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Tumor Necrosis Factor (TNF)-α Inhibits Insulin Signaling through Stimulation of the p55 TNF Receptor and Activation of Sphingomyelinase*

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Tumor necrosis factor (TNF)-α plays a central role in the state of insulin resistance associated with obesity. It has previously been shown that one important mechanism by which TNF-α interferes with insulin signaling is through the serine phosphorylation of insulin receptor substrate-1 (IRS-1), which can then function as an inhibitor of the tyrosine kinase activity of the insulin receptor (IR). However, the receptors and the signaling pathway used by TNF-α that mediate the inhibition of IR activity are unknown. We show here that human TNF-α, which binds only to the murine p55 TNF receptor (TNFR), is as effective at inhibiting insulin-dependent tyrosine phosphorylation of IR and IRS-1 in adipocytes and myeloid 32D cells as murine TNF-α, which binds to both p55 TNFR and p75 TNFR. Likewise, antibodies that are specific agonists for p55 TNFR or p75 TNFR demonstrate that stimulation of p55 TNFR is sufficient to inhibit insulin signaling, though a small effect can also be seen with antibodies to p75 TNFR. Exogenous sphingomyelinase and ceramides, known to be formed by activation of p55 TNFR, inhibit IR and IRS-1 tyrosine phosphorylation and convert IRS-1 into an inhibitor of IR tyrosine kinase *in vitro*. Myeloid 32D cells expressing IR and IRS-1 are sensitive to this inhibition, but cells expressing IR and IRS-2 are resistant, pointing to an important difference in the biological function between IRS-1 and IRS-2. These data strongly suggest that TNF-α inhibits insulin signaling via stimulation of p55 TNFR and sphingomyelinase activity, which results in the production of an inhibitory form of IRS-1.

Insulin resistance is defined as a smaller than normal response to a given dose of insulin. It is also a ubiquitous correlate of obesity and a central component of non-insulin-dependent diabetes mellitus, likely representing a major causal link between these two disorders (1). Insulin resistance has been implicated in a wide range of pathological states such as dyslepidemia, atherosclerosis, and cardiovascular disorders (2). Since as many as 30% of the adult population in the United States are obese, a better understanding of insulin resistance is an important scientific and medical goal. However, our understanding of the molecular basis of the close association between obesity and insulin resistance is very incomplete.

Several lines of evidence indicate that TNF-α[1] plays a central role in the insulin resistance observed in obesity (3). Adipocytes from most if not all obese animals overexpress TNF-α relative to their lean counterparts (4–6). This observation has been extended to humans where expression of TNF-α is in strong positive correlation with the degree of obesity and the level of hyperinsulinemia, often taken as an indirect measure of insulin resistance (7, 8). More recently, using reverse transcriptase-polymerase chain reaction, TNF-α has also been shown to be overexpressed in muscle during obesity (9). TNF-α plays a causal role in the insulin resistance of experimental animals since neutralization of TNF-α in obese rats increases their insulin sensitivity (4), probably due to the concomitant increase in the tyrosine kinase activity of the IR in adipose tissue and muscle (10). TNF-α has been shown to interfere with insulin signaling by inhibiting IR tyrosine kinase activity in cell culture (11, 12). At the molecular level, it has recently been shown that TNF-α induces serine phosphorylation of IRS-1 (13, 14), and this modified form of IRS-1 can function as an inhibitor of the IR tyrosine kinase activity *in vitro* and in intact cells (13).

Despite the considerable evidence for a key role for TNF-α, the early steps by which this cytokine inhibits insulin signaling are unknown. TNF-α binds with high affinity to two receptors that possess totally different intracellular domains (15). These receptors, p55 TNFR and p75 TNFR, are glycoproteins with a single transmembrane domain. Both proteins are devoid of any enzymatic activity but can associate with several different intracellular proteins (16–20). Although it seems clear that associated proteins play a role in the signal transduction by these receptors, their precise functions are unknown.

