTPase inhibitors and wortmannin. To assess effects on downstream signaling, tyrosine phosphorylation of the following substrate proteins of the insulin recepter was determined: insulin receptor substrate-1, the coupling protein Shc, focal adhesion kinase (FAK^{125}), and unidentified proteins of 130 kD, 60 kD, and 44 kD. Hyperglycemia (25 mM glucose) and TNF-α showed analogous (> 50% inhibition) effects on tyrosine phosphorylation of insulin receptor substrate-1, Shc, p60, and p44, whereas opposite effects were observed for tyrosine phosphorylation of FAK^{125}, which is dephosphorylated after insulin stimulation. Whereas TNF-α did not prevent insulin-induced dephosphorylation of FAK^{125}, 25 mM glucose blocked this insulin effect completely. In summary, the data suggest that TNF-α and high glucose modulate insulin receptor-signaling through different mechanisms: (*a*) TNF-α modulates insulin receptor signals by PTPase activation, whereas glucose acts through activation of PKC. (*b*) Differences in modulation of the insulin receptor signaling cascade are found with TNF-α and high glucose: Hyperglycemia-induced insulin receptor inhibition blocks both insulin receptor-dependent tyrosine phosphorylation and dephosphorylation of insulin receptor substrate proteins. In contrast, TNF-α blocks only substrate phosphorylation, and it does not block insulin-induced substrate dephosphorylation. The different effects on FAK^{125} regulation allow the speculation that long-term cell effects related to FAK^{125} activity might develop in a different way in hyperglycemia- and TNF-α–dependent insulin resistance. (*J. Clin. Invest.* 1996. 97:1471–1477.) Key words: non–insulin-dependent diabetes mellitus • obesity • insulin receptor • protein kinase C • phosphotyrosine phosphatase

Introduction

Non–insulin-dependent diabetes mellitus (NIDDM)[1] is characterized by abnormalities of insulin secretion and by insulin resistance of the major target tissues (1). In particular, insulin resistance of the skeletal muscle appears to play a pivotal role in the pathogenesis of the disease (2). Several studies have previously investigated whether an impaired signaling capacity of the insulin receptor contributes to the pathogenesis of skeletal muscle insulin resistance. Most studies have reported that autophosphorylation or substrate phosphorylation of the insulin receptor tyrosine kinase isolated from diabetic skeletal muscle is reduced (3–9). The molecular mechanisms responsible for the reduced activation of the insulin receptor kinase (IRK) in NIDDM patients have not yet been identified. However, the defect appears to be acquired rather than inherited, as insulin receptor mutations are extremely rare in common forms of NIDDM (10). Modulators of insulin receptor function are therefore probably of predominant importance for the pathogenesis of insulin resistance in NIDDM. Conditions modulating the signaling function of the IRK have been studied in many different cell systems. It has been shown that factors such as hyperinsulinemia and hypoinsulinemia as well as agonists, including catecholamines, adenosine, and phorbolesters, are able to regulate insulin receptor function (for review see reference 11). There is also evidence for a regulatory role of G-proteins (12) and other membrane proteins (13). In addition, insulin receptor inhibition by high glucose levels was demonstrated in different cell models (14–17), and, more recently, a number of studies have shown that the cytokine TNF-α might be another important regulator of insulin receptor function (18–21). Hyperglycemia- and TNF-α–induced receptor modulation are of particular interest because they may medi-

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1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; HIR, human insulin receptor; IRK, insulin receptor kinase; IRS-1, insulin receptor substrate-1; NIDDM, non–insulin-dependent diabetes mellitus; PI3, phosphatidylinositol-3; PKC, protein kinase C; PTPase, phosphotyrosine phosphatase.

ate two quantitatively important components of acquired skeletal muscle insulin resistance, i.e., the so-called metabolic insulin resistance and obesity-linked resistance.

Glucose clamp studies have shown that, among factors that cause metabolic insulin resistance in NIDDM, elevated glucose levels are of predominant importance. An inhibition of insulin receptor function most likely contributes to hyperglycemia-induced insulin resistance of the skeletal muscle. Whereas the importance of hyperglycemia-induced insulin resistance in NIDDM patients is well accepted, the role of TNF-α in the pathogenesis of insulin resistance in humans is still a matter of speculation. The hypothesis that TNF-α might play a role in obesity-linked insulin-resistance was proposed by Spiegelman and co-workers based on their observations in obese, insulin resistant animal models (18). This group has demonstrated that (*a*) the expression of TNF-α is increased in the adipose tissue of these animal models; (*b*) the insulin resistance is ameliorated when the animals are treated with a soluble TNF-α receptor construct that neutralizes the effects of TNF-α; and (*c*) the reduced tyrosine kinase activity of insulin receptors in the skeletal muscle of these animals is fully reversed after treatment with a TNF-α antagonist. In summary, these studies have strongly suggested that TNF-α might provide a link between obesity and skeletal muscle insulin resistance.

