Sphingomyelinase and Ceramide Suppress Insulin-induced Tyrosine Phosphorylation of the Insulin Receptor Substrate-1*

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The sphingomyelin pathway is a newly described signal transduction pathway mediating the action of several cytokines including tumor necrosis factor-α (TNF). TNF was recently shown to interfere with insulin-induced tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1). In this work we examined the possible effect of direct activation of the sphingomyelin pathway on insulin-induced tyrosine phosphorylation of IRS-1. Incubation of the insulin-sensitive rat hepatoma Fao cells with bacterial sphingomyelinase (SMase) that causes membrane hydrolysis of sphingomyelin led to a time- and dose-dependent decrease in insulin-induced tyrosine phosphorylation of IRS-1. The effect was apparent after 10 min of incubation and with a dose of 10 milliunits/ml SMase. It was not associated with a decrease in insulin receptor autophosphorylation. In addition, SMase treatment interrupted the association of the 85-kDa catalytic subunit of phosphatidylinositol 3-kinase with IRS-1. A similar impact on IRS-1 tyrosine phosphorylation was observed after addition of cell-permeable ceramide analogs (C2 and C6). Comparable changes in IRS-1 tyrosine phosphorylation and electrophoretic mobility were found after exposure of cells to either TNF, SMase, or ceramide. Our findings suggest that TNF may utilize the sphingomyelin pathway in its effect on the insulin-stimulated tyrosine phosphorylation of IRS-1.

The sphingomyelin pathway is a new signal transduction pathway mediating the action of the cytokines, tumor necrosis factor-α (TNF), interleukin-1, and interferon-γ (reviewed in Refs. 1–3). This pathway is initiated by activation of a neutral sphingomyelinase (SMase), which hydrolyzes membrane sphingomyelin to ceramide and phosphocholine. In addition, ceramide can be generated through the action of an acidic sphingomyelinase (SMase) activated by 1,2-diacylglycerol as a result of stimulation of a ligand-responsive phosphatidylinositol-specific phospholipase C (PC-PLC). Ceramide functions as a second messenger and can stimulate a specific proline-directed Ser/Thr protein kinase as well as a specific protein phosphatase (1–3). The role of the sphingomyelin pathway in TNF action has been documented in several cell lines including hepatocytes (4–8). TNF led to rapid breakdown of sphingomyelin by SMases, while addition of neutral SMase or cell-permeable analogs of ceramide to cells mimicked TNF actions.

We and others have recently demonstrated that TNF leads to suppression of early events in insulin signal transduction (9–11). Specifically, it inhibited the insulin-induced tyrosine phosphorylation of the major insulin receptor substrate, IRS-1, and impaired the association of IRS-1 with phosphatidylinositol (PI) 3-kinase (11). In the current work we examined the involvement of the sphingomyelin pathway in this cross-talk between TNF and insulin transmembrane signaling mechanisms.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Rat hepatoma Fao cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Biological Industries, Beit Haemek, Israel). Recombinant murine TNF was a gift from Boehringer (Ingelheim, FRG), and recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). SMase (neutral, Staphylococcus aureus) and PC-PLC (Bacillus cereus) were from Sigma. N-Acetylsphingosine (C2-ceramide) and N-hexanoylsphingosine (C6-ceramide) were from Biomol Inc. (Plymouth Meeting, PA). Stock solutions (20 mM) of the lipid analogs were prepared in ethanol or dimethyl sulfoxide, and before use aliquots were sonicated into medium as required. The final concentration of ethanol and dimethyl sulfide in the incubations was 0.025%. Protein G- and protein A-Sepharose were from Pharmacia (Uppsala, Sweden). A14-mono-1235-insulin (2000 CI/mmol) was from Amersham (Aylesbury, Buckinghamshire, United Kingdom). All other reagents were from Sigma.

Antibodies—Rabbit polyclonal insulin receptor antibodies were generated against a synthetic peptide corresponding to positions 1309–1324 of the human insulin receptor (12). Rabbit polyclonal IRS-1 antibodies were generated against a synthetic peptide corresponding to the carboxy-terminal 14 amino acids of rat IRS-1 (13) were a gift from Y. Zick (Weizmann Institute of Science, Rehovot, Israel). Polyclonal antibody directed against the p85 regulatory subunit of PI 3-kinase was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Phosphotyrosine antibodies were a gift from M. White (Joslin Diabetes Center, Boston, MA).