The ability of p55 TNFR and p75 TNFR individually to mediate TNF-α signaling has been an area of active study. p75 TNFR binds TNF-α with a higher affinity and with a higher dissociation rate than p55 TNFR (K_d of 100 pM *versus* 500 pM and $t_{1/2}$ of 10 min *versus* 3 h) (15). The p55 TNFR has been implicated in many biological processes including lipopolysaccharide- and D-galactosamine-induced lethality and production of interleukin-6 and granulocyte macrophage-colony-stimulat-

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[1] The abbreviations used are: TNF, tumor necrosis factor; IR, insulin receptor; IRS, insulin receptor substrate; TNFR, TNF receptor; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis.

ing factor in fibroblasts (15). The p75 TNFR has been associated with several biological activities, including inhibition of early hematopoiesis, induction of cytokine production, activation of NF-κB, and at high abundance of p75 TNFR, cell death (15). At present, the role played by the individual TNF receptors in insulin resistance is not known.

We show here that TNF-α inhibits insulin-induced IR and IRS-1 tyrosine phosphorylation in two cell lines that express the genes for both p55 and p75 TNF receptors. Using two different approaches, stimulation of p55 TNFR strongly inhibits its insulin-stimulated tyrosine phosphorylation of IR and IRS-1. An inhibition of IR and IRS-1 tyrosine phosphorylation was also observed after treatment of the cells with sphingomyelinase and synthetic analogous of ceramide, mediators that have been linked to p55 TNFR. Similar to TNF-α, sphingomyelinase and ceramide convert IRS-1 into an inhibitor of the IR tyrosine kinase activity *in vitro*. Interestingly, this effect is very specific for IRS-1, since IRS-2 cannot serve as an inhibitor in this assay.

MATERIALS AND METHODS

Reagents—Antibodies to insulin receptor were a gift from Drs. B. Cheatam and C. R. Kahn, anti-phosphotyrosine antibody was provided by Dr. T. Roberts, and PDGF BB and antibodies to PDGFβ receptor were gifts from Dr. C. Stiles. Human and mouse TNF-α were from Biosource International. C2 ceramide (*N*-acetylsphingosine), C6 ceramide (*N*-hexanoylsphingosine), and sphingomyelinase from *Staphylococcus aureus* were from Sigma.

Cell Culture—3T3-L1 cells were grown and differentiated into adipocytes as described previously (21). After maximal differentiation (at least 90% of cells differentiated) the medium was replaced with Dulbecco's modified Eagle's medium with 0.2% bovine serum albumin for 2 days and then supplemented with appropriate ligands. 32D-IR/IRS-1 and 32IR/IRS-2 cells were grown in suspension in RPMI 1640 supplemented with bovine calf serum (10%) and WEHI-3-conditioned medium (5%) as a source of interleukin-3 (22, 23).

Preparation of Cell Extracts, Immunoprecipitations, and Western Blots—After treatment with TNF-α, antibodies, ceramide, or sphingomyelinase (6 h for 3T3-L1 adipocytes and 4 h for 32D-IR/IRS-1), cells were stimulated for 3 min with insulin (10^{-7} M). Cells were washed in ice-cold phosphate-buffered saline and solubilized in stop buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3V0_4, 100 mM NaF, 1% Triton X-100 (v/v), 10 μg/ml aprotinin, 20 mM leupeptin, and 0.18 mg/ml phenylmethylsulfonyl fluoride). IR, IRS-1, and PDGFβ receptor were immunopurified from cell extracts with specific antibodies preabsorbed to protein A-Sepharose (Pharmacia Biotech Inc.) for 90 min at 4 °C. After three washes in stop buffer, Laemmli buffer was added to the beads, which were boiled for 3 min, and proteins were submitted to SDS-PAGE under reducing conditions on a 7.5% polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore), and Western blot analysis was performed using the ECL Western blot kit (Amersham Corp.) according to the manufacturer's instructions.