In this study, we compare the effects of high glucose and TNF-α on the insulin receptor and early components of the insulin signaling chain in cells overexpressing the human insulin receptor (HIR). The first aim of the study was to clarify whether glucose and TNF-α act through the same mechanisms for insulin signaling. We have earlier obtained evidence that hyperglycemia-induced insulin receptor inhibition involves activation of protein kinase C (PKC) (14, 15), and we therefore tested first the role of PKC in TNF-α–mediated insulin resistance. Because we found evidence that TNF-α acts through a different mechanism, we tested, in the second part of the study, whether this causes differences in downstream signaling.

Methods

Materials. Cell culture reagents and FCS were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, PMSF, benzamidine, bacitracin, Na_3VO_4, Triton X-100, Tween 20, and DTT were from Sigma (Munich, Germany). The reagents for SDS-PAGE and Western blotting were obtained from Roth (Karlsruhe, Germany) and BioRad (Munich, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). All other reagents were of the best grade commercially available. Visualization of immunocomplexes after Western blotting was performed with the nonradioactive enhanced chemiluminescence system (Amersham International, Little Chalfont, UK) and with diagnostic film (X-OMAT AR5; Eastman Kodak Co., Rochester, NY). The polyclonal anti-pTyr antibody against insulin receptor substrate-1 (IRS-1) was kindly provided by Morris F. White (Joslin Diabetes Center, Boston, MA). Rat-1 fibroblasts overexpressing the HIR, the cDNAs for the wild-type receptor, and a polyclonal rabbit antibody against insulin receptor β-subunit (CT104/anti-β-subunit) were gifts from Axel Ullrich (Max Planck Institut, Martinsried, Germany). Polyclonal antibodies against focal adhesion kinase (FAK)[125] and Shc were obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

Stable cell lines. Mouse NIH3T3 cells transfected with the HIR have been described before (22). The cells were maintained in DME supplemented with 10% FCS.

Whole-cell extract. Cells were grown to confluence in 94-mm dishes and starved in a serum-free medium (MEM Earle's salts from Gibco, supplemented with 1 mM glucose) for 12 h. Cells were incubated with or without TNF-α (0.5–10 nM) at 37°C before stimulation with 10^{-7} M insulin for 3 min. Subsequently, the cells were lysed in 700 μl lysis buffer (50 mM Hepes, pH 7.2, supplemented with 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 100 μM sodium orthovanadate, 100 mM NaF, 10 mM $Na_4P_2O_7$) on ice. The lysates were centrifuged for 10 min at 4°C with 12,000 *g*. In dephosphorylation experiments, cells were stimulated with insulin for 3 min in the absence or presence of 10 nM TNF-α. After stimulation, cells were washed with serum-free medium and lysed after different times as described above. Protein concentration was measured by BCA reagent (Pierce, Rockford, IL). 50 μg of protein was mixed with 5× Laemmli buffer, boiled at 95°C for 3 min, and analyzed by 7.5% SDS-PAGE or 7–15% gradient gels.

Plasma membrane preparation. Cells were cultured in 94-mm culture dishes until confluency and starved in a serum-free medium (MEM with Earle's salts from Gibco, supplemented with 1 mM glucose) 12 h before the experiments. Cells were then preincubated with 10 nM TNF-α, 25 mM glucose, 40 μM orthovanadate, or 35 μM phenylarsenoxid (final concentrations) at 37°C for various times (0–30 min) before insulin (10^{-7} M final concentration) was added for 3 min. Cells were washed once with ice-cold PBS, solubilized in 2 ml of a solution containing protease and phosphatase inhibitors (25 mM Hepes, 50 mM EDTA, 10 μg/ml aprotinin, 15 mM benzamidine, 54 U/ml bacitracin, 100 mM NaF, 15 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 2 mM ATP, 2 mM PMSF, 1 mM $MnCl_2$, 10% glycerol, pH 7.5) and centrifuged for 5 min at 860 *g*. Pellets were resuspended in 1 ml of the above solution and lysed by three freeze-thaw steps. Subsequently, cells were centrifuged for 30 min at 16,000 *g*, and the pellets were resuspended in 30 μl of the above buffer containing 1% (vol/vol) Triton X-100 and solubilized for 30 min at 4°C. Solubilized membranes were then centrifuged for 30 min at 14,000 *g*, and supernatants were diluted 1:2 with 25 mM Hepes, pH 7.4. After the addition of 5× Laemmli buffer (50 mM DTT) and boiling at 95°C, samples were applied to 7.5% SDS-PAGE.