Ligand Treatment of Intact Cells, Immunoprecipitation, and Western Immunoblotting—Confluent monolayers of Fao cells, grown in 60-mm dishes, were serum deprived for 6–16 h prior to each experiment. Medium was removed, and cells were incubated at 37 °C with SMase, PC-PLC, C2- or C6-ceramide, or TNF in serum-free medium at the indicated concentrations and times. Cells were then stimulated 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. Cells were solubilized at 4 °C with lysis buffer (50 mM Hepes, pH 7.5, 80 mM β-glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM each of EGTA, EDTA, and sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μM trypsin inhibitor). Cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. Aliquots of the supernatants were normalized for protein, mixed with concentrated (10%) Laemmli sample buffer, boiled for 5 min, and resolved on 7.5% SDS-PAGE under reducing conditions. In addition, cell extracts were immunoprecipitated for 4 h at 4 °C with IRS-1 antibodies preabsorbed on protein G-Sepharose beads or with insulin receptor antibodies coupled to protein A-Sepharose beads as described previously (11). Immunocomplexes were washed four times with lysis buffer containing 0.1% Triton X-100, suspended in Laemmli sample buffer, and

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¶ The abbreviations used are: TNF, tumor necrosis factor; PC-PLC, phosphatidylinositol-specific phospholipase C; IRS-1, insulin receptor substrate-1; SMase, sphingomyelinase; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol.

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Sphingomyelinase Suppresses IRS-1 Phosphorylation

Inhibition by Sphingomyelinase of Insulin-stimulated Tyrosine Phosphorylation of IRS-1 in Intact Fao Cells—Insulin receptor is a protein tyrosine kinase, which upon stimulation by insulin phosphorylates the receptor itself and its major intracellular substrate IRS-1 on tyrosine residues (14, 15). To examine the effect of Sphingomyelinase (SMase) on insulin-stimulated tyrosine phosphorylation, Sphingomyelinase-treated Fao cells were stimulated for 1 min by insulin. Cell extracts were analyzed by SDS-PAGE, blotted onto nitrocellulose filters, and probed with antibodies directed against IRS-1 and PI 3-kinase subunits. The effect was seen already after 10 min of incubation with 100 milliunits/ml SMase but peaked after a 30-min exposure (Fig. 2A). The identity of the lower component of the 180–185-kDa broad band as IRS-1 (9, 16, 17) was confirmed by immunoprecipitation with a specific IRS-1 antibody (Fig. 2B). To underscore the specificity of SMase effect, cells were exposed to another phospholipase under the same conditions. Equivalent concentrations of PC-PLC (100–300 milliunits/ml) had no effect on insulin-stimulated IRS-1 tyrosine phosphorylation.

After tyrosine phosphorylation, IRS-1 binds to several SH2 domain proteins including the 85-kDa regulatory subunit of PI 3-kinase (14, 15). To evaluate the SMase effect on this interaction, IRS-1 immunoprecipitates were immunoblotted with antibodies directed against the 85-kDa subunit of PI 3-kinase. Preincubation with SMase, 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. 2C, lanes b–e). This decrease did not result from a reduction in IRS-1 content, probed by immunoblotting with IRS-1 antibodies (not shown), but rather from a decrease in its tyrosine phosphorylation (Fig. 2B). While incubation with SMase for 30 min, without insulin stimulation, did not induce tyrosine phosphorylation of IRS-1 or association with PI 3-kinase, it caused a dose-dependent tyrosine phosphorylation of an unidentified 200–210-kDa band (Fig. 2, lane f, ppTyr).

Inhibition by Ceramide of Insulin-stimulated Tyrosine Phosphorylation of IRS-1 in Intact Fao Cells—Ceramide, the breakdown product of sphingomyelin, serves as the second messenger in the sphingomyelin pathway (1–3). To evaluate its effect on insulin-induced phosphorylation similar experiments to those described above for SMase were repeated with the cell-permeable C2- and C6-ceramide analogs. As shown in Fig. 3, preincubation of Fao cells for 10 min with the C2 analog of ceramide led to a maximal effect with 300 milliunits/ml. The effect was seen already after 10 min of incubation with 100 milliunits/ml SMase but peaked after a 30-min exposure (Fig. 2A). The identity of the lower component of the 180–185-kDa broad band as IRS-1 (9, 16, 17) was confirmed by immunoprecipitation with a specific IRS-1 antibody (Fig. 2B). To underscore the specificity of SMase effect, cells were exposed to another phospholipase under the same conditions. Equivalent concentrations of PC-PLC (100–300 milliunits/ml) had no effect on insulin-stimulated IRS-1 tyrosine phosphorylation.

