Tumor Necrosis Factor α-induced Phosphorylation of Insulin Receptor Substrate-1 (IRS-1)

POSSIBLE MECHANISM FOR SUPPRESSION OF INSULIN-STIMULATED TYROSINE PHOSPHORYLATION OF IRS-1*

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Tumor necrosis factor-α (TNF), a cytokine produced primarily by activated macrophages, mediates various detrimental manifestations seen in sepsis and cancer (1, 2). In addition to other TNF effects, it is involved in inducing insulin resistance in these disease states (3–6). Recently, accumulating data suggest that TNF may be the cause and link of obesity-induced insulin resistance (7–9). We have shown that in Fao hepatoma cells TNF leads to a decrease in insulin-stimulated tyrosine phosphorylation of the insulin receptor (IR) and its major substrate IRS-1 (10). In a rodent model of obesity, blocking TNF effects led to increased insulin sensitivity (7, 11) in parallel with correction of the impaired insulin-induced tyrosine phosphorylation of IRS-1 (11). In addition, insulin sensitizing agents of the thiazolidinediones family were able to reverse TNF-induced decrease in tyrosine phosphorylation of IRS-1 (12). These observations underline the role of impaired IRS-1 tyrosine phosphorylation in TNF impact on insulin action.

TNF acts through specific membrane receptors whose mechanism of signal transduction has not yet been fully elucidated (13–15). Several pathways activated by TNF may influence insulin-induced tyrosine phosphorylation. For example, TNF-induced activation of multiple Ser/Thr protein kinases (16–23) or inactivation of Ser/Thr phosphatases (24, 25), may inhibit kinase activity of IR and/or the tyrosine phosphorylation of IRS-1 through multisite phosphorylation on Ser/Thr residues (26–29). Alternatively, TNF inhibitory effect may be mediated through the activation of specific tyrosine phosphatases (30). This study tries to elucidate whether these mechanisms are involved in the TNF effect on insulin signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Recombinant murine TNF was kindly provided by Boehringer Ingelheim, FRG. Recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). Carrier-free [32P]orthophosphate was from Rotem Industries (Beer-Sheva, Israel) and [γ-32P]ATP from Amersham (Aylesbury, Buckinghamshire, United Kingdom). Protein G- and protein A-Sepharose were from Pharmacia Biotech Inc., and wheat germ agglutinin coupled to agarose was from Bio-Makor (Rehovot, Israel). All other reagents were from Sigma.

Antibodies—Rabbit polyclonal IR antibodies were generated against a synthetic peptide corresponding to positions 1309–1324 of the human IR (31). IRS-1 immunoprecipitation was performed with antibodies from Upstate Biotechnology Inc. (Lake Placid, NY) and immunoblotting with rabbit polyclonal antibodies raised against a synthetic peptide corresponding to the carboxyl-terminal 14 amino acids of rat IRS-1 (gift from Y. Zick, Weizmann Institute of Science, Israel). Polyclonal antibody directed against the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) was purchased from Upstate Biotechnology Inc. Phosphotyrosine (Tyr(P)) antibodies used in immunoblotting experiments were a gift from M. White (Boston, MA), while for immunoprecipitation, monoclonal Tyr(P) antibodies (PY-20) from ICN were used.

Cell Culture—Rat hepatoma Fao cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Biological Industries, Beth Haemek, Israel).

Ligand Treatment of Intact Cells—Confluent monolayers of Fao cells, grown in 60-mm dishes, were deprived of serum for 16 h prior to each experiment. The medium was removed, and cells were incubated with or without 5 nM TNF in serum-free medium for the indicated time interval. Fao cells were then incubated without or with 100 nM insulin for 1 min at 37 °C. The reaction was terminated by removing the medium and freezing cell monolayers with liquid nitrogen. In some experiments, cells were preincubated with 50 μM sodium orthovanadate.
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or 10–50 nM staurosporine for 16 h or 30 min, respectively. Cells were solubilized at 4°C with 0.4 ml/dish of buffer A (50 mM Hepes, pH 7.6, 150 mM sucrose, 2 mM sodium orthovanadate, 80 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium EDTA, 2 mM sodium EDTA, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor). The solubilized cells were scapped and sedimented by centrifugation for 15 min at 4°C at 12,000 × g. Aliquots of the supernatants were normalized for protein, mixed with concentrated (5×) Laemmli sample buffer, boiled for 5 min, and resolved on 7.5% or 6% SDS-PAGE under reducing conditions.

