Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 11-18

INHIBITION OF TYROSINE AUTOPHOSPHORYLATION OF THE SOLUBILIZED INSULIN RECEPTOR BY AN INSULIN-STIMULATING PEPTIDE DERIVED FROM BOVINE SERUM ALBUMIN

Akemichi Ueno[1]*, Naokatu Arakaki[1], Yoshiro Takeda[1], and Hajime Fujio[2]

[1]Department of Biochemistry, School of Dentistry,
The University of Tokushima, Tokushima 770, Japan

[2]Department of Immunochemistry, Research Institute for
Microbial Diseases, Osaka University, Osaka 565, Japan

Received January 20, 1987

SUMMARY: A polypeptide from a tryptic digest of bovine serum albumin potentiates glucose oxidation stimulated by insulin in isolated rat adipocytes. We studied whether this effect is related to a modification of the insulin receptor kinase. In a solubilized rat adipocytes receptor system, the peptide caused dose-dependent inhibition of the stimulation by insulin of phosphorylation of the 95,000 dalton subunit of insulin receptor. The peptide also inhibited stimulation by vanadate of tyrosine autophosphorylation of the β subunit of the receptor, though it enhanced vanadate-stimulated glucose oxidation. During the phosphorylation reaction, no phosphorylated forms of the peptide could be detected. The peptide had no effect on dephosphorylation of the phosphorylated β subunit of the insulin receptor. These results strongly suggest that the inhibition of phosphorylation by the peptide is due not to either simple substrate competition or activation of phosphoprotein phosphatase, but to specific inhibition of tyrosine-specific protein kinase.

An insulin-stimulating peptide (ISP) was isolated from a tryptic digest of bovine serum albumin (BSA) to study modulation of the action of insulin (1). It is a two-chain polypeptide consisting of 71 amino acid residues (calculated Mr=8,496), corresponding essentially to residues 115-143 and 144-184(185) of BSA connected to each other by a disulfide bond (2). The peptide increases both the sensitivity and the maximal responses of the cells to insulin on insulin-stimulated glucose oxidation, 2-deoxyglucose transport, and lipid synthesis from glucose in isolated rat adipocytes in an insulin-dependent manner. ISP has little effect on the specific binding of labeled insulin to

*Present address: Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637.

Abbreviations: ISP, insulin-stimulating peptide; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; p-APMSF, (p-amidinophenyl) methanesulfonyl fluoride hydrochloride.

0006-291X/87 $1.50

the receptor in rat adipocytes, and effectively protects insulin from degradation (3).

Several lines of evidence have indicated that the insulin receptor itself is a tyrosine-specific protein kinase, and that insulin (4,5) and some mimickers of insulin (6,7) stimulate the kinase activity of the receptor to phosphorylate itself as well as other proteins in intact cells or in a cell-free system.

In the present work, we used partially purified insulin receptors to study the correlation of the insulin-potentiating action and receptor phosphorylation. Results showed that ISP greatly decreased the extent of phosphorylation stimulated by insulin of the 95,000 dalton receptor subunit and that it also inhibited vanadate-enhanced phosphorylation, though it potentiated the effects of vanadate and insulin on glucose oxidation.

MATERIALS AND METHODS

Materials The following materials were purchased: crystalline porcine insulin (25.9 U/mg), BSA essentially free of fatty acid and globulin, and aprotinin from Sigma; wheat germ agglutinin coupled to agarose from Miles; collagenase from Cooper; N-acetyl-D-glucosamine and all reagents for NaDodSO$_4$/polyacrylamide gel electrophoresis from Nakarai (Kyoto); (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (p-APMSF), Triton X-100, and sodium orthovanadate from Wako (Osaka); D-[1-^{14}C]glucose from the Radiochemical Centre; [γ-^{32}P]ATP from ICN. All other chemicals were of the highest commercial grade available.

Preparation of ISP and assay of glucose oxidation ISP was purified as described previously (2). Preparation of isolated adipocytes (8) and assay of glucose oxidation (3,9) were performed by standard methods using epididymal fat pads of fed Sprague-Dawley rats weighing 180-200 g.