Binding of ^{125}I-insulin to insulin receptor on intact cells. Monolayers in 145-mm culture dishes were grown to confluence. 18 h before the experiments, the medium was changed to DME/F12 mix without FCS. Cells were then gently scraped from the plates in binding buffer (50 mM Tris-HCl, 1% BSA, 10 mM $MgSO_4$, pH 7.5) and centrifuged for 5 min at 860 *g*. Equal amounts of cells were incubated with radioactive labeled ^{125}I-insulin (0.0035 nM) and unlabeled insulin (0.1–1,000 nM). After incubation for various times (0–120 min) at 21°C, the cell pellets were washed two times with binding buffer to remove free ligand. After various times, pellets were washed three times again with the same buffer, and the radioactivity was measured in a gamma counter. To obtain a value for the unspecific binding, cell pellets were incubated simultaneously with ^{125}I-insulins and unlabeled insulins at a final concentration of 2×10^{-5} M. All other values were corrected for this amount.

Competitive binding of unlabeled insulin at steady state was measured by overnight incubation at 4°C with ^{125}I-insulin (30,000 cpm/1,000 μl cell suspension) and various concentrations of unlabeled insulin. Incubation was performed at 4°C to inhibit endocytosis of receptor-bound insulin. Nonspecific binding was determined by incubating cells with labeled insulin plus 10^{-6} M unlabeled insulin. Pellets were washed three times with binding buffer. The amount of insulin bound to the receptor was determined in a gamma counter, and Scatchard analysis was performed.

Western blotting. Denaturated proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting (transfer buffer: 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 8.8). After transfer, the filters were blocked with 5% nonfat dried milk in Tris-buffered saline (1.5 M NaCl, 0.1 M Tris-HCl, pH 7.4) or with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100, 0.25% gelatine, pH 7.4) for 1 h. Subsequently, they were incubated with the

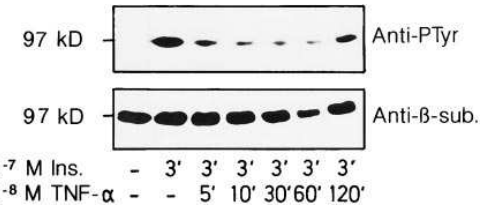

Figure 1. TNF-α effect on insulin-induced receptor autophosphorylation. Whole-cell lysate was prepared from NIH3T3-HIR B fibroblasts. Cells were incubated with 10 nM TNF-α for different times and subsequently stimulated with 10^{-7} M insulin for 3 min. Western blots were probed with antibodies against αPY (antiphosphotyrosine) or insulin receptor (anti-β-sub), respectively. A representative immunoblot is shown. This result was reproduced in four independent experiments.

first antibody (for anti-β-subunit (CT104) in TBS and 5% dried milk, for all other antibodies in NET buffer) overnight at 4°C. After incubation with the specific antibody, nitrocelluloses were washed several times with either PBS, containing 0.05% Tween 20, or NET buffer. Immunocomplexes were detected by the addition of horseradish peroxidase–coupled anti–rabbit IgG or anti–mouse IgG and addition of chemiluminescence reagent (ECL).

Results

Effect of PKC and phosphotyrosine phosphatase (PTPase) inhibitors on TNF-α–modulated receptor function. Fig. 1 shows the effect of TNF-α on NIH-3T3 cells overexpressing the HIR isoform B. TNF-α inhibits insulin-induced receptor autophosphorylation after 5 min. The inhibitory effect is not retained at longer incubation (120 min) of the cells with TNF-α. Inhibitory effects of TNF-α were obtained at concentrations between 0.5 and 10 nM (data not shown). To evaluate the potential mechanism of the inhibitory TNF-α effect, we tested the influence of different potential inhibitors. Two principal mechanisms of an inhibitory effect of TNF-α on the insulin receptor kinase activity seem possible. In analogy to catecholamine-, phorbol ester–, and hyperglycemia-induced insulin resistance, TNF-α might inhibit the insulin receptor function through stimulation of serine/threonine kinase activity.

