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REGULATION OF INSULIN RECEPTOR KINASE ACTIVITY BY INSULIN MIMICKERS AND AN INSULIN ANTAGONIST

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Three agents which mimic insulin action in intact cells (concanavalin A, wheat germ agglutinin, and polyclonal insulin receptor antibody), mimicked insulin's ability to stimulate the kinase activity of purified insulin receptors. In contrast, monoclonal insulin receptor antibody, an antagonist of insulin action, did not stimulate the phosphorylation of the insulin receptor either in intact IM-9 cells or in purified receptor preparations. This antibody, however, antagonized the ability of insulin to stimulate the phosphorylation of the receptor both in intact cells and in the purified receptor. These studies with insulin mimickers and an insulin antagonist are consistent with a role for the kinase activity of the receptor mediating the actions of insulin.

The initial event in the action of insulin is its binding to a specific receptor protein located on the surface of target cells (1). After binding to its receptor, insulin elicits a variety of biological responses. The mechanism(s), however, whereby insulin elicits these responses is unknown. Recently, Kasuga et al reported that the β subunit of the insulin receptor is phosphorylated in the intact cell, and that insulin enhances this phosphorylation reaction (2). Subsequently, several lines of evidence have indicated that the insulin receptor is itself a tyrosine specific protein kinase and that insulin stimulates the kinase activity of the receptor to phosphorylate itself as well as other proteins (3-13). Since insulin regulates various enzymes via phosphorylation-dephosphorylation mechanisms (1,14), it has been hypothesized that the kinase activity of the receptor mediates some or all of the actions of insulin (2-13). For this hypothesis to be valid, known insulin mimickers which act through the insulin receptor should

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directly increase the kinase activity of the receptor, whereas insulin antagonists should inhibit insulin-stimulated kinase activity. The present study examines the effects on receptor kinase activity of an insulin antagonist, a monoclonal anti-receptor antibody, (15) and three insulin agonists, concanavalin A (16-19), wheat germ agglutinin (19) and polyclonal anti-insulin receptor antibody (20). In these studies, we find that the insulin mimickers, like insulin, stimulate receptor kinase activity. In contrast, the insulin antagonist has no effect by itself, but when added with insulin it antagonizes the stimulatory effects of the hormone.

MATERIALS AND METHODS

Materials:

The following were purchased: porcine insulin (27.3 USP units per mg) from Elanco; [^{32}P]orthophosphate from New England Nuclear; [γ-^{32}P]ATP from Amersham; wheat germ agglutinin coupled to agarose from Miles; concanavalin A, wheat germ agglutinin, N-acetyl-D-glucosamine and phenylmethylsulfonyl fluoride from Sigma.

Methods:

Intact cell experiments. IM-9 cells (20×10^6) were incubated with 2 mCi of [^{32}P]orthophosphate in 20 ml of RPMI 1640 medium without phosphate for 60 min at 37°. Cells were divided into four 5 ml portions and incubated for 5 min at 37° with the indicated additions. The cells were washed, lysed in the presence of phosphatase inhibitors and the insulin receptors were enriched on a wheat germ agglutinin affinity column as previously described (2). The material from this column was absorbed with monoclonal antibody to the receptor bound to Staphylococcus aureus through an affinity-purified rabbit anti-mouse Ig bridge (15). The immunoprecipitated proteins were solubilized and electrophoresed on a 7.5% polyacrylamide gel (15). After electrophoresis, the gels were stained, dried and autoradiographed with Kodak X-OMAT AR film using Dupont lightning plus enhancing screens.

Purified receptor experiments. Insulin receptors were purified from IM-9 cells after solubilization of the cells in 1% Triton X-100 in 50 mM Hepes, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 units/ml of bacitracin as previously described (3). In brief, the solubilized cells were centrifuged at 100,000 x g for 1 hour and the supernatant was passed over an 8 ml column of monoclonal anti-receptor antibody coupled to Affi-Gel 15. The column was washed sequentially with 100 ml of 50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF and 100 units/ml bacitracin, then 20 ml of the same buffer with the addition of 1 M NaCl, and then the receptor was eluted with 1.5 M $MgCl_2$ in 120 mM borate buffer, pH 6.5 with 0.1% Triton X-100. The receptor was further purified on a 5 ml column containing wheat germ agglutinin coupled to agarose. The receptor was purified from human placenta via the same procedure after dissociating 30 g of tissue in 60 ml of the solubilizing buffer in a Brinkman polytron.

The kinase activity of the purified receptor was measured after incubating the receptor with the indicated ligands for 60 min at 24° in 30 µl of a buffer containing 50 mM Hepes, pH 7.6, 0.1% Triton X-100, 150 mM NaCl and 2

mM $MnCl_2$. Then 5 μM [γ-^{32}P]ATP (20 Ci/mmole) was added and the reaction was allowed to proceed for 60 min at 24°. The samples were then made 1% in SDS and 5% in mercaptoethanol, heated to 100° for 1 min and electrophoresed on a 7.5% polyacrylamide gel. The gel was dried, the β subunit band identified by autoradiography and the appropriate portion of the gel was excised and the radioactivity determined in a liquid scintillation counter.

RESULTS

Initial studies were carried out with intact cultured human lymphocytes (IM-9 cells), a cell type with well-characterized insulin receptors (21). Insulin at 30 nM, as previously reported (2), caused a two-fold stimulation of the phosphorylation of the β subunit (M_r 95,000) of its receptor (Fig. 1). Next, an antagonist of insulin action, a monoclonal receptor antibody (15), was studied. In contrast to insulin, this antibody at 1 μM did not stimulate phosphorylation of the receptor in three separate experiments (Fig. 1). Moreover, this antibody inhibited the ability of insulin tc stimulate receptor phosphorylation (Fig. 1).