Preparation of a solubilized insulin receptor fraction Insulin receptors were solubilized and purified by wheat germ agglutinin affinity chromatography as described by Häring et al. (10). Adipocytes (50 ml of a suspension of approximately 3 X 10^6 cells/ml) were washed four times with phosphate-free Krebs-Ringer phosphate Hepes (KRPH) buffer (3) containing 0.5% BSA, 12 mM $NaHCO_3$, and 0.55 mM glucose, and then immediately frozen. The cells were lysed by three cycles of freeze-thawing in liquid nitrogen and a water bath, respectively, and the resultant cell lysate was centrifuged for 90 min at 200,000 X g. The precipitate was dissolved in 25 mM Hepes, 1% Triton X-100, 2 mM p-APMSF, and aprotinin (1 U/ml) and centrifuged for 60 min at the same speed as before. The supernatant (a solubilized membranous fraction) was then applied to a column of wheat germ agglutinin coupled to agarose and recycled five times through the column. The column was washed extensively with 25 mM Hepes buffer (pH 7.4) containing 0.1% Triton X-100, and then bound material was eluted in a final volume of 2 ml with buffer supplemented with 0.3 M N-acetyl glucosamine and used as the solubilized insulin receptor fraction within two days after chromatography.

Phosphorylation and dephosphorylation of insulin receptor Partially purified insulin receptors were incubated with or without insulin and/or vanadate in the presence or absence of ISP at 25°C for 1 h. Then the phosphorylation reaction was induced by incubating the receptors with 5 μM [γ-^{32}P]-ATP in 50 mM Hepes buffer, pH 7.4, containing 10 mM $MgCl_2$ and 2 mM $MnCl_2$ at 4°C for 60 min (10). The reaction was terminated by adding NaF (100 mM), sodium pyrophosphate (10 mM), EDTA (5 mM), and ATP (5 mM). Tyrosine-O-phosphorylated proteins containing the β subunit of the insulin receptor were identified by immunoprecipitation with antibodies against phosphotyrosine (11). After 16 h at 4°C, 150 μl of Protein A (Pansorbin; 10% w/v) was added and incubation was continued for 1 h at 4°C. The precipitate was then collected by centrifugaiton at 10,000 X g for 5 min at 4°C and washed twice with RIPA buffer (12) and once with 1% Triton and 0.1% $NaDodSO_4$. Immunoprecipitates were boiled for 3 min in Laemmli's sample buffer (13) in the presence or absence of 2-mercaptoethanol. The solubilized samples were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with one of the following gel systems: 7.5% acrylamide gel (13), a 4-18% linear gradient of acrylamide (acrylamide:bis-acrylamide=20:1), and 20.9% acrylamide containing a trace amount of NaCl (14). After electrophoresis, the slab gels were stained with Coomassie brilliant blue R250, destained, dried, and autoradiographed with Fuji RX film for 0.5 to 2 days at -70°C.

Dephosphorylation was studied by incubating the phosphorylated (for 15 min at 4°C), partially purified receptors in the presence of unlabeled ATP (1 mM) with or without ISP (2.5 μM) at 4°C, as described by Tamura et al. (15). At suitable times the reaction was terminated by adding a solution of NaF, EDTA, sodium pyrophosphate, and ATP at final concentrations of 100 mM, 5 mM, 10 mM, and 25 mM, respectively. Immunoprecipitation, $NaDodSO_4$/polyacrylamide gel electrophoresis, and autoradiography were then carried out as described above.

RESULTS AND DISCUSSION

As previously described, ISP stimulated $^{14}CO_2$ production from [1-^{14}C]-glucose by isolated rat adipocytes in the presence of various concentrations of insulin (3), though it had little effect alone. To determine whether the potentiation of insulin-stimulated glucose oxidation by ISP was correlated with alteration of the tyrosine kinase activity of the insulin receptor, we studied the effect of ISP on the state of phosphorylation of the insulin receptor. For this we incubated solubilized rat adipose insulin receptors with an equal amount of wheat germ-purified proteins and a non-saturating concentration of [γ-^{32}P]ATP. Fig. 1A shows autoradiograms of the phosphorylated glycoproteins. The major phosphorylated Mr 95,000 protein has been tentatively identified as the β subunit of the insulin receptor of rat adipocytes (10,16). On incubation of the solubilized fraction with insulin (10 nM), the incorporation of ^{32}P into this protein increased approximately 15-fold, as determined by scanning densitometry (Fig. 1A, lanes 1 and 3). On incubation with ISP (2.5 μM), the basal and insulin-dependent phosphorylations were both

