A Peptide-based Protein-tyrosine Phosphatase Inhibitor Specifically Enhances Insulin Receptor Function in Intact Cells*

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Hemanta K. Kole, Michael J. Garant, Sutapa Kole, and Michel Bernier

From the Diabetes Section, Laboratory of Clinical Physiology, NIA, National Institutes of Health, Baltimore, Maryland 21224

3S-peptide-I is a synthetic tris-sulfotyrosyl dodecapeptide corresponding to the major site of insulin receptor autophosphorylation that potently inhibits dephosphorylation of the insulin receptor in a cell-free system and in digitonin-permeabilized Chinese hamster ovary (CHO) cells overexpressing the human insulin receptors (CHO/HIRc cells) (Liotta, A. S., Kole, H. K., Fales, H. M., Roth, J., and Bernier, M. (1994) J. Biol. Chem. 269, 22996–23001). In the present study, we found that 3S-peptide-I was not capable of inhibiting dephosphorylation of the epidermal growth factor (EGF) receptors in digitonin-permeabilized CHO cells that overexpress human EGF receptors (CHO/EGF-R cells). Moreover, the addition of epidermal growth factor (EGF) receptors in digitonin-permeabilized CHO cells that overexpress human EGF receptors (CHO/EGF-R cells) resulted in a concentration-dependent increase in insulin-stimulated phosphorylation of the insulin receptor, with a maximum effect (2.7-fold) at 50 μM. In contrast, ligand-stimulated EGF receptor phosphorylation in CHO/EGF-R cells was not affected by the presence of stearyl 3S-peptide-I. Furthermore, treatment of CHO/HIRc cells with this N-stearyl peptide led to a significant enhancement of the insulin-induced association of phosphatidylinositol (PI) 3-kinase activity with insulin receptor substrate 1 and the activation of mitogen-activated protein kinase. However, stearyl 3S-peptide-I had no effect on the EGF-stimulated activation of PI-3-kinase and mitogen-activated protein kinase in CHO/EGF-R cells. These data indicate that this tris-sulfotyrosyl dodecapeptide selectively enhances insulin signal transduction by specifically inhibiting dephosphorylation of the insulin receptor in intact cells.

The binding of insulin to its cell surface receptor induces phosphorylation of specific tyrosyl residues within the intracellular domain of the receptor β-subunit. This autophosphorylation reaction activates the receptor’s intrinsic tyrosine kinase activity toward various cellular substrates including IRS-1, IRS-2, and Shc proteins (1–3) and thereby plays a key role in the metabolic and mitogenic signaling pathways of insulin (2).

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‡ To whom correspondence should be addressed: Diabetes Section, Laboratory of Clinical Physiology, Gerontology Research Center, NIA, NIH, 4940 Eastern Ave., Baltimore, MD 21224, Tel.: 410-558-8416; Fax: 410-558-8381; E-mail: Hemantak@vax.grc.nia.nih.gov.

1 The abbreviations used are: IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; EGF, epidermal growth factor; PI, phosphatidylinositol; CHO, Chinese hamster ovary; MAP, mitogen-activated protein; MBP, myelin basic protein; PVDF, polyvinylidene difluoride; PTPase, protein-tyrosine phosphatase; PMSF, phenylmethylsulfonyl fluoride; CHO/HIRc, CHO cells overexpressing the human insulin receptor; CHO/EGF-R, CHO cells overexpressing the human EGF receptor.

Dephosphorylation of the insulin receptor by cellular protein-tyrosine phosphatases (PTPases) attenuates the receptor kinase activity and, hence, the effects of insulin (4–6). Thus, PTPases may oppose tyrosine kinase-mediated insulin signaling and contribute to insulin resistance. Indeed, altered PTPase activity has been noted in different tissues from diabetic rats (7–9) and humans (10–12). Therefore, the development of PTPase inhibitors that act as specific modulators of insulin receptor functions may provide novel ways to treat diabetes. It has been reported previously that vanadate and pervanadate cause a marked improvement of glucose homeostasis in streptozotocin-treated rats (13, 14) by exerting insulin-like effects on peripheral tissues. Both compounds are broad spectrum PTPase inhibitors (15, 16) that appear to function via a mechanism distal to the insulin receptor (17–19). Because vanadate and pervanadate affect various systems under physiological conditions at relatively high doses (20, 21), they are not likely to become useful therapeutic agents. It has become apparent that PTPases are selective among different phosphotyrosine-containing proteins (22, 23) and synthetic peptides (24–26), indicating an interaction between PTPase and the specific amino acid sequence of the protein substrate. This behavior has provided the basis for the synthesis of non-hydrolyzable phosphotyrosine peptide analogs (27–31) with the goal of selective inhibition of tyrosine phosphatases acting on specific protein substrates. We have reported previously that a tris-sulfotyrosyl dodecapeptide analogue of the insulin receptor autocatalytic domain (3S-peptide-I) potently inhibits the dephosphorylation of the insulin receptor in vitro (30). The same study has also shown that the conjugation of 3S-peptide-I to stearic acid leads to an enhanced insulin-stimulated receptor autophosphorylation in intact cells (30).