In Vitro Reconstitution Experiments—Wheat germ agglutinin-purified IR was treated with 10^{-6} M insulin for 30 min at 4 °C in kinase buffer (30 mM Hepes, pH 7.2, 30 mM NaCl, 0.1% Triton (v/v)) and incubated with immunopurified IRS-1 or IRS-2 obtained as described above but washed twice in stop buffer and three times in kinase buffer. Phosphorylation of the IR was initiated by adding 15 μM [γ-^{32}P]ATP, 8 mM $MnCl_2$, and 4 mM $MgCl_2$ for 1 h at room temperature. The reaction was stopped by addition of Laemmli buffer and analyzed by SDS-PAGE.

RESULTS

TNF-α Inhibits Insulin Signaling in 3T3-L1 Adipocytes and Myeloid 32D Cells—In order to investigate the TNF receptors involved in insulin resistance, we wanted to make use of agonist antibodies that have been used as specific activators of p55 TNFR and p75 TNFR. However, our previous work in 3T3-F442A adipocytes indicated that a robust effect on insulin receptor signaling took several days to develop in these cells (11). Since antibodies applied to the cells can be taken up via Fc receptors or destroyed, many cell lines were surveyed to find those that show a relatively rapid effect of TNF-α on insulin signaling. Fig. 1 shows that 6 h of treatment of 3T3-L1 adipocytes with murine TNF-α strongly inhibits the insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 at doses from 10 to 50 ng/ml. We have also investigated this effect in myeloid 32D-IR/IRS-1 cells. The parent 32D cells have low levels of IR and no IRS-1 or IRS-2 and have been used as a model system where these components have been genetically engineered into cells to study specific biochemical mechanisms related to insulin action (22, 23). Fig. 1 illustrates that 4 h of TNF-α treatment (from 1 to 25 ng/ml) inhibits IR and IRS-1 tyrosine phosphorylation stimulated by insulin in these cells. In both cell lines, the absolute quantities of IR and IRS-1 were not affected, indicating that TNF-α induces a specific defect in the stoichiometry of tyrosine phosphorylation.

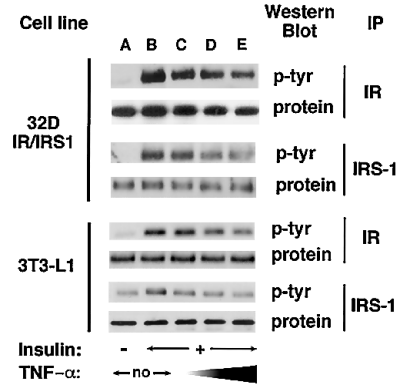

FIG. 1. **TNF-α inhibits IR and IRS-1 tyrosine phosphorylation in adipocytes and myeloid cells.** *Upper panels*, 32D cells were incubated in serum-free complete Cellgro medium and treated for 4 h with 1, 5, and 25 ng/ml (*lanes C, D,* and *E*, respectively) or without (*lanes A* and *B*) recombinant mouse TNF-α. Cells were treated for 3 min with (*lanes B–E*) or without (*lane A*) insulin (10^{-7} M), and IR and IRS-1 were immunoprecipitated (*IP*) for 90 min at 4 °C. After washes, proteins were analyzed by Western blot using anti-phosphotyrosine (*p-tyr*), anti-IR, or anti-IRS-1 (protein) antibodies. *Lower panels*, 3T3-L1 were starved in complete Cellgro medium for 48 h and treated for 6 h with 10, 25, and 50 ng/ml (*lanes C, D,* and *E*, respectively) or without (*lanes A* and *B*) TNF-α. IR and IRS-1 were then analyzed as described above. Results are representative of experiments performed at least three times.

