Communication

Tumor Necrosis Factor Promotes Phosphorylation and Binding of Insulin Receptor Substrate 1 to Phosphatidylinositol 3-Kinase in 3T3-L1 Adipocytes*

(Received for publication, August 10, 1995, and in revised form, November 17, 1995)

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Chronic incubation of 3T3-L1 adipocytes with tumor necrosis factor (TNF) induces a state of insulin resistance characterized by a diminished ability of insulin to induce phosphorylation of the β subunit of its own receptor and insulin receptor substrate 1 (IRS-1). When adipocytes are briefly pretreated with TNF and then stimulated with insulin, tyrosine phosphorylation of IRS-1 increases above the level induced by insulin alone. By itself, TNF induces the time-dependent tyrosine phosphorylation of proteins in 3T3-L1 adipocytes. Among these is IRS-1, a docking protein with tyrosine phosphorylation sites that bind cytoplasmic signaling molecules that contain Src homology 2 (SH2) domains. TNF stimulation of 3T3-L1 adipocytes also promotes the association of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) with IRS-1 and also its tyrosine phosphorylation. In murine 3T3-L1 adipocytes, IRS-1 and PI 3-kinase phosphorylation and the association of these proteins are promoted by murine TNF, which interacts with the type 1 and type 2 TNF receptors. Human TNF, which binds to the murine type 1 TNF receptor selectively, also promotes IRS-1 phosphorylation and binding of IRS-1 to PI 3-kinase. This is the first demonstration that a member of the TNF/nerve growth factor receptor superfamily can use an IRS-1 signaling system as a component of its cellular response and provides a mechanism through which TNF receptors may engage downstream elements in signaling pathways.

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EXPERIMENTAL PROCEDURES

Materials—Recombinant murine TNF (mTNF) was from R & D Systems (Minneapolis, MN). Recombinant human TNF (hTNF) was a gift from Genentech (San Francisco, CA). Horseradish peroxidase-conjugated monovalent anti-phosphotyrosine antibody (RC20) and agarose-conjugated monoclonal antiphosphotyrosine antibody (PY20) were from Transduction Laboratories (Lexington, KY). Polyclonal antiserum to the 85-kDa regulatory subunit of rat phosphatidylinositol 3-kinase (PI 3-kinase) (PI 3-kinase) was from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antiserum to human IRS-1 and monoclonal antiserum to the murine insulin receptor were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Culture—3T3-L1 fibroblasts from the American Type Culture Collection were grown in 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, l-glutamine, penicillin (500 units/ml), and streptomycin (100 units/ml) in a humidified incubator under 5% CO2 at 37°C. Differentiation to adipocytes was conducted as described (6). Maximal adipose conversion was achieved 10–12 days after initiation of differentiation.

Treatment of 3T3-L1 Adipocytes with TNF—For chronic cytokine treatments, fully differentiated adipocytes maintained in DMEM, 10% fetal bovine serum and 100 ng/ml insulin were incubated in the absence or presence of 0.1 nM TNF for 5 days. The cells were then cultured in serum-free DMEM containing 0.5% bovine serum albumin, with or without 0.1 nM TNF, for 16 h and stimulated with 100 nM insulin for 5 min. For acute cytokine treatments, 3T3-L1 adipocytes were cultured in DMEM containing 1% fetal bovine serum for 38 h and then in serum-free DMEM containing 0.5% bovine serum albumin for 5 h before treatment with TNF as described in the legends to Figs. 2–5.