Immunoprecipitation from Fao Cell Extracts—Cells were treated with ligands as described above and solubilized with buffer B (20 μl/ml) without SDS. Cell extracts were immunoprecipitated for 4 h at 4°C with antibodies to IRS-1 (15 μg/ml) or Tyr(P) (10 μg/ml) preadsorbed on protein G-Sepharose beads or with antibodies to IR (1:200 dilution) coupled to protein A-Sepharose beads. The immunocomplexes were pelleted by centrifugation at 12,000 × g and washed twice with buffer B and twice with buffer C containing 0.1% Triton X-100. The pellets were then suspended in Laemmli sample buffer and resolved on 7.5% or 6% SDS-PAGE.

Western Immunoblotting—Electrophoretic transfer of proteins to nitrocellulose papers was carried out as described previously (10). Blots were incubated with the appropriate antibodies, and proteins were detected by Enhanced Chemiluminescence using either horseradish peroxidase-labeled Protein A or horseradish peroxidase-labeled antibody mouse IgG (Amersham).

32P Labeling of Fao Cells—Fao cells in phosphate-free RPMI medium were labeled for 3 h at 37°C with [32P]orthophosphate (0.1 mCi/ml). Cells were then incubated for 20 min without or with TNF or calyculin A or for 1 min with 100 nM insulin. Cells were rapidly washed in ice-cold phosphate-free medium and solubilized for 40 min in 0.1 M of buffer C (20 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 2 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor). After centrifugation at 10,000 × g for 15 min, supernatants were subjected to immunoprecipitation at 4°C with IRS-1 or Tyr(P) antibodies preadsorbed on protein G-Sepharose beads for 4 h. The immune pellets were washed twice with buffer C, twice with buffer C without Nonidet P-40 supplemented with 0.5 mM NaCl, and once with buffer C. Immunoprecipitated proteins were treated with Laemmli sample buffer, boiled for 5 min, and resolved on 7.5% SDS-PAGE, and exposed to autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described by Boyle et al. (32). Briefly, immunoprecipitated [32P]phosphate-labeled IRS-1 was separated on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The IRS-1 phosphorylated bands were excised from the membrane and hydrolyzed in 6 N HCl at 110°C for 1 h. The hydrolysates were dried by Speed-vac, or once with buffer C. Immunoprecipitated proteins were treated with Laemmli sample buffer and resolved on 7.5 or 6% SDS-PAGE.

X-100, 50 mM Hepes buffer, pH 7.5, in the presence of 5 μM [γ-32P]ATP, 10 mM MgCl2, and 100 mM insulin. Autophosphorylation was terminated by adding 10 mM EDTA and 1 mM ATP. Immunoprecipitates were washed and resuspended in 25 mM Hepes buffer, pH 7.5, containing 5 mM EDTA, 1 mM dithiothreitol and protease inhibitors. Particulate and cytosolic fractions from Fao cells (50 μg) were subjected to SDS-PAGE to evaluate protein-tyrosine phosphatase activity. After incubation for 20 min at 30°C the dephosphorylation reaction was terminated by adding Laemmli sample buffer and boiling for 5 min. Samples were analyzed by SDS-PAGE and autoradiography. The degree of dephosphorylation was evaluated by densitometry and expressed as density of the IR β-subunit band after incubation with buffer alone or with the appropriate hepatic fraction.

RESULTS

TNF Effect on Insulin-stimulated Tyrosine Phosphorylation—Fao cells were treated for 60 min with 5 nM TNF prior to stimulation for 1 min by 100 nM insulin. Cell extracts and Tyr(P) antibody immunoprecipitates were analyzed by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with Tyr(P) antibodies. Insulin treatment caused tyrosine phosphorylation of proteins at 95–100, 180–185 kDa (Fig. 1, upper and lower left panels, lane b). Preincubation with TNF alone did not induce any tyrosine phosphorylation of these bands (Fig. 1, upper and lower left panels, lane c). Moreover, TNF treatment led to a delay in the electrophoretic mobility of the 185-kDa band. The identity of the lower component of the 180–185-kDa broad band as IRS-1 (10, 33) and the 95-kDa band as the β-subunit of the IR was confirmed by immunoprecipitation with their respective specific antibodies prior to electrophoresis. (Fig. 1, upper and lower right panels). TNF inhibitory influence was more pronounced on the insulin-induced tyrosine phosphorylation of IRS-1 than on IR β-subunit and was apparent already after 15 min of incubation (Fig. 2). Insulin-stimulated increase in receptor tyrosine kinase activity, when assessed in vitro with an artificial substrate, was not significantly decreased after treatment with TNF (Fig. 3).