In earlier studies we have obtained evidence that activation of PKC and phosphorylation of the insulin receptor at serine residues is involved in the hyperglycemia-induced inhibition of the IRK in these cell systems (14, 15). To test whether such a mechanism might also be related to the TNF-α effect, we investigated whether the PKC inhibitor H7 is able to suppress insulin receptor inhibition. Fig. 2, *A* and *B*, shows the TNF-α– and glucose-induced inhibition of the receptor autophosphorylation. While H7 prevents the inhibitory effect of glucose, it has no influence on the TNF-α–induced inhibition, suggesting that PKC might possibly not be involved in the inhibitory TNF-α effect. In addition, the effect of troglitazone (CS045), which acts as an insulin sensitizer, was tested. As reported earlier (23), troglitazone prevents the inhibitory effect of glucose. In contrast, TNF-α–induced IRK inhibition is not modulated by troglitazone. It was recently demonstrated that phosphatidylinositol-3 (PI3) kinase contains serine/threonine kinase activity (24, 25) and that IRS-1 is a substrate that is phosphorylated by PI3 kinase (26). To test whether PI3 kinase might be involved in the TNF-α–mediated IRK inhibition, we used the PI3 kinase inhibitor wortmannin. The effectiveness of wortmannin was tested in PI3 kinase assays performed in parallel experiments. Fig. 2, *A* and *B* shows that wortmannin did not prevent the inhibitory effect of TNF-α on the receptor autophosphorylation. Fig. 2, *A* and *B* shows further that inhibition of PI3 kinase with wortmannin can prevent neither the glucose- nor the TNF-α–induced IRK inhibition. None of the tested inhibitors (H7, troglitazone, and wortmannin) had an effect on insulin receptor phosphorylation in neither the basal nor the insulin-stimulated state.

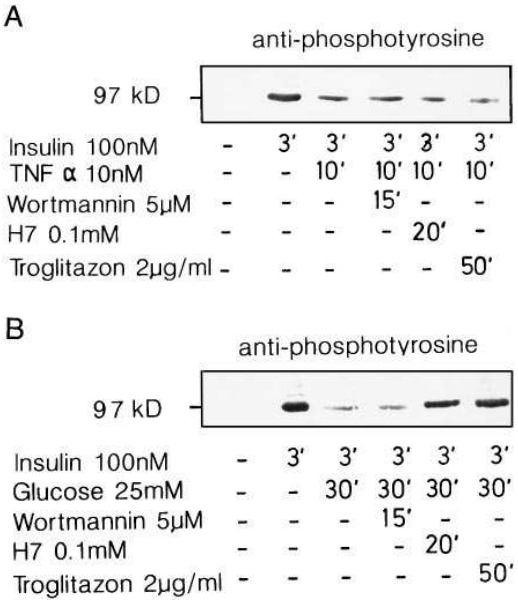

Figure 2. Effect of wortmannin, H7 and CSO45 on the TNF-α– and glucose-induced inhibition of the IRK. NIH3T3-HIR B fibroblasts were preincubated with 5 μM wortmannin, 0.1 mM H7, or 2 μg/ml troglitazone (CS045) and then incubated with either 10 nM TNF-α for 10 min (Fig. 2 *A*) or with 25 mM glucose for 30 min (Fig. 2 *B*) before stimulation with 10^{-7} M insulin for 3 min. Whole-cell extracts were prepared as described above. Tyrosine phosphorylation of the β-subunit was detected by immunoblotting with αPY antibody. A representative immunoblot is shown. These results were reproduced six times.

Another potential mechanism is stimulation of tyrosine phosphatases by TNF-α. To investigate a possible role of tyrosine phosphatases for the TNF-induced IRK inhibition, we used orthovanadate and phenylarsenoxide as tyrosine phosphatase inhibitors. Orthovanadate (Fig. 3) and phenylarsenoxide (data not shown) prevented the inhibitory effect of TNF-α, whereas they did not influence IRK inhibition of glucose. The data suggest that activation of tyrosine phosphatases might be involved in the TNF-α–induced receptor inhibition, while this is obviously not the case for glucose-induced receptor inhibition.

The data are consistent with the idea that TNF-α stimulates tyrosine phosphatases, which are able to dephosphorylate the insulin receptor. If this is a valid conclusion, an increased rate of receptor dephosphorylation in the presence of TNF-α should be expected. Fig. 4 shows a time course of dephosphorylation of the insulin receptor. *A* shows a dephosphorylation kinetic in the absence of TNF-α, and *B* in the presence of TNF-α. *C* shows the dephosphorylation kinetic of insulin receptor in cells that were insulin stimulated, washed, and then

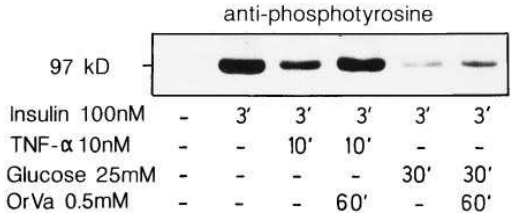

Figure 3. Effect of orthovanadate on the TNF-α– and glucose-induced IRK phophorylation. NIH3T3-HIR B cells were incubated with 10 nM TNF-α or 25 mM glucose and stimulated with 10^{-7} M Insulin for 3 min, in the presence or absence of 40 μM orthovanadate (*OrVa*). Plasma membranes were prepared as described above. The phosphorylation of the β-subunit was detected by immunoblotting with αPY antibody. The same results were reproduced in six independent experiments.