In this study, we extended this observation by comparing the levels of phosphorylation and signaling of the insulin receptor with that of the epidermal growth factor receptor following ligand stimulation in the presence of stearyl 3S-peptide-I. Our results show that while stearyl 3S-peptide-I specifically enhanced the immediate phosphorylation of the insulin receptor at tyrosine residues and subsequent stimulation of PI-3-kinase and MAP kinase activities in insulin-treated cells, it had no effect on the EGF receptor activation and signaling.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of the highest purity commercially available. EGF, myelin basic protein (MBP), monodonal and polyclonal anti-phosphotyrosine antibodies, polyclonal anti-EGF receptor antibody, polyclonal MAP kinase antibody, and polyclonal anti-IRS-1 antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyvinylidene difluoride (PVDF) membrane and precast 4–12 and 4–20% gradient polyacrylamide gels were purchased from Novex (San Diego, CA). Protein G-plus/Protein A-agarose beads and monoclonal anti-insulin receptor antibody were from Oncogene Science, Inc. (Uniondale, NY), and polyclonal ERK-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Sodium orthovanadate, Tween-20, Nonidet P-40, and Triton X-100 were purchased from Sigma.
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Cell Lines—The Chinese hamster ovary (CHO) cell line transfected with an expression plasmid encoding the normal human insulin receptor (CHO/HIRc) was a generous gift from Dr. Morris F. White, Joslin Diabetes Center, Boston, MA. CHO cells overexpressing human EGF receptor (CHO/EGF-R) was a generous gift from Dr. Roger J. Davis, University of Massachusetts Medical School, Worcester, MA. The cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum and were cultured to confluence in 35-mm dishes.

Peptide Synthesis—Peptide-I (TRDIYETDYYRK-amide) is a decapeptide whose primary sequence is identical to the 1142–1153 region of insulin-like growth factor-I, tyrosine sulfation of resin-bound peptide-I, and its modifications. The purity of each amino acid was confirmed by mass spectrometric analyses.

Autophosphorylation and Dephosphorylation of EGF Receptors in Permeabilized CHO/EGF-R Cells—Both reactions were evaluated following permeabilization with digitonin, essentially as described previously (33). Briefly, serum-starved cells were permeabilized with 35 μg/ml digitonin for 20 min at room temperature, transferred to 6°C, and then treated with 5 mM EGF for 5 min. Thereafter, the phosphorylation reaction was started by the addition of 100 μM ATP and 4 mM MnCl₂. 10 min later, the dephosphorylation of EGF receptors was initiated by the addition of 20 mM EDTA in the presence of 1 mM sodium orthovanadate or 35-μg peptide-I at the indicated concentrations. The reaction was stopped after 5 min by immersing the dishes in liquid nitrogen. The cells were lysed in radiolabeled precipitation buffer containing 20 mM Tris·Cl (pH 7.5), 137 mM NaCl, 1 mM sodium orthovanadate, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% NaN₃, 0.2 mM PMSE, 1 mM benzamidine, 8 μg/ml aprotinin, and 2 μg/ml leupeptin. The lysates were centrifuged at 17,000 × g for 10 min at 4°C. The clarified lysates were incubated with a monoclonal anti-phosphotyrosine antibody. After overnight incubation at 4°C, the immune complexes were precipitated with Protein G-plus/Protein A-agarose beads and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions (34) followed by electrotransfer on PVDF membranes. Western blotting was performed with a monoclonal anti-phosphotyrosine antibody, as described recently (35). The blots were developed using the ECL chemiluminescence detection system (Amerham Corp.), and the immune complexes were collected by mixing with Protein G-plus/Protein A beads. Immunoprecipitates were subjected to immunoprecipitation with polygonal anti-MAP kinase antibody. After collecting the immune complexes by the addition of Protein G-plus/Protein A-agarose, the beads were washed twice with lysis buffer, twice with kinase buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl₂, and 1 mM diithiore- 