TNF Effect on the Interaction of IRS-1 and PI 3-Kinase—Cells were incubated at 37°C without or with 5 nM TNF for different periods of time. Insulin was also added (100 nM final concentration) to the indicated plates for the final 10 min at 37°C. Cells were lysed and then immunoprecipitated with IR antibody for 4 h at 4°C, and IR tyrosine kinase activity was determined as described by Chin et al. using poly(Glu,Tyr) (4:1) as an exogenous substrate (28). To identify the amount of receptor in each reaction was comparable, the immunoprecipitates were electrophoresed on SDS-PAGE and immunoblotted with antibodies to IR as described above.

Preparation of Subcellular Fractions from Fao Cells—Cells were incubated at 37°C with or without 5 nM TNF for different periods of time, rinsed with phosphate-buffered saline, and homogenized in a Dounce homogenizer in buffer D (10 mM Hepes, pH 7.0, 5 mM EDTA, 0.1% 2-mercaptoethanol, 250 mM sucrose, 0.5 mM benzamidone, 1 mM phenylmethylsulfonfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1% Nonidet P-40 (0.1% Nonidet P-40). Homogenates were centrifuged at 100,000 × g for 60 min, and the supernatants after this procedure were retained as the “cytosolic fraction.” Pellets were resuspended in buffer D supplemented with 1% Triton X-100 and were retained as the “particulate fraction.”

Dephosphorylation Assay—IR was purified from homogenate rat liver on wheat germ agglutinin-agarose as described previously (34), and solubilized with immunoprecipitation with specific IR antibodies coupled to protein A-Sepharose for 4 h at 4°C. The immunocomplexes were allowed to undergo insulin-induced autophosphorylation in 0.1% Triton X-100.

FIG. 1. Effect of TNF on insulin-stimulated protein tyrosine phosphorylation in intact Fao cells. Fao cells were incubated with 5 nM TNF for 1 h and stimulated with 100 nM insulin for 1 min at 37°C. Cell extracts were subjected to immunoprecipitation with antibodies to phosphotyrosine (pTyr Ab), to IRS-1 (IRS-1 Ab), or to the IR β-subunit (IR-Ab). Total cell extracts and immune pellets were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose papers, and phosphotyrosine-containing proteins were probed with Tyr(P) antibodies. These proteins were visualized using a chemiluminescent peroxidase substrate and autoradiography.

TNF increases phosphorylation of IRS-1.
induced association between IRS-1 and one of these enzymes, PI 3-kinase. Preincubation with TNF prior to insulin stimulation led to a decrease in the amount of the 85-kDa subunit of PI 3-kinase that coprecipitated with IRS-1 (Fig. 4, right panel). This decrease did not result from a reduction in IRS-1 content (Fig. 4, left panel) but rather from a decrease in its tyrosine phosphorylation state (Fig. 4, middle panel).

Role of Tyrosine Phosphatases in TNF Effect—To evaluate protein-tyrosine phosphatase involvement we reexamined TNF effect on insulin-induced phosphorylation in the absence (Fig. 5, left six lanes) or presence (Fig. 5, right six lanes) of vanadate, a protein-tyrosine phosphatase inhibitor. TNF inhibitory effect was unchanged by preincubation with vanadate under conditions where vanadate markedly increased both IRS-1 and IR tyrosine phosphorylation through inactivation of protein-tyrosine phosphatases. In addition, TNF did not alter either cytosolic or particulate protein-tyrosine phosphatase activity as measured with the tyrosine-phosphorylated β-subunit of the IR as a substrate (Fig. 6).

TNF-induced Ser/Thr Phosphorylation of IRS-1—The delay in electrophoretic mobility of IRS-1 in TNF-treated cells and its decreased tyrosine phosphorylation can be attributed to an increase in Ser/Thr phosphorylation of this protein. To evaluate this possibility 32P-labeled Fao cells were stimulated with TNF, and cell extracts were subjected to immunoprecipitation with IRS-1 antibody to determine total phosphorylation of this protein (Fig. 7, upper left panel) and in parallel with Tyr(P) antibodies to detect any TNF-induced tyrosine phosphorylation of IRS-1 and of IR (Fig. 7, upper right panel). IRS-1 was phosphorylated already in the basal state (Fig. 7, upper left panel, first lane) but not on tyrosine residues (Fig. 7, upper right panel, third lane). TNF alone led to a 60% increase in total phosphorylation of IRS-1 as well as to a mild delay in its electrophoretic mobility (Fig. 7, upper left panel, second lane).
Insulin but not TNF led to tyrosine phosphorylation of IRS-1 and the IR β-subunit, implying that the increased phosphorylation with TNF stimulation is on Ser/Thr residues (Fig. 7, upper right panel). This suggestion was further supported by phosphoamino acid analysis showing only phosphoserine in IRS-1 from cells stimulated with TNF (Fig. 7, lower panel).