Stimulation of p55 TNFR Is Sufficient to Inhibit Insulin-induced Tyrosine Phosphorylation of IR and IRS-1—It is now well established that human TNF-α binds to murine p55 TNFR but not murine p75 TNFR (24). Thus, using human *versus* mouse TNF-α has provided a tool to compare the effect of the stimulation of only one type of receptor with the activation of both receptors. As a first step we verified that mRNA encoding both receptors was present in 32D-IR/IRS-1 cells and 3T3-L1 adipocytes (data not shown). Mouse and human TNF-α were titrated on both cell lines, and IR and IRS-1 were analyzed by anti-phosphotyrosine Western blot. As observed in Fig. 2, human and mouse TNF-α inhibit IR and IRS-1 phosphorylation with a very similar potency in both cell lines. This result suggests that stimulation of p55 TNFR by itself is sufficient to mimic the full effect of TNF-α on IR tyrosine kinase inhibition.

It has recently been shown that polyclonal antibodies directed toward p55 TNFR or p75 TNFR can act as agonist on these receptors, with no cross-reaction (25). Thus, these agents provide a means to activate both of these receptors individually. 32D-IR/IRS-1 and 3T3-L1 cells were incubated with 5 and 15 μg/ml polyclonal antibodies directed toward p55 TNFR (Ab55) or p75 TNFR (Ab75), or both. Cells were then treated with insulin, and IR and IRS-1 were immunoprecipitated and analyzed by anti-phosphotyrosine Western blot. As observed in Fig. 3, Ab55 inhibits IR and IRS-1 tyrosine phosphorylation in a dose-dependent way in 32D-IR/IRS-1 (7 and 49% for IR and 10 and 45% for IRS-1) and in 3T3-L1 adipocytes (5 and 55% for

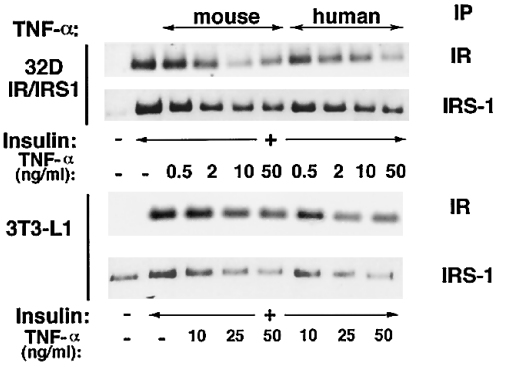

FIG. 2. **Inhibition of insulin signaling by human and mouse TNF-α.** 32D-IR/IRS-1 cells and 3T3-L1 adipocytes were treated for 4 and 6 h, respectively, with the indicated concentration of mouse or human recombinant TNF-α. Cells were stimulated with insulin for 3 min. At the end of the incubation IR and IRS-1 were immunoprecipitated (*IP*) and analyzed by Western blot using anti-phosphotyrosine antibodies. These results are representative of experiments performed two (3T3-L1) to four (32D cells) times with comparable results.