RESULTS

Effect of 3S-35-Peptide-I on EGF Receptor Dephosphorylation in Permeabilized CHO Cells—We have shown previously (30) that the trisulfotyrosyl dodecapeptide 35-peptide-I potently inhibits insulin receptor dephosphorylation in digitonin-permeabilized CHO/HIRc cells. Under these conditions, membrane architecture remains intact while allowing the rapid entry of ATP, peptides, and small molecules into the intracellular compartment (30). Here, we investigated the effect of 3S-35-peptide-I on the dephosphorylation of the EGF receptors after permeabilization of CHO/EGF-R cells with digitonin. To study dephosphorylation of the EGF receptor, phosphorylation reaction was carried out in the presence of EGF and ATP, and then was stopped by the addition of EDTA. The amount of tyrosine-phosphorylated EGF receptors was analyzed using an anti-phosphotyrosine antibody immunoblotting method. The phosphotyrosine content of the EGF receptor decreased by ~76% after 5 min of dephosphorylation at 6°C in control cells (Fig. 1). 3S-35-peptide-I treatment did not alter EGF receptor dephosphorylation; however, vanadate strongly inhibited dephosphorylation of the EGF receptor. These results show that under these experimental conditions, 3S-35-peptide-I, whose sequence is unrelated to the EGF receptor (37), does not interact with PTPases acting on the EGF receptor.

Effect of Stearyl 3S-35-Peptide-I on Receptor Phosphorylation in Intact Cells—We next examined the effect of stearyl 3S-35-peptide-I on the ligand-stimulated phosphorylation of insulin receptor and EGF receptor in intact cells. For these studies, CHO cells expressing large numbers of insulin receptors (CHO/HIRc cells) or EGF receptors (CHO/EGF-R cells) were incubated with a range of concentrations of stearyl 3S-35-peptide-I or vanadate (1 mM) for 1 h and stimulated with their respective ligand. The extent of receptor tyrosine phosphorylation was then determined by Western immunoblotting. Fig. 2A shows that stearyl 3S-35-peptide-I (100 μM) was capable of increasing phosphoryla-
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**Fig. 1. Effect of 3S-peptide-I on the dephosphorylation of the EGF receptors in permeabilized CHO/EGF-R cells.** Confluent monolayers of CHO/EGF-R cells were incubated for 16 h in serum-free Ham's F-12 medium before permeabilization with digitonin. Permeabilized cells, cooled to 6 °C, were treated with EGF (5 nM), and the phosphorylation reaction was initiated by the addition of 100 μM ATP. 10 min later, the phosphorylation reaction was stopped (time = 0) by incubating the cells with 20 mM EDTA in the absence or presence of 3S-peptide-I or vanadate for 5 min at 6 °C. Cell extracts were prepared and then immunoprecipitated with a monoclonal anti-phosphotyrosine antibody. Proteins were separated by SDS-polyacrylamide gels under reducing conditions and then treated with their respective ligand. Cell lysate from CHO/HIRc cells was incubated with a polyclonal anti-IRS-1 antibody while monoclonal anti-phosphotyrosine antibody was used to detect phosphorylation. Insulin receptors in cell lysates were immunoprecipitated with a monoclonal anti-insulin receptor antibody and electrophoresed on SDS-polyacrylamide gels under reducing conditions. After electrophoresis, PVDF membranes were probed with a polyclonal anti-phosphotyrosine antibody. The blots were developed using the ECL chemiluminescence detection system, and autoradiograms were quantified by scanning laser densitometry. The results are means ± S.D. of two to three experiments in which each treatment was performed using two culture dishes.