Immunoprecipitation and Immunoblotting—After treatments, 3T3-L1 adipocytes were washed twice with ice-cold phosphate-buffered saline and lysed by incubation in 50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM dithiothreitol, PAGE, polyacrylamide gel electrophoresis; TNFR-1, 55-kDa type 1 TNF receptor; TNFR-2, 75-kDa type 2 TNF receptor.
sodium pyrophosphate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml peptatin A, and 1 mM sodium orthovanadate for 30 min at 4°C. The lysate was centrifuged (150,000 × g, 1 h), and the infranatant was removed from the fat and passed through a 22-μm filter. Extracts (1–1.5 mg of protein) were shaken with antiserum overnight at 4°C, 40 μl of a slurry of protein G-plus/protein A-agarose was added, and incubation was continued for 2 h at 4°C. The incubate was centrifuged briefly at 10,000 × g, and the agarose was washed with 20 mM Heps, pH 7.0, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, 1 mM sodium orthovanadate and recentrifuged. This was repeated four times after which Laemmli medium was added, and the agarose was heated at 100°C for 5 min. Immunoprecipitated proteins were fractionated on 7.5% acrylamide gels and blotted to Immobilon-P (Millipore) during an overnight transfer. Immunoblots were blocked by incubation in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 1% bovine serum albumin for 2 h, incubated with TBST containing 1% bovine serum albumin and the primary antibody for 1–2 h at room temperature, washed three times with TBST, and in experiments not using a primary antibody conjugated to horseradish peroxidase, incubated with anti-mouse or anti-rabbit secondary antibodies for 1 h. The enhanced chemiluminescent detection system was used for protein detection. To probe immunoblots with a second antiserum, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50°C. The blots were then incubated with antiserum and processed as described above.

RESULTS

Chronic incubation of 3T3-L1 adipocytes with mTNF has been reported to inhibit the ability of insulin to induce tyrosine phosphorylation of its receptor and IRS-1 (5). We have confirmed that TNF was capable of inducing a state of insulin resistance in the 3T3-L1 adipocytes employed in the present study. To accomplish this, 3T3-L1 adipocytes incubated for 5 days in the absence or presence of 0.1 nM mTNF were tested for responsiveness to insulin, based on the ability of insulin to induce tyrosine phosphorylation of the β subunit of its own receptor and of IRS-1. Since high concentrations of TNF induce dedifferentiation of adipocytes (3, 7), experiments testing for chronic effects of TNF on insulin signaling were conducted with a low concentration of the cytokine (0.1 nM TNF) that causes no general reduction of mRNAs associated with the adipocyte phenotype (3). As shown in Fig. 1, mTNF inhibited the ability of insulin to promote the tyrosine phosphorylation of its own receptor and of IRS-1 (Fig. 1A), without affecting expression of either the insulin receptor or IRS-1 (Fig. 1B). Quantitative analysis using a densitometer showed that, relative to control, TNF diminished the ability of insulin to promote phosphorylation of its receptor and IRS-1 by 46 and 61%, respectively.

Acute incubation of 3T3-L1 adipocytes with mTNF did not inhibit the ability of insulin to promote IRS-1 phosphorylation; rather, when adipocytes were briefly treated with mTNF for various times and then stimulated with insulin, tyrosine phosphorylation of IRS-1 and a group of proteins that immunoprecipitate with IRS-1 increased above the level induced by insulin alone (Fig. 2A). Quantitative analysis (Fig. 2B) showed that the maximal effect on IRS-1 phosphorylation was observed when cells were treated with mTNF for 15 min, insulin being present during the last 3 min of incubation. One explanation for this observation is that mTNF may induce the tyrosine phosphorylation of proteins in 3T3-L1 adipocytes, including IRS-1.

To determine whether tyrosine phosphorylation of cellular proteins is induced by TNF, phosphoproteins from control and mTNF-stimulated 3T3-L1 adipocytes were immunoprecipitated with antiserum to phosphotyrosine, fractionated by SDS-PAGE, and analyzed by Western blotting using antiphosphotyrosine antibodies. As illustrated in Fig. 3, mTNF promoted the tyrosine phosphorylation of a group of cellular proteins. Phosphorylation of some proteins was evident within 5 min, reached a maximal level at 15 min, and then diminished. The M<sub>v</sub> values of several of the tyrosine-phosphorylated proteins were consistent with those of signaling molecules implicated in the actions of some growth factors. Among these were 180-kDa IRS-1 and the 85-kDa subunit of PI-3 kinase.