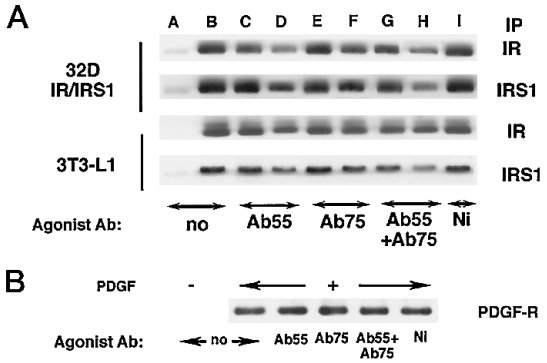

FIG. 3. **Inhibition of insulin-stimulated IR and IRS-1 tyrosine phosphorylation by antibody activation of p55 TNFR and p75 TNFR.** *Panel A*, 32D-IR/IRS-1 cells and 3T3-L1 adipocytes were treated in the presence of 5 and 15 μg/ml Ab55 (*lanes C* and *D*), Ab75 (*lanes E* and *F*), Ab55 and Ab75 (*lanes G* and *H*), or 30 μg/ml non-immune antibodies (*Ni, lane I*) for 4 and 6 h, respectively. *Ab*, antibody. Cells were then treated without (*lane A*) or with (*lanes B–I*) insulin for 3 min, rinsed in ice-cold phosphate-buffered saline, and lysed. IR and IRS-1 were immunoprecipitated (*IP*) and analyzed by anti-phosphotyrosine Western blot. *Panel B*, 3T3-L1 adipocytes were treated with 15 μg/ml Ab55, Ab75, both Ab55 and Ab75, or 30 μg/ml non-immune antibodies for 6 h. Cell were then stimulated by PDGF BB for 10 min, and the PDGFβ receptor was immunoprecipitated and analyzed by anti-phosphotyrosine Western blot. These results are representative of experiments performed three (3T3-L1) to six (32D cells) times with similar results. Films were scanned on an Abaton scanner and were quantified using NIH-Image v.1.44.

IR and 15 and 60% for IRS-1). In 32D cells and 3T3-L1 adipocytes, Ab75 induces a slight decrease of IR and IRS-1 phosphorylation but only at the highest dose. In 32D-IR/IRS-1 the inhibition induced by Ab75 reached 15 ± 2% and 20 ± 4% for IR and IRS-1, respectively, and in the presence of Ab75, the ability of Ab55 to inhibit insulin signaling was increased in an additive manner (from 49 ± 5% to 68 ± 4% for IR, and from 45 ± 6% to 63 ± 6% for IRS-1 at the higher concentration, mean ± S.E. of three different experiments). Incubations of cells in the presence of non-immune antibodies (Ni) did not modify the ability of insulin to stimulate IR and IRS-1 tyrosine phosphorylation.

These results do not rule out the possibility that the inhibition of the IR by agonist antibodies might be due to a general inhibition of all the receptors endowed with tyrosine kinase activity. To approach this question 3T3-L1 adipocytes were incubated with Ab55 and Ab75 as described above and were treated with PDGF BB for 10 min. The PDGFβ receptor was immunoprecipitated and analyzed by anti-phosphotyrosine Western blot. As observed in Fig. 3*B*, the tyrosine phosphorylation of the PDGFβ receptor was not altered after stimulation of p55 TNFR or p75 TNFR. This indicates that inhibition of tyrosine kinase activity by Ab55 is accomplished with some specificity for the IR.

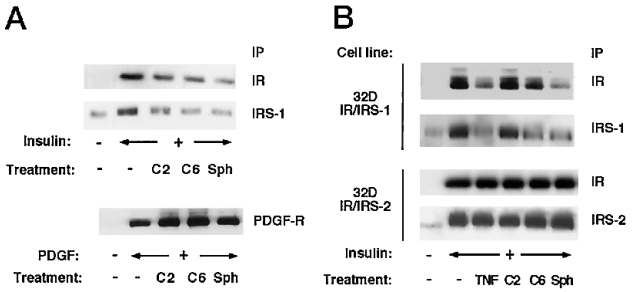

FIG. 4. **Ceramide- and sphingomyelinase-induced inhibition of IR and IRS-1 tyrosine phosphorylation.** *A*, 3T3-L1 adipocytes were incubated in the presence of C2 (1 μM), C6 (1 μM), or sphingomyelinase (*Sph*, 1 unit/ml) for 6 h. After a 3-min insulin treatment or 10 min of PDGF BB treatment, IR and IRS-1 or PDGFβ receptor were immunoprecipitated (*IP*), and proteins were separated by SDS-PAGE on a 7.5% acrylamide gel and analyzed by anti-phosphotyrosine Western blot. *B*, 32D-IR/IRS-1 and 32D-IR/IRS-2 were treated with TNF-α (50 ng/ml), C2 (1 μM), C6 (1 μM), or sphingomyelinase (1 unit/ml). After a 3-min insulin treatment (10^{-7} M) cells were lysed, and IR, IRS-1, or IRS-2 were immunoprecipitated and analyzed by anti-phosphotyrosine Western blot. All these results are representative of experiments performed at least three times with similar results.