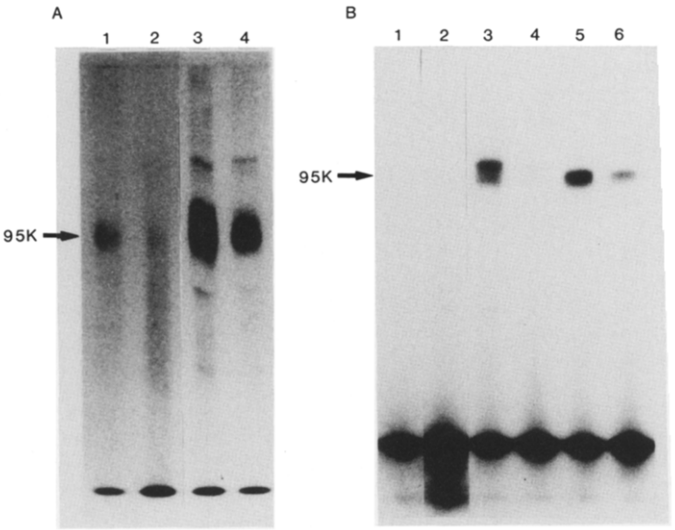

Fig. 1. Effect of ISP on phosphorylation of the solubilized insulin receptor fraction. Isolated rat adipocytes were solubilized and the receptor fraction was enriched by wheat germ agglutinin affinity chromatography as described in the text. Aliquots of the partially purified receptors were preincubated with or without 10 nM insulin or 0.2 mM vanadate in the presence or absence of 2.5 μM ISP at 25°C for 1 h. The phosphorylation assay was carried out as described in the text using approximately 9 μg protein and 0.1 mCi [γ-^{32}P]ATP per lane. Immunoprecipitation was performed (B) or not performed (A) with anti-phosphotyrosine antibodies. A, lane 1, no addition; lane 2, ISP (2.5 μM); lane 3, insulin (10 nM); lane 4, insulin (10 nM) and ISP (2.5 μM). B, lane 1, no addition; lane 2, ISP (2.5 μM); lane 3, vanadate (0.2 mM); lane 4, vanadate (0.2 mM) and ISP (2.5 μM); lane 5, insulin (10 nM); lane 6, insulin (10 nM) and ISP (2.5 μM).

clearly inhibited (Fig. 1A). To reduce the background of other proteins and clarify the tyrosine-specific phosphorylation, we used anti-phosphotyrosine antibodies in following experiments. These antibodies were highly specific for phosphotyrosine and did not cross-react with phosphoserine or phosphothreonine (11).

Preincubation of the same receptor fraction with insulin plus ISP (2.5 μM) caused 61% decrease in the extent of phosphorylation of the Mr 95,000 receptor subunit (Fig. 1B, lanes 5 and 6). The decrease in autophosphorylation of the β subunit of the insulin receptor was not attributable to either decreased insulin binding or decreased immunoprecipitation, since ISP itself did not affect insulin binding to adipocyte receptors (3) and the inhibition was also observed without antibodies (Fig. 1A).

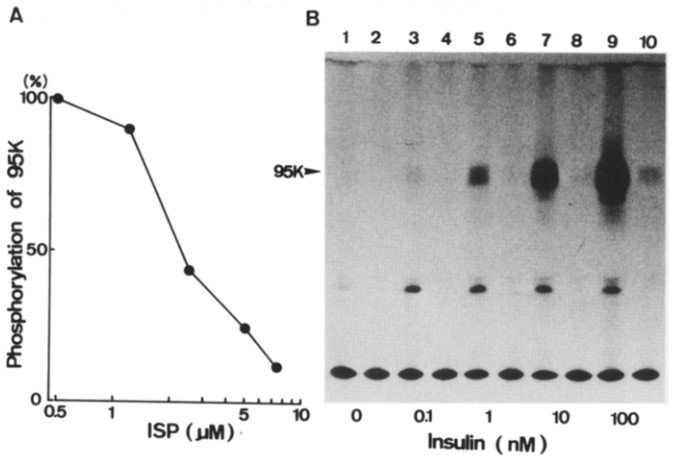

Fig. 2. Effects of the concentration of ISP or insulin on phosphorylation of the insulin receptor. (A) Dose-response curve for ISP. Aliquots of wheat germ agglutinin-purified insulin receptors (9 μg) were preincubated with 10 nM insulin in the presence of increasing concentrations of ISP. Phosphorylation, immunoprecipitation, $NaDodSO_4$/polyacrylamide (7.5%) gel electrophoresis, and autoradiography were performed as described in the text. The 95K subunit of the receptor was determined by densitometry scanning. The integral of the density peak in the absence of ISP is expressed as 100%. Values are means for two separate experiments. (B) Effect of insulin. After preincubation for 1 h with the indicated concentrations of insulin in the absence or presence of ISP (10 μM), the solubilized insulin receptor (9 μg) was incubated for 1 h with 5 μM [γ-^{32}P]ATP, 2 mM $MnCl_2$, and 10 mM $MgCl_2$. Other experimental conditions are described in the text.