Comparison of TNF and Ser/Thr Phosphatase Inhibitors Effects—It has recently been shown that inhibition of Ser/Thr phosphatases increases IRS-1 Ser/Thr phosphorylation in 3T3-L1 adipocytes (29). In our system, 20-min incubation with calyculin A, a specific Ser/Thr phosphatase inhibitor, mimicked the effect of TNF and led to an apparent increase in the degree of IRS-1 phosphorylation and to a decrease in its electrophoretic mobility (Fig. 7, upper left panel, third lane). We further compared the effects of both calyculin A and okadaic acid, another inhibitor of the protein phosphatases 2A and 1, to the TNF effect on IRS-1 tyrosine phosphorylation. Preincubation of Fao cells with these agents decreased insulin-induced tyrosine phosphorylation of IRS-1 as did incubation with TNF (Fig. 8). Moreover, both treatments led to a comparable lag in the electrophoretic mobility of IRS-1. A dose- and time-dependent effect of calyculin A inhibition was demonstrated (Fig. 8, lanes d–h).

Staurosporine and TNF Effect—To evaluate whether TNF-induced Ser/Thr phosphorylation of IRS-1 is mediated by activation of the calcium-phospholipid-dependent kinase, protein kinase C, the original experiment depicted in Fig. 1 was repeated in Fao cells pretreated with staurosporine, a protein kinase C inhibitor (36). Incubation with 50 nM of staurosporine

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this was linked to a decrease in IRS-1 electrophoretic mobility due to phosphorylation on Ser/Thr residues (29). Our similar data in Fao cells confirm the uniquely parallel pattern induced by TNF and Ser/Thr phosphatase inhibitors on IRS-1 phosphorylation and suggest that TNF-induced increase in serine phosphorylation may occur through inactivation of these phosphatases.

An alternative pathway leading to increased Ser/Thr content of IRS-1 may be TNF activation of serine kinases. Indeed, TNF has been shown to activate multiple serine kinases including protein kinase C, protein kinase A, β-casein kinase, and mitogen-activated protein (MAP) kinases (16–23). Protein kinase C activation and/or overexpression has been shown to hamper insulin-induced tyrosine phosphorylation as well as insulin action in intact cells (26–28). In addition, TNF activates protein kinase C in several cell lines (22, 23), making it a candidate for conveying the TNF effect. The possibility to down-regulate protein kinase C was not utilized in our system, since phorbol esters can potentially lead to shedding of TNF receptors (40). Use of staurosporine, a potent nonspecific protein kinase C inhibitor, augmented IRS-1 tyrosine phosphorylation but did not alter TNF effect. It is noteworthy that in FS-4 fibroblasts TNF induction of heat shock protein 28 phosphorylation was also not inhibited by staurosporine and other protein kinase C inhibitors, and the increased serine phosphorylation of this protein was attributed to inhibition of serine phosphatases (41). Other well characterized kinases whose activity was shown to be increased by TNF in several cells including hepatocytes are the MAP kinase isoforms (17–20). Recently, IRS-1 phosphorylation by MAP kinase was suggested, making it a plausible candidate to be involved in TNF-induced phosphorylation of IRS-1 (34, 35). Moreover, in 3T3-L1 adipocytes Kletzien et al. (12) underlined the complex potential interactions between the multiple MAP kinase species, TNF and insulin. While TNF obliterated the insulin-induced phosphorylation and activation of p44 MAP kinase, it stimulated a p38 MAP kinase.

Serine phosphorylation of IRS-1 may not be the only mechanism mediating TNF inhibition of insulin-induced tyrosine phosphorylation. In this study obliteration of protein-tyrosine phosphatase activity by vanadate did not reverse TNF inhibition, implying that protein-tyrosine phosphatases are not involved in this effect. Moreover, TNF did not alter protein-tyrosine phosphatase activity in Fao cells when measured in vitro using the phosphorylated IR β-subunit as a substrate.

In summary, the phenomenon investigated in this work constitutes an interesting example for the complex cross-talk between cytokines and growth factors that operate through receptors with intrinsic tyrosine kinase activity. This may represent one of the delicate physiological regulatory mechanisms of insulin action that when exaggerated may lead to insulin resistance. The immediate clinical significance of this interaction was recently underlined by Hotamisligil and Spiegelman (10), who suggested that TNF is the cause and link in obesity-induced insulin resistance. According to their hypothesis, TNF overproduction by adipose tissue inhibits insulin action and is connected to the development of non-insulin-dependent diabetes mellitus. Better understanding of the cascade of events involved in obesity-linked non-insulin-dependent diabetes mellitus may form the basis for reversal of this prevalent deleterious disease.

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