**Fig. 2. Effect of stearyl 3S-peptide-I on the autophosphorylation of the insulin receptors in intact CHO/HIRc cells.** A, time-course of insulin receptor autophosphorylation in the absence or presence of 100 μM stearyl 3S-peptide-I, B, dose-response curve for insulin-stimulated receptor phosphorylation by stearyl 3S-peptide-I. CHO/HIRc cells were serum-starved for 16 h after which stearyl 3S-peptide-I (0–100 μM) or 1 mM vanadate was added for 1 h. Cells were then treated with 100 nM insulin for the indicated times to induce receptor autophosphorylation. Insulin receptors in cell lysates were immunoprecipitated with a monoclonal anti-insulin receptor antibody and electrophoresed on SDS-polyacrylamide gels under reducing conditions. After electrophoresis, PVDF membranes were probed with a polyclonal anti-phosphotyrosine antibody. The blots were developed using the ECL chemiluminescence detection system, and autoradiograms were quantified by scanning laser densitometry. The results are means ± S.D. of two to three experiments in which each treatment was performed using two culture dishes.
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Effect of Stearyl 3S-Peptide-I on Ligand-Stimulated MAP Kinase Activity in Intact Cells—MAP kinase activity is rapidly stimulated in response to insulin and other growth factors (41, 42) via a mechanism that involves both tyrosine and serine/threonine phosphorylation of the enzyme itself (43). MAP kinase activity was assayed in the anti-MAP kinase immunoprecipitates prepared from cells treated or not with stearyl 3S-peptide-I by measuring the level of phosphorylation of an exogenous substrate, MBP. Low levels of MAP kinase activity were detected in the immunoprecipitates prepared from unstimulated cells. When cells were treated with their respective ligand, a large increase in MAP kinase activity was observed (Fig. 5, A and B). In CHO/HIRc cells, stearyl 3S-peptide-I (50 μM) increased insulin-stimulated MAP kinase activity by 2.3-fold (\( p < 0.0001 \)) while having no significant effect on EGF-induced activation of MAP kinase in CHO/EGF-R cells (Fig. 5, A and B). Thus, it appears again that 3S-peptide-I is capable of interacting with specific components of insulin signal transduction pathways.

**DISCUSSION**

Protein tyrosine phosphorylation plays a determinant role in regulating many cellular processes. The level of phosphotyrosine in the cell is a balance between the actions of protein tyrosine kinases and PTPases. It has become apparent that there is a large number of PTPases with distinct specificities, some of which for tyrosine kinase–linked receptors. The mechanism that govern PTPase substrate specificity in cells is largely unknown. Cellular compartmentalization (44), interaction with closely associated regulatory proteins, levels of PTPase inhibitors and activators (45, 46), as well as posttranslational modifications (47–49) might provide such possible mechanism. In addition, features in the primary structure surrounding the dephosphorylation site may contribute to substrate specificity.

The experiments described in this paper were designed to assess whether 3S-peptide-I displays any specificity as an inhibitor for the PTPases acting on the insulin receptor. 3S-Peptide-I has three nonhydrolyzable sulfotyrosine residues inserted during the chemical synthesis and which correspond to the major autophosphorylation site of the insulin receptor. The incorporation of stearyl moieties to 3S-peptide-I enables its entry in intact cells and thereby allows access of the peptide to the intracellular milieu. We have shown that stearyl 3S-peptide-I stimulated insulin-induced autophosphorylation of the insulin receptor β-subunit, which, in turn, increased the association of PI-3-kinase activity with IRS-1 and MAP kinase activation in response to insulin. These stimulatory effects of stearyl 3S-peptide-I were specific for insulin signaling, as receptor autophosphorylation levels, PI-3-kinase, and MAP kinase activation in response to EGF were not affected. Whether the sulfotyrosine-containing peptides corresponding to the autophosphorylation site of other growth factor receptors (e.g., receptor for EGF, PDGF, and fibroblast growth factor) can show specificity toward their respective receptor phosphorylation and signaling remains to be determined.