ISP also potentiated vanadate-stimulated glucose oxidation. Incubation of adipocytes with 0.2 mM vanadate increased glucose oxidation to 19.0 ± 0.8 (±S.E., n=3) nmol CO_2 production per 10^6 cells per h, from the control level of 14.0 ± 0.9 (n=3). The addition of ISP together with vanadate strikingly enhanced the effect of vanadate, increasing glucose oxidation to 30.3 ± 1.0 (n=3) nmol/10^6 cells/h. This supports our previous conclusion that ISP increases the effects of insulin by some other mechanism besides inhibition of insulin degradation (3). When the receptor fraction was preincubated with 0.2 mM vanadate for 1 h at 4°C, the extent of phosphorylation of the β subunit of the insulin receptor was also greatly increased, confirming the reports of Tamura et al. (7,15). ISP (2.5 μM) also strongly inhibited (92%) vanadate-stimulated phosphorylation (Fig. 1B, lanes 3 and 4). Thus ISP may affect some step(s) common to the stimulations of phosphorylation by insulin and vanadate.

ISP inhibited 10 nM insulin-stimulated autophosphorylation of the β subunit dose-dependently (Fig. 2A), and was inhibitory at concentrations of over

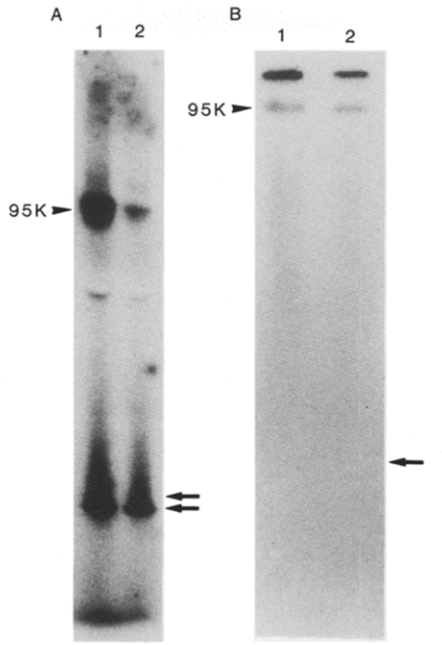

Fig. 3. Phosphorylation of ISP by the partially purified insulin receptor. The solubilized insulin receptor fraction was preincubated with 10 nM insulin in the presence (2) or absence (1) of 2.5 μM ISP. Then, the phosphorylated and immunoprecipitated samples were analyzed in one of the two following systems: A), treatment with sample buffer containing 5% (v/v) 2-mercaptoethanol followed by $NaDodSO_4$/polyacrylamide gel electrophoresis on a 4-18% gradient of acrylamide; B), treatment with sample buffer without 2-mercaptoethanol and then electrophoresis on 20.9% acrylamide containing NaCl. Arrows indicate bands of ISP.

1.2 μM. Using the same system, we examined the influence of insulin concentration on the effect of 10 μM ISP. Insulin caused marked and dose-dependent stimulation of ^{32}P incorporation into the Mr 95,000 protein, as observed by Häring et al. using anti-receptor antibodies (10). As shown in Fig. 2B, ISP (10 μM) almost completely blocked the stimulation of phosphorylation by insulin at the concentrations tested (Fig. 2B).

Next, we examined whether the inhibitory effect of ISP could be because it was a preferable substrate for phosphorylation, since it has seven possible sites per molecule that might be phosphorylated by a tyrosine kinase. However, we could not detect any phosphorylated ISP in two $NaDodSO_4$/polyacrylamide gel systems, NaCl-gel in the absence of 2-mercaptoethanol (Fig. 3B) and 4-18% gradient gel in the presence of the reductant (Fig. 3A), though both gel systems resolved ISP clearly (2). These results exclude the possibility that