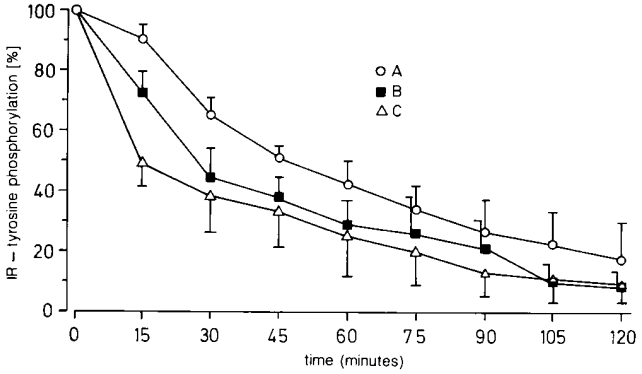

Figure 5. Kinetics of the dephosphorylation experiments shown in Fig. 4. Scanning data (arbitrary units) were obtained from four experiments. The results are shown as mean±SEM. The tyrosine phosphorylation of the insulin receptor after 3 min of insulin stimulation was taken as 100%. The curves represent: (*A*) the dephosphorylation of the insulin receptor after 3 min of insulin stimulation in the absence of TNF-α; (*B*) the insulin receptor dephosphorylation of insulin-stimulated cells in the presence of TNF-α; (*C*) the insulin recepter dephosphorylation of insulin-stimulated cells washed with serum-free medium before the addition of TNF-α.

incubated with TNF-α for different times. The dephosphorylation was quantified by scanning densitometry, and mean values are shown in Fig. 5. A significant difference in the dephosphorylation rate of the insulin receptor is seen at time points 15′ and 30′, whereas the curves are not significantly different at later time points. Furthermore, there is a difference in the initial dephosphorylation rate between conditions B and C, most likely due to the fact that dephosphorylation after preincubation with TNF-α (condition B) starts from an already lower phosphorylation level (Fig. 4). ^{125}I-Insulin binding kinetics and equilibrium binding were determined in parallel experiments. TNF-α did not modulate insulin binding; in particular, TNF-α did not enhance the rates of dissociation of insulin for the receptor. The increased rate of receptor dephosphorylation is therefore compatible with the proposed role of a PTPase stimulator.

Comparison of TNF-α to hyperglycemia effects on tyrosine phosphorylation patterns. Because these data suggest that TNF-α and hyperglycemia inhibit the insulin receptor signal by different mechanisms, we investigated whether this has consequences for postreceptor signaling. Fig. 6 shows an immunoblot in which tyrosine phosphorylation of insulin receptor and IRS-1 is detected. Both TNF-α and high glucose are able to inhibit IRS-1 phosphorylation. Thus, as far as receptor autophosphorylation and IRS-1 phosphorylation are concerned, inhibition is seen with both TNF-α and high glucose. A combination of TNF-α and glucose appears to cause a more pronounced inhibition of insulin and IRS-1 phosphorylation; however, this effect did not reach statistical significance (data not shown). The TNF-α and glucose effects on the phosphorylation of the insulin receptor and of IRS-1 were quantified by scanning densitometry. Mean values are shown in Fig. 9 *A*. Effects of TNF-α and high glucose levels on other substrates of the insulin receptor kinase in the M_r range between 40 and 100 kD are shown in Fig. 7. Some of the proteins that are tyrosine phosphorylated in response to insulin are not affected by high glucose levels and TNF-α. On the other hand, several proteins revealed a reduced tyrosine phosphorylation in the presence of TNF-α as well as high glucose. One of these proteins is identified by immunoblotting as the coupling protein Shc. In addition, TNF-α and high glucose reduce tyrosine phosphorylation of unidentified insulin receptor substrates with ~ 60 kD and 44 kD. The anti–MAP kinase antibody failed to recognize the pp44. The TNF-α–dependent inhibition of Shc phosphoryla-