To test whether IRS-1 is involved in TNF signaling, 3T3-L1 adipocytes were incubated with 5 nM mTNF for various times at 37°C. IRS-1 in cell lysates was then immunoprecipitated and separated from other proteins by SDS-PAGE. The phosphorylation of IRS-1 isolated from control and mTNF-treated cells was then evaluated by Western blotting using antiserum to phosphotyrosine. As illustrated in Fig. 4A, phosphorylation of IRS-1 and proteins that associate with IRS-1 was stimulated by mTNF without any effect on the cellular content of IRS-1 (Fig. 4B). Analysis of autoradiographs with a densitometer showed that IRS-1 phosphorylation induced by mTNF reached a maximal level after 15 min and then diminished (Fig. 4C). TNF also appeared to promote a very modest increase in the tyrosine phosphorylation of the β subunit of the insulin receptor (data not shown).

We next tested whether the phosphorylation induced by mTNF led to coupling of IRS-1 with cytoplasmic signaling molecules that contain SH2 domains, which bind phosphoty-
FIG. 3. **TNF-promoted tyrosine phosphorylations.** 3T3-L1 adipocytes were treated with 5 nM mTNF at 37 °C for various times. Phosphorylated proteins were immunoprecipitated from cell lysates using antiserum to phosphotyrosine, fractionated by SDS-PAGE, and transferred to Immobilon-P. The Western blot was incubated with antiserum to phosphotyrosine.

FIG. 4. **TNF-promoted phosphorylation of IRS-1.** A, 3T3-L1 adipocytes were treated with 5 nM mTNF at 37 °C for various times. Proteins in cell lysates were immunoprecipitated with antiserum to IRS-1, fractionated by SDS-PAGE, and transferred to Immobilon-P. The Western blot was incubated with antiserum to phosphotyrosine. B, the Western blot was stripped and reprobed with antiserum to IRS-1. C, the Western blot was scanned with a densitometer, and the effect of TNF on IRS-1 phosphorylation is reported in arbitrary scanning units.

One such is PI 3-kinase, a heterodimer composed of 85-kDa regulatory and 110-kDa catalytic subunits (10). To determine whether IRS-1 associates with PI 3-kinase during stimulation with mTNF, IRS-1 was immunoprecipitated from control 3T3-L1 adipocytes or adipocytes stimulated with TNF or insulin for 15 min. After SDS-PAGE, Western blots were probed with antiserum to the 85-kDa regulatory subunit of PI 3-kinase. Also, proteins in a cell lysate were immunoprecipitated with antiserum to PI 3-kinase, fractionated by SDS-PAGE, and blotted to Immobilon-P, which was probed with antiserum to PI 3-kinase, which definitively identified the regulatory subunit of the enzyme. B, mTNF-promoted phosphorylation of PI 3-kinase. 3T3-L1 adipocytes were incubated in the absence or presence of 5 nM mTNF for 15 min at 37 °C. Proteins in cell lysates were immunoprecipitated with antiserum to PI 3-kinase, fractionated by SDS-PAGE, and transferred to Immobilon-P, which was probed with antiserum to phosphotyrosine. Bottom, phosphorylations induced by hTNF. Cells were incubated with 5 nM hTNF or 100 nM insulin for 15 min at 37 °C. C, association of IRS-1 and PI 3-kinase was examined as described in the legend to A. D, tyrosine phosphorylation of IRS-1 was evaluated as described in the legend to Fig. 4.