Ceramides and Sphingomyelinase Mimic the Effect of TNF-α on Insulin Signaling—Among the many activities of the p55 TNFR is the activation of sphingomyelinase, which leads to the production of ceramides and phosphocholine (15). Ceramides induce the activation of ceramide-activated kinases and phosphatases (26, 27). To ask whether this pathway may be relevant to insulin signaling, 3T3-L1 adipocytes were treated with sphingomyelinase and two cell-permeant ceramides (*N*-acetylsphingosine (C2) and *N*-hexanoylsphingosine (C6)). Cells were then stimulated with insulin, and IR and IRS-1 tyrosine phosphorylation was analyzed (Fig. 4*A*). C2 and C6 reduce IR and IRS-1 phosphorylation equivalently (about 50%), while sphingomyelinase appeared to yield a stronger inhibition than either (about 70%). We investigated the specificity of this effect of the ceramides and sphingomyelinase for IR signaling by determining their effect on PDGF receptor tyrosine phosphorylation. 3T3-L1 adipocytes were treated with sphingomyelinase and ceramides and stimulated with PDGF BB, and the PDGFβ receptor was immunoprecipitated and analyzed by anti-phosphotyrosine Western blot. As observed in Fig. 4*A*, the tyrosine phosphorylation of the PDGFβ receptor was not inhibited by this treatment, indicating that the inhibition observed on the IR exhibits some specificity.

It has previously been shown that IRS-1 plays a very important role in mediating the inhibition of IR induced by TNF-α (13) (see below). It was therefore determined whether sphingomyelinase- and ceramide-mediated IR signaling inhibition functioned through IRS-1. To do this, we compared the ability of TNF-α, sphingomyelinase, and ceramide to inhibit IR signaling in 32D-IR/IRS-1 cells and in 32D cells expressing IR and IRS-2 (32D-IR/IRS-2). IRS-2 is a protein that possesses significant homology with IRS-1 and that has been shown to be an alternative substrate of the IR in IRS-1-deficient cells (40, 41). So far, no important differences in the biological function of these two proteins have been described. 32D-IR/IRS-1 and 32D-IR/IRS-2 cells were treated with TNF-α, sphingomyelinase, C2, and C6 for 4 h. Cells were then stimulated by insulin and IR, and IRS-1 and IRS-2 tyrosine phosphorylation was assessed by

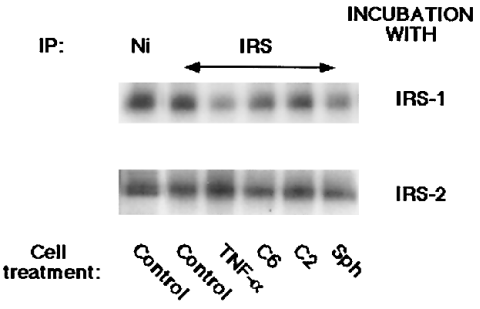

FIG. 5. **TNF-α, sphingomyelinase, and ceramide convert IRS-1 but not IRS-2 into an inhibitor of the IR tyrosine kinase activity.** 32D-IR/IRS-1 and 32D-IR/IRS-2 were treated for 4 h with TNF-α (10 ng/ml), C2 (1 μM), C6 (1 μM), or sphingomyelinase (1 unit/ml). IRS-1 and IRS-2 were then immunoprecipitated and after several washes were incubated in the presence of wheat germ agglutinin-purified IR, which has been stimulated by insulin (10^{-6} M) for 30 min at 4 °C. The kinase reaction was initiated by the addition of [γ-^{32}P]ATP, $MgCl_2$, and $MnCl_2$ and continued for 1 h at room temperature. The IR was then analyzed by SDS-PAGE. These results are representative of experiments performed three times with comparable results.

immunoprecipitation followed by anti-phosphotyrosine Western blot. As observed in Fig. 4*B*, in 32D-IR/IRS-1 cells, C2 caused a modest inhibition (10% for IR and 20% for IRS-1), while C6 and sphingomyelinase inhibited IR (50 and 80%, respectively) and IRS-1 tyrosine phosphorylation (60 and 70%). However, TNF-α, sphingomyelinase, and ceramide do not modify IR and IRS-2 tyrosine phosphorylation in 32D-IR/IRS-2 cells. This indicates that IRS-1 but not IRS-2 is involved in the mechanism by which TNF-α, sphingomyelinase, and ceramides inhibit insulin signaling in 32D cells.