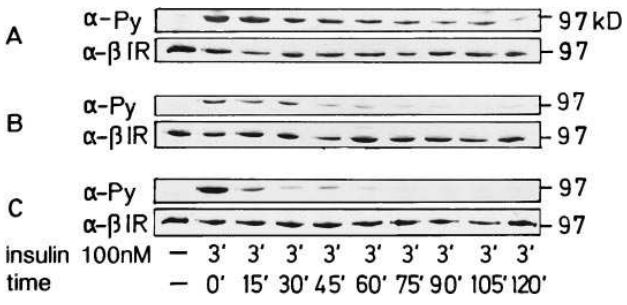

Figure 4. Dephosphorylation of the insulin-stimulated insulin receptor in absence and presence of TNF-α. NIH3T3-HIR B cells were stimulated with 10^{-7} M insulin in the absence (*A*) or presence of 10 nM TNF-α (*B*). After 3 min of insulin stimulation, the cells were washed with serum-free medium and lysed at indicated times. (*C*) The cells were incubated for 3 min with insulin in the absence of TNF-α, washed with a serum-free medium, incubated with 10 nM TNF-α, and lysed at the indicated times. Western blots were probed with αPY antibody. A representative immunoblot is shown. These results were reproduced four times.

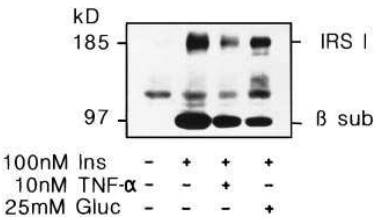

Figure 6. TNF-α and glucose effect on the IRS-1 phosphorylation. NIH3T3-HIR B cells were incubated with 10 nM TNF-α or 25 mM glucose before stimulation with 10^{-7} M insulin. Whole-cell lysates were separated on a 7.5% SDS PAGE and blotted against αPY antibody. A representative immunoblot is shown. This result was reproduced six times.

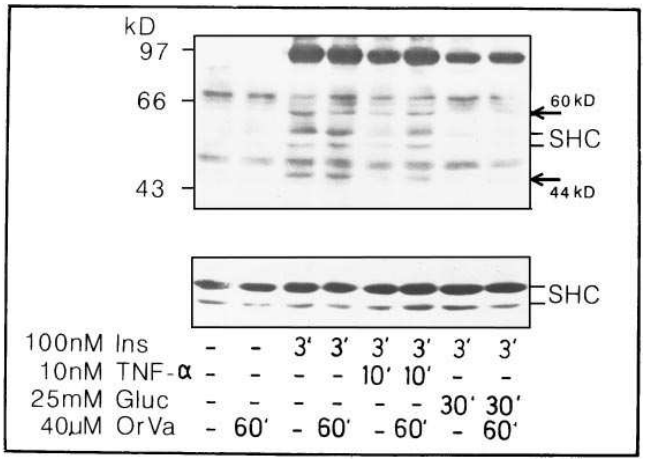

Figure 7. Tyrosine phosphorylation of endogenous proteins in NIH3T3 fibroblasts overexpressing the insulin receptor: effect of high glucose and TNF-α. NIH3T3-HIR B cells were incubated with 10 nM TNF-α or 25 mM glucose in the presence or absence of 40 μM orthovanadate (*OrVa*). (*top*) Whole-cell lysates were separated by a 7–15% SDS-PAGE and blotted against αPY antibody. (*bottom*) The same blot was reprobed with αSHC antibody. This result was reproduced four times.

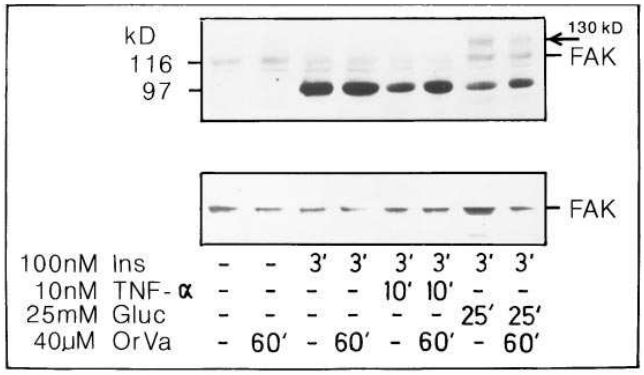

Figure 8. Influence of glucose and TNF-α on the tyrosine phosphorylation of the focal adhesion kinase FAK in NIH3T3 fibroblasts overexpressing the HIR. NIH3T3-HIR B cells were incubated with 10 nM TNF-α or 25 mM glucose in the presence or absence of 40 μM orthovanadate. (*top*) Whole-cell lysates were separated on a 7–15% SDS-PAGE and blotted against αPY antibody. (*bottom*) The same blot was detected with FAK^{125} antibody. This result was reproduced four times.

tion and unidentified proteins at 60 and 44 kD is abolished by incubation with the phosphatase inhibitor orthovanadate (Fig. 7). Again, orthovanadate did not affect the glucose-induced inhibition of Shc, pp60, and pp44 phosphorylation. Furthermore, this supports the hypothesis that TNF-α acts through inhibition of PTPases whereas glucose did not. The phosphorylation of these bands was quantified by scanning densitometry; mean values are shown in Fig. 9 *A*.