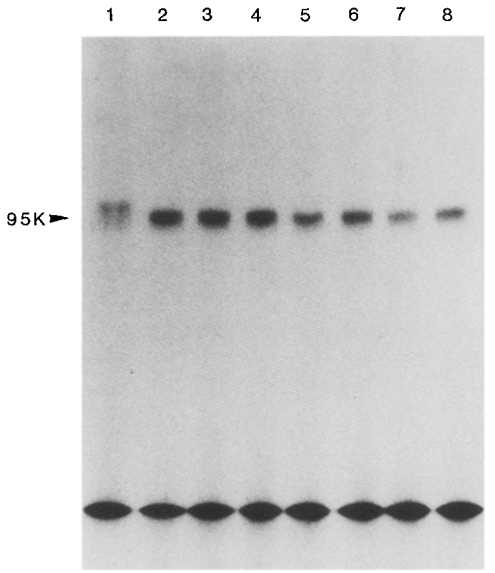

Fig. 4. Effect of ISP on dephosphorylation of insulin receptor. 9-μg portions of insulin receptor fraction were incubated without (lane 1) or with (lanes 2 to 8) insulin (100 nM) at 25°C for 1 h, and then incubated with [γ-^{32}P]ATP (5 μM, 160 μCi/nmol), $MnCl_2$ (2 mM), and $MgCl_2$ (10 mM) for 15 min at 4°C. ATP was then added to a final concentration of 1 mM with (lanes 4,6 and 8) or without (lanes 1,2,3,5 and 7) ISP (2.5 μM) and the mixtures were incubated for 0 min (lanes 1 and 2), 15 min (lanes 3 and 4), 30 min (lanes 5 and 6) or 60 min (lanes 7 and 8) at 4°C. The reaction was terminated as described in the text.

ISP inhibits autophosphorylation of the solubilized receptor fraction by competing as a substrate for the receptor tyrosine kinase. Another possibility was that ISP might activate phosphotyrosine phosphatase, thus lowering the extent of phosphorylation of the insulin receptor. To examine this possibility, we stopped the incorporation of ^{32}P into the receptor protein by adding a high concentration of unlabeled ATP and then tested the effect of ISP on dephosphorylation. As shown in Fig. 4, ISP did not stimulate dephosphorylation of the phosphorylated β subunit of the insulin receptor, but infact was rather inhibitory. These results strongly suggest that ISP inhibited tyrosine-specific protein kinase associated with the insulin receptor.

The physiological meaning of the present results is not clear at present. However, if activation of receptor-associated tyrosine kinase is one of the integrated events in the action of insulin (20), ISP might inhibit the

phosphorylation through a feedback mechanism, since it potentiates the action of insulin (1) and acts as an insulin mediator in the activation of pyruvate dehydrogenase both in intact adipocytes and in mitochondrial fractions in the absence of added insulin (Ueno, A., Arakaki, N., Nishikawa, S. and Takeda, Y. to be published).

ACKNOWLEDGEMENT

This work was supported in part by Grant-in-Aid for Scientific Research (nos. 59370012 and 59480139) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Ueno, A., Arakaki, N., Inoue, H., Oribe, T., and Takeda, Y. (1984) Biochem. Biophys. Res. Commun. 122, 1179-1185.
2. Ueno, A., Hong, Y-M., Arakaki, N., and Takeda, Y. (1985) J. Biochem. 98, 269-278.
3. Ueno, A., Arakaki, N., Oribe, T., and Takeda, Y. (1986) Mol. Cell. Biochem. 70, 121-130.
4. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) Science 215, 185-187.
5. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., and Kahn, C. R. (1982) Nature 298, 667-669.
6. Roth, R. A., Cassell, D. J., Maddux, B. A., and Goldfine, I. D. (1983) Biochem. Biophys. Res. Commun. 115, 245-252.
7. Tamura, S., Brown, T. A., Dubler, R. E., and Larner, J. (1983) Biochem. Biophys. Res. Commun. 113, 80-86.
8. Rodbell, M. (1964) J. Biol. Chem. 239, 375-380.
9. Fain, J. N., Reed, N., and Saperstein, R. (1967) J. Biol. Chem. 242, 1887-1894.
10. Häring, H.-U., Kasuga, M., and Kahn, C. R. (1982) Biochem. Biophys. Res. Commun. 108, 1538-1545.
11. Seki, J., Owada, M. K., Sakato, N., and Fujio, H. (1986) Cancer Res. 46, 907-916.
12. Brugge, J. S., and Erikson, R. L. (1977) Nature 269, 346-348.
13. Laemmli, U. K. (1970) Nature 227, 680-685.
14. Ito, K., Date, T., and Wickner, W. (1980) J. Biol. Chem. 255, 2123-2130.
15. Tamura, S., Brown, T. A., Whipple, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K., and Larner, J. (1984) J. Biol. Chem. 259, 6650-6658.
16. Häring, H., Kirsch, D., Obermaier, B., Ermel, B., and Machicao, F. (1986) J. Biol. Chem. 261, 3869-3875.
17. Simpson, I. A., and Hedo, J. A. (1984) Science 223, 1301-1304.
18. Pilch, P. F., Axelrod, J. D., and Czech, M. P. (1981) in Current Views on Insulin Receptors (Andreani, D., DePirro, R., Lauro, R., Olefsky, J., and Roth, J. eds) pp. 255-260, Academic Press, London.
19. Green, A. (1986) Biochem. J. 238, 663-669.
20. Kahn, C. R. (1985) Ann. Rev. Med. 36, 429-451.