It has recently been shown that TNF-α converts IRS-1 to an inhibitor of the IR autophosphorylation *in vitro* (13). We therefore assessed whether sphingomyelinase and ceramide could mediate the same effect. 32D-IR/IRS-1 cells were treated with TNF-α, sphingomyelinase, C2, and C6 for 4 h. IRS-1 was immunoprecipitated and incubated in the presence of ligand-stimulated IR. The autophosphorylation of IR was performed for 1 h in the presence of [γ-^{32}P]ATP, and proteins were separated on SDS-PAGE (Fig. 5). As compared with the control incubation (non-immune immunoprecipitation) or with the incubation with IRS-1 from untreated cells, IRS-1 from TNF-α-treated cells decreases the ability of the IR to autophosphorylate. Interestingly, sphingomyelinase and C6 were also able to induce such an inhibition, and C2 had no or little effect on the IR tyrosine kinase activity. This correlates the ability of these compounds to inhibit IR and IRS-1 tyrosine phosphorylation *in vivo* and their ability to convert IRS-1 into an inhibitor of the IR. This suggests that TNF-α, sphingomyelinase, and ceramide inhibit IR signaling through the same mechanism. We also measured the ability of IRS-2 from cells treated with TNF-α, sphingomyelinase, and ceramide to inhibit the IR (Fig. 5). Consistent with the data obtained in intact cells, no modification of the ability of the IR to autophosphorylate was observed. These results taken together strongly suggest a role for sphingomyelinase and subsequent production of ceramides in the mechanism by which TNF-α induces a defect in insulin signaling.

DISCUSSION

Several recent lines of evidence now indicate that TNF-α is a very important link between insulin resistance and obesity. In addition to the data showing overexpression of TNF-α from adipose tissue of obese and insulin-resistant rodents (4–6), new evidence indicates a tight correlation between obesity, insulin resistance, and TNF-α expression in humans (7, 8). That the relationship between TNF-α and insulin resistance is causal has been shown directly in animal neutralization studies. Using a soluble TNF receptor-IgG fusion protein designed to neutralize TNF-α, it has been shown that insulin resistance could be greatly reduced in obese Zucker fatty rats, along with an improvement in hyperglycemia, hyperinsulinemia, and hyperlipidemia (10). This correlated with a large increase in the tyrosine phosphorylation of both the IR and IRS-1, which are known to be affected during obesity and insulin resistance (10). Reciprocally, treatment of adipocytes in culture with TNF-α induces a decrease in the tyrosine kinase activity of IR (11). An inhibition of insulin-induced tyrosine phosphorylation of IR and IRS-1 has also been shown in cultured hepatoma cells (12). It is very likely that this inhibition of IR tyrosine kinase activity by TNF-α is a major mechanism by which TNF-α induces insulin resistance in obesity. Indeed, although several defects located at a postinsulin receptor level have been detected in obese animals, such as a decrease in the quantity of IR and of the insulin-sensitive glucose transporter Glut4, it has been noted that none of them can explain the extent of insulin resistance in this disease (1, 28). On the other hand, reduced tyrosine kinase activity of the IR, such as occurred under the action of TNF-α, has been noted in both animals and human non-insulin-dependent diabetes mellitus (29, 30). Clearly, induced tyrosine kinase activity would be expected to affect all subsequent actions of insulin (31). Understanding the molecular mechanisms by which TNF-α inhibits IR tyrosine kinase activity and subsequent tyrosine phosphorylation of IRS-1 will provide insight into the the molecular basis of TNF-α-induced insulin resistance in obesity.