Change in the fatty acid composition of membrane phospholipids by the stearyl moiety might provide a mechanism by which stearyl 3S-peptide-I enhances the transmission of insulin signal. Fatty acids are normal constituents of biological membranes, influencing the physicochemical state of lipid domains (50). It has been proposed that exogenous addition of fatty acids perturbs the bilayer structure of the plasma membrane, leading to alterations in membrane-cytoskeleton interactions and the modification of the physical state of transmembrane receptors and regulatory proteins (51). Consistent with this hypothesis are reports demonstrating that fatty acids can inhibit transmembrane signaling within minutes (52, 53) through changes in membrane fluidity or permeability (54, 55).
The lack of effect of free stearic acid on both insulin-stimulated tyrosine phosphorylation of the insulin receptor and activation of PI-3-kinase in intact cells (data not shown) suggests that the primary action of stearyl 3S-peptide-I may not be the result of a nonspecific alteration of membrane phospholipid properties. Our previous study suggests that the effect of 3S-peptide-I is not mediated through direct stimulation of the insulin receptor β-subunit autophosphorylation when assayed in vitro (Ref. 30 and data not shown), but rather is the result of an alteration in tyrosine dephosphorylation. Under the same experimental conditions whereby cells were semipermeabilized with digitonin, we did not observe the inhibition of EGFr receptor dephosphorylation by 3S-peptide-I. Therefore, the lack of effect of 3S-peptide-I on the level of tyrosine phosphorylation and dephosphorylation of the EGFr receptor and its downstream signaling molecules suggests that cellular PTPases can be selectively inhibited by nonhydrolyzable phosphotyrosyl-containing peptide analogs based, in part, on the primary structure of the protein substrate. On the other hand, 3S-peptide-I does not affect the activity of members of serine/threonine phosphatase family or alkaline phosphatase, but has been shown to partially inhibit a recombinant PTPase, PTP-1B (30). This effect may occur due to the accessibility of the sulfotyrosyl residues per se and not because of the nature of the amino acid residues adjacent to the three sulfotyrosines in the above peptide.

This study demonstrates that 3S-peptide-I is an effective agent that increases the action of insulin on two important signaling mediators. This effect appears to occur as a result of action at a step proximal to the tyrosine phosphorylation of the insulin receptor and the IRS-1. Numerous proteins bind to tyrosine-phosphorylated IRS-1 through their SH2 domains after insulin stimulation. This interaction appears to be an activation step for several intracellular enzymes containing SH2 motifs. Using anti-IRS-1 antibody, we showed that stearyl 3S-peptide-I increased insulin-stimulated PI-3-kinase activity. Because of the multifunctional role played by PI-3-kinase within the cell, i.e. in cell growth, activation of pp70 S6 kinase, and in GLUT-4 translocation (38), experiments are being designed in an attempt to elucidate the impact 3S-peptide-I may have on insulin signal transduction in a number of insulin responsive cells. We observed an increase in insulin-induced MAP kinase activation in response to stearyl 3S-peptide-I. MAP kinase participates in a phosphorylation cascade in cells that plays an important role in coordinating the regulation of a number of kinases and phosphatases involved in glycogen synthesis and nuclear signaling. It is known that the association of SH-PTP2 (Syp) to tyrosine-phosphorylated IRS-1 (56) participates in insulin-stimulated MAP kinase activation (57–59). Because the formation of a SH-PTP2/IRS-1 complex has been shown to function as a potent activator of the Ras-Raf-MAP kinase cascade (60, 61), our results suggest that 3S-peptide-I may not alter the catalytic activity of SH-PTP2. Although more studies are necessary to evaluate the effect of 3S-peptide-I in the activity of several enzymes in vitro and on various metabolic and mitogenic responses of insulin, these data suggest that selective inhibition of the insulin receptor dephosphorylation by 3S-peptide-I causes a specific activation of downstream components of insulin signal transduction pathways. There are divergent views regarding which of the three phosphotyrosyl residues (1146, 1150, or 1151) contained in the catalytic domain of the insulin receptor is the primary target of the physiologically relevant PTPase(s) action in situ (4, 24, 25, 62–64). The selective sulfation of individual tyrosyl residues within a given peptide sequence is possible by using protected side-chains on the tyrosine(s) that we do not wish to modify. This methodology allows the development of monosulfated analogs of 3S-peptide-I and should demonstrate whether dephosphorylation of the insulin receptor is preferentially inhibited by the relative position of sulfotyrosine residues in peptide-I. Likewise, variations of the peptide scanning approach, which include the synthesis of a series of sulfotyrosylated peptides with different length or the construction of peptide libraries where individual amino acids are replaced by alanine (26, 28) shall define the optimal structure needed for the rational design of an insulin receptor-specific PTPase inhibitor.

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REFERENCES
1. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) Science 215, 185–187
2. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
3. Sun, X., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glashen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) Nature 377, 173–177
4. King, M. J., and Sale, G. J. (1993) Biochem. J. 292, 2435–2438
5. Meyerovitch, J., Backer, J. M., and Kahn, C. R. (1989) J. Clin. Invest. 84, 976–983
6. Tappia, P. S., Atkinson, P. G., Sharma, R. P., and Sale, G. J. (1993) Biochem. J. 292, 1–5
7. Ahmad, F., and Goldstein, B. J. (1995) Am. J. Physiol. 268, E932–E940