**DISCUSSION**

The tyrosine phosphorylation of growth factor receptors leads to their interaction with cytoplasmic signaling molecules that contain a conserved motif of about 100 amino acids called the SH2 domain (8, 9). The SH2 domain binds phosphorylated tyrosine and promotes interactions of phosphorylated receptors with signaling molecules. Some receptors, such as those for insulin and IGF-1, do not interact strongly with most of the SH2 proteins known to be components of their signaling pathways (17). These receptors stimulate tyrosine phosphorylation of an accessory protein, IRS-1, which serves as an intermediary and interacts with signaling proteins that contain SH2 domains and play a role in promoting cellular responses.

The present study is the first to demonstrate that members of the TNF/nerve growth factor receptor superfAMILY utilize a signaling mechanism employing IRS-1. TNF acutely induces tyrosine phosphorylation of IRS-1 and its association with other proteins. One of the proteins with which IRS-1 binds in response to TNF stimulation is PI 3-kinase, a dual specificity lipid and serine kinase consisting of an 85-kDa regulatory subunit that contains two SH2 domains and a 110-kDa cata-
lytic subunit (10, 11). Activation of PI 3-kinase is believed to be initiated by association of its regulatory subunit with phosphotyrosines in activated mitogen receptors or in accessory proteins such as IRS-1 (11, 25). These interactions promote relocalization of PI 3-kinase to the plasma membrane, bringing the enzyme closer to its substrates, which may account for activation of the enzyme (11). Once activated, PI 3-kinase catalyzes production of phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (26). The functions of these lipid second messengers are unknown; however, they may be important in intracellular protein trafficking and activation of specific isoforms of protein kinase C (27, 28). Activation of PI 3-kinase is associated with insulin-induced glucose transport and with the growth stimulatory activity of mitogens (29, 30). PI 3-kinase also promotes the serine phosphorylation of IRS-1 (31); however, it is not known whether this activity relates to the ability of TNF to induce insulin resistance during long term incubation with 3T3-L1 adipocytes. In any event, our observations show that TNF induces an acute insulin- or growth factor-like response in cells. This observation may, in part, explain the proliferative response induced by TNF in fibroblasts and some transformed cells (32–34).

The responses induced by TNF through IRS-1 need not be identical to those of other factors that utilize this docking protein. IRS-1 contains at least 20 potential tyrosine phosphorylation sites, at least eight of which are phosphorylated in response to insulin (35). Differences in the specificity of the as yet unidentified tyrosine kinase activated by TNF, compared with those utilized by insulin and other growth factors and cytokines, may result in phosphorylation of distinct sites in IRS-1, binding to a unique array of signaling proteins and induction of distinct responses.

TNF initiates its actions by binding to either of two receptors (15). The extracellular domains of the receptors share homologies with one another and with other members of the TNF/nerve growth factor receptor superfamily (36). The intracellular domains do not display sequence similarities, which accounts for the distinct responses induced by the receptors; TNFR-1 induces cytotoxicity, fibroblast proliferation, antiviral responses, and the host defense against pathogens, whereas TNFR-2 plays a role in cytotoxicity that is still being defined, inhibits early hematopoiesis, and is involved in the proliferation of monocytes and T cells (2, 37–42). Neither receptor contains protein tyrosine kinase activity or any motif, which suggests a mechanism through which signals can be transmitted into the cell (15, 36). The present study demonstrates that TNFR-1 activates a tyrosine kinase that promotes phosphorylation of IRS-1 and its engagement with cytoplasmic signaling molecules; we cannot exclude the possibility that TNFR-2 also uses this mechanism.

Elaboration of TNF is associated with the insulin resistance of cancer, infection, and non-insulin-dependent diabetes mellitus (19). The ability of TNF to impair signaling through the insulin receptor and IRS-1 during chronic treatments suggests sites at which metabolic abnormalities may initiate. Acutely, TNF activates tyrosine kinase activity and induces phosphorylation of IRS-1 and its interaction with PI 3-kinase. These observations suggest a mechanism through which TNF receptors can couple with cytoplasmic signaling molecules, thereby forming complexes that may promote responses to TNF.