One mechanism by which TNF-α interferes with the function of the IR appears to involve TNF-α-induced serine phosphorylation of IRS-1 (13, 14). This serine-phosphorylated IRS-1 acts as an inhibitor of IR *in vitro* and is also associated with reduced IR activity in intact cells (13). On the other hand, the first steps stimulated by TNF-α, which lead to the inhibition of IR tyrosine phosphorylation, were unknown. By using human TNF-α we observed that stimulation of p55 TNFR alone was sufficient to inhibit IR and IRS-1 tyrosine phosphorylation with the same potency of stimulation of both TNF receptors. Agonist antibodies to p55 TNFR induced a very similar effect. Activation of p75 TNFR alone by specific agonist antibodies also resulted in an inhibition of the IR signaling, though this effect is smaller compared with that induced by antibodies to p55 TNFR. Of course, it is possible that the difference in the magnitude of the effects of p55 TNFR and p75 TNFR antibodies resides in differences in their respective affinities for their ligands or in their stability. However, the fact that the two approaches yield similar results strongly suggests that stimulation of p55 TNFR is mainly responsible for the effect of TNF-α on IR and IRS-1 tyrosine phosphorylation in these cells. However, it is important to note that there is some reason to believe that p75 TNFR could play some role *in vivo*. First, the mRNA for p75 TNFR is dramatically increased (4.5-fold) (5) in the adipose tissue of obese/insulin-resistant mice. Second, since the absolute levels of TNF-α are rather low in obesity (less than 100 pg/ml) (4) and the p75 TNFR has a higher affinity for TNF-α, it could play a relatively more important role. The definitive role of these two receptors must include an analysis of insulin resistance in obese mice containing appropriate null alleles for both receptors (32–34).

One of the signaling events triggered by p55 TNFR is the stimulation of a membrane-bound neutral sphingomyelinase, which hydrolyzes sphingomyelin to ceramide and choline (26, 27). We show here that ceramides and sphingomyelinase can also induce a decrease in the tyrosine phosphorylation of IR and IRS-1. At the molecular level, ceramide and sphingomyeli-

nase convert IRS-1 into an inhibitor of IR tyrosine kinase activity, as does TNF-α. These compounds have some specificity for IR signaling since they do not inhibit PDGF tyrosine phosphorylation in adipocytes nor do they modify IR and IRS-2 tyrosine phosphorylation in 32D-IR/IRS-2 cells. These data indicate that activation of sphingomyelinase and production of ceramides is likely to be a major pathway used by p55 TNFR to mediate IR inhibition. Ceramides directly activate various enzymes such as PKC-ζ (35, 36), a membrane-associated kinase (37) that phosphorylates and activates Raf-1 (38), and a ceramide-activated protein phosphatase that is a subtype of heterotrimeric phosphatase 2A (39). This leads to the activation of a cascade of phosphorylation/dephosphorylation events (26). It is likely that stimulation of these enzymes leads to modification of IRS-1 and subsequent inhibition of the IR.

Since IRS-1 is a central component in the mechanism by which TNF-α inhibits IR signaling, it was of potential interest to investigate a role for IRS-2 in this process. IRS-2, also known as 4PS, exhibits substantial structural homology with IRS-1 (40), and they also share similar biological function. Indeed, as does IRS-1, IRS-2 binds to Grb2, to the p85 subunit of the phosphatidylinositol 3-kinase, and possesses potential binding sites for PTP-2C (40, 41). Moreover, IRS-2 has been shown to be the alternative substrate of the IR in IRS-1-deficient mice (41). However, it is clear from Figs. 4 and 5 that IRS-2 cannot replace IRS-1 in this inhibition. This is the first important difference observed in the function of IRS-1 and IRS-2, and this could be an important tool to understand the mechanism by which IRS-1 inhibits the IR upon TNF-α treatment of the cells.

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