Fig. 8 shows the effect of TNF-α and high glucose on tyrosine phosphorylation of proteins in the M_r range between 95 and 150 kD. In unstimulated cells we found a 125-kD protein phosphorylated on tyrosine residues. Insulin stimulates the dephosphorylation of this protein, which was identified by immunoblotting as FAK^{125}. It was recently reported that insulin induces dephosphorylation of FAK^{125} (27). This effect of insulin is clearly shown in this immunoblot. TNF-α and high glucose modulate the insulin-induced dephosphorylation of FAK^{125} in different ways. Whereas hyperglycemia blocked insulin-dependent FAK^{125} dephosphorylation completely, TNF-α rather seemed to enhance the insulin-induced dephosphorylation of FAK^{125} (Fig. 8). The phosphorylation of FAK^{125} was quantified by scanning densitometry and mean values are shown in Fig. 9 *B*. Incubation of cells with TNF-α or high levels of glucose did not have any effect on the basal phosphorylation status of FAK^{125} or any other protein (data not shown). In addition, high glucose induced tyrosine phosphorylation of an unidentified 130-kD protein (Fig. 8), which was not affected in the presence of TNF-α. The effect of high glucose on FAK^{125} and 130-kD protein phosphorylation was not antagonized by TNF-α (data not shown).

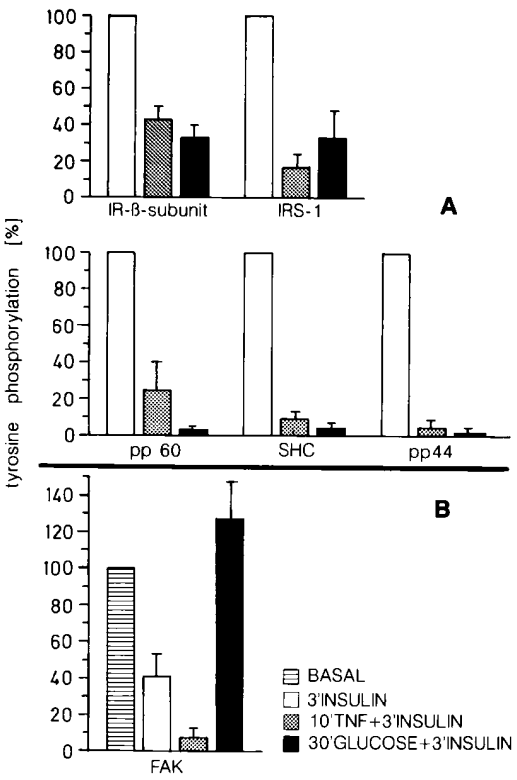

Figure 9. TNF-α and glucose effect on the tyrosine phosphorylation of different phosphoproteins in NIH3T3 fibroblasts. (*A*) The tyrosine phosphorylation of the indicated proteins after 3 min of insulin stimulation was taken as 100% (*open box*). The shaded box shows the percentage of tyrosine phosphorylation after 10 min of incubation with 10 nM TNF-α and 3 min of insulin stimulation. The solid box shows the percentage of tyrosine phosphorylation after incubation with 25 mM glucose and 3 min of insulin stimulation ($n = 4$). (*B*) The tyrosine phosphorylation of FAK in the basal state was taken as 100% (*striped box*). The tyrosine phosphorylation of FAK is shown as follows: (*open box*) after 3 min of insulin stimulation; (*shaded box*) after incubation for 10 min with 10 nM TNF-α and 3 min of insulin stimulation; (*solid box*) after incubation for 30 min with 25 mM glucose and 3 min insulin stimulation ($n = 4$).

Discussion

TNF-α effect on receptor autophosphorylation. Our results are in good agreement with the proposed role of TNF-α as a mediator of insulin resistance as it was suggested by Spiegelman

and co-workers (18–20). TNF-α–induced inhibition of HIR function can be clearly demonstrated. However, we find different time courses compared with earlier reports. TNF-α induces in our cell system a rapid but transient inhibition of the IRK activity. This result contrasts with the earlier reported data of Spiegelman and co-workers in isolated adipocytes (18–20). In adipocytes, a prolonged incubation over several days with TNF-α was required to induce an inhibition of insulin receptor signaling. However, the time course of the TNF-α effect in our cells is in good agreement with the studies of Karasik and coworkers (21) in rat hepatoma cells. This group observed an inhibitory effect of TNF-α within the first 60 min of incubation. The reason for the different time courses of TNF-α effects in different cells is unclear at present. It is conceivable that the rapid effects observed in our cells and in hepatoma cells might occur through mechanisms that are different from those that cause the chronic effects of TNF-α.