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ceramide, prior to 1 min of insulin stimulation, led to a decrease in IRS-1 tyrosine phosphorylation. This effect was time-dependent and peaked after 30 min of exposure to the analog, associated by a small shift in electrophoretic mobility. The effect was less pronounced after 60 min of ceramide exposure. A small decrease in the amount of the 85-kDa subunit of PI 3-kinase associated with IRS-1 was also seen after 10–20 min of exposure to C2-ceramide (not shown). Incubation with ceramide alone induced no tyrosine phosphorylation of IRS-1 or a higher Mr band (Fig. 3, left 2 lanes). Similar results were seen with the C6 analog of ceramide (Fig. 4, left panel).

As TNF is known to activate SMase and to interfere with insulin-induced tyrosine phosphorylation, cells were exposed in parallel to TNF, ceramide, and SMase. A high degree of similarity between the effect of all three agents on insulin-induced IRS-1 tyrosine phosphorylation and electrophoretic mobility was observed (Fig. 4), though consistently the effects of SMase were the most pronounced.

**DISCUSSION**

In the current work we supply evidence that activation of the sphingomyelin pathway interferes with the early events of insulin transmembrane signaling. Addition of SMase that hydrolyzes sphingomyelin in the plasma membrane to ceramide and phosphocholine, or of cell-permeable ceramide analogs to Fao cells, both led to a decrease in insulin-induced tyrosine phosphorylation of IRS-1. This was not associated with inhibition of the insulin receptor intrinsic kinase activity as insulin receptor autophosphorylation of its β-subunit was not reduced. IRS-1 is the major substrate for the insulin receptor, and upon tyrosine phosphorylation it transduces insulin effects through association with multiple SH2-containing molecules (14, 15). This downstream step was also disrupted by activation of the sphingomyelin pathway as demonstrated with PI 3-kinase.

Similar effects were previously reported by us in the same system exposed to TNF, which utilizes the sphingomyelin pathway for signal transduction (9, 11). The similarity between the TNF effect on insulin induction of IRS-1 tyrosine phosphorylation and that achieved by directly activating the sphingomyelin pathway suggests that TNF may employ this pathway in its interference with IRS-1 tyrosine phosphorylation. This observation may fit into a working model explaining TNF action in this system. TNF increases IRS-1 phosphorylation on serine residues leading to a decrease in its electrophoretic mobility (11). TNF-induced serine phosphorylation of IRS-1 may interfere with insulin-stimulated tyrosine phosphorylation of the substrate and impair insulin action. Ceramide acts as the second messenger in the sphingomyelin pathway and activates proline-directed Ser/Thr kinases including a specific membrane-associated protein kinase (18, 19), mitogen-activated protein kinases (6, 20), and stress-activated protein kinases (8, 21, 22). IRS-1 has multiple X-Ser/Thr-Pro-X motifs and is a substrate for the mitogen-activated protein kinase (14, 15).

Thus, activation by the sphingomyelin pathway of these proline-directed Ser/Thr kinases may mediate the TNF effect on IRS-1, leading both to a delay in its electrophoretic mobility as well as impaired tyrosine phosphorylation. A similar scenario where TNF activates neutral SMase, which in turn activates a cascade of Ser/Thr phosphorylations, has been described in other cell lines (2, 3, 6, 22, 23). It is noteworthy that other cytokines, namely interleukin-1 and interferon-γ, which activate the sphingomyelin pathway (1), have also been shown to decrease insulin-induced tyrosine phosphorylation of IRS-1 (10).

Combined with previous reports the current data help portray a more detailed picture of the possible interplay between cytokines and insulin transmembrane signaling pathways. The clinical relevance of this cross-talk is underscored by the ample evidence supporting the possible role of TNF as a key player in the mode of action of insulin resistance in cancer, obesity (24).

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