Effects of PTPase inhibitors and PCK inhibitors. The phosphorylation state of the insulin receptor and the substrate proteins in intact cells is the result of an equilibrium determined by kinase and phosphatase activity. Therefore, our experiments cannot clearly distinguish between TNF-α effects on kinase activity and phosphatase activity. However, the observation that the acute effects of TNF-α may be prevented by vanadate and phenylarsenoxide points towards a role of tyrosine phosphatase activation. The increased rate of receptor dephosphorylation in the presence of TNF-α is compatible with this idea. At present it is unclear which tyrosine phosphatase is responsible for insulin receptor dephosphorylation. Therefore, it is difficult to speculate on candidates mediating TNF-α effects. The PKC inhibitor H7 efficiently prevents the inhibitory effect of glucose on receptor phosphorylation, which is evidence that the glucose-induced inhibition of the insulin receptor involves activation of PKC and serine phosphorylation of the receptor (14). However, H7 is inefficient in blocking the effect of TNF-α. Even though PKC activation by TNF-α was described earlier and we observed a TNF-α–induced translocation of the PKC isoform ϵ (data not shown) that parallels the inhibitory kinetic, the negative results with H7 argue, rather, against a role of PKC-dependent serine phosphorylation mediating the TNF-α–induced IRK inhibition. However, this does not exclude a possible role of PKC in other TNF-α–mediated effects.

Comparison of phosphotyrosine protein patterns in TNF-α to high glucose–treated cells. Hyperglycemia- and TNF-α–induced inhibition of insulin receptor autophosphorylation is paralleled by inhibition of IRS-1 and Shc phosphorylation as well as a phosphorylation inhibition of unidentified proteins with 44 and 60 kD. These effects might be explained as direct consequences of the reduced receptor kinase activity even though direct effects of TNF-α or hyperglycemia on these substrate proteins cannot be excluded. Discordant effects of TNF-α and hyperglycemia are observed on the insulin-induced dephosphorylation of FAK^{125}. It was recently reported that insulin stimulates dephosphorylation of FAK^{125}, and it was suggested that this is due to activation of a tyrosine phosphatase (27). If dephosphorylation of FAK^{125} is due to insulin receptor–mediated activation of a PTPase, our results with glucose and TNF-α inhibition would suggest that high glucose but not TNF-α interferes with activation of this phosphatase by stimulated insulin receptor. Furthermore, we found that TNF-α inhibits insulin receptor autophosphorylation and signaling to the substrates IRS-1, Shc, pp60, and pp44. These data suggest that TNF-α acts at least at two steps in the insulin signaling chain; i.e., at the receptor level and at a postreceptor level. TNF-α might activate a phosphatase that is able to dephosphorylate FAK^{125}. It is possible that a TNF-α–activated phosphatase and an insulin receptor–activated phosphatase are identical. The conclusion that TNF-α might act through activation of a tyrosine phosphatase is also consistent with the results obtained with different pharmacological tools used in our study.

Potential consequences of different modulation of focal adhesion kinase (FAK^{125}) in hyperglycemia- and TNF-α–induced insulin resistance. Recent studies have suggested that FAK^{125} is involved in mitogenic signaling (28–30). FAK^{125} is activated by G-protein–coupled neuro peptide receptors and by different receptor and nonreceptor tyrosine kinases (30–33). The recent observation that insulin causes dephosphorylation of FAK^{125} suggests a balance between inhibitory insulin effects and different stimulatory effects. Such a balance was very recently demonstrated for insulin and PDGF. It was shown that dephosphorylation of FAK^{125} by insulin is paralleled by a decrease in the cellular content of actin stress fibers. In contrast to insulin, PDGF stimulation increased actin stress fiber content and enhanced FAK^{125} tyrosine phosphorylation (33). It is of particular interest that angiotensin II, which might play an important role in the development of vascular complications in diabetic patients as well, is a potent stimulator of FAK^{125} (32). A disturbed balance between insulin and angiotensin II might be relevant for the development of diabetic complications, e.g., nephropathy and retinopathy. If this is true, different modulation of insulin signaling to FAK^{125} in hyperglycemia- and TNF-α–induced insulin resistance might have different consequences for the long-term development of microvascular complications in NIDDM.

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