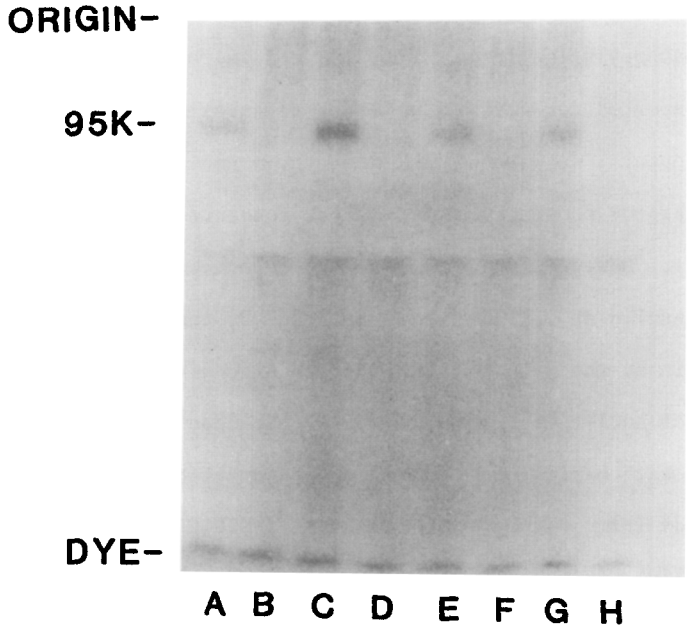


Figure 1 Effect of monoclonal antibody and insulin on receptor phosphorylation in intact IM-9 cells. Cells prelabeled with [^{32}P]orthophosphate were divided into aliquots and either buffer (A,B), insulin (30 nM) (C,D), anti-receptor antibody (1 μM) (E,F) or insulin (30 nM) together with anti-receptor antibody (1 μM) (G,H) was added. The lysates of these cells were precipitated with either monoclonal anti-insulin receptor (A,C,E,G) or normal IgG (B,D,F,H) and the precipitates were analyzed by SDS gel electrophoresis.

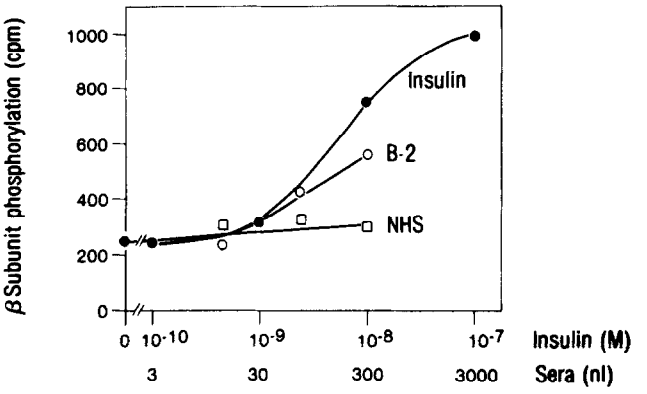
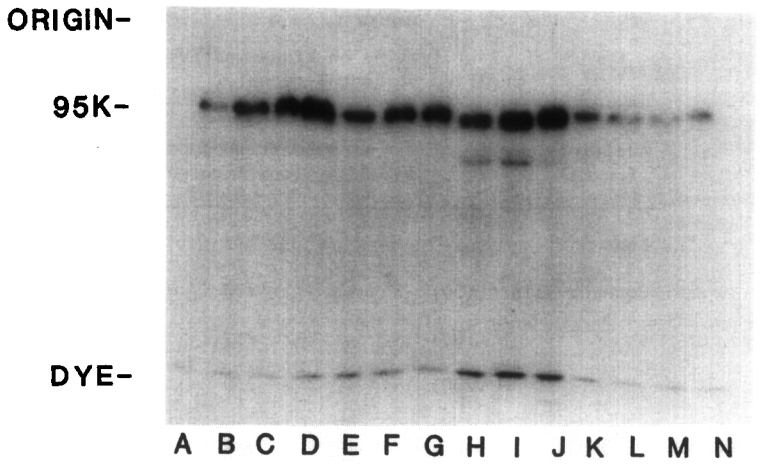


Figure 2 Stimulation of kinase activity of purified receptors by insulin and polyclonal anti-receptor antibody. Purified IM-9 insulin receptor was incubated with either insulin, antisera (B-2) or normal human sera (NHS) and the samples were analyzed by SDS gel electrophoresis.

To further study the regulation of insulin receptor kinase activity, purified insulin receptor preparations obtained from IM-9 lymphocytes and human placenta were utilized. With these preparations, as with whole cells, insulin stimulated receptor phosphorylation. Detectable effects of insulin were observed at 1 nM, and at 100 nM there was a 5-fold increase in receptor phosphorylation (Figs. 2 and 3), a dose response similar to those previously reported for purified receptor preparations (5-8,11,12). With purified receptors, as with whole cells, the monoclonal antibody did not stimulate receptor phosphorylation even at concentrations as high as 1 μM (Fig. 3, top). However, the antibody at 1 μM antagonized the stimulatory effects of insulin at 1, 10, and 100 nM (Fig. 3, top); in contrast, control immunoglobulin at the same concentrations had no effect (Fig. 3, top). This effect of anti-receptor antibody was dose-dependent; with insulin at 10 nM, a one-half maximal inhibition of phosphorylation was observed with 10 nM antibody (Fig. 3, bottom) and maximal inhibition (to control levels without insulin) was observed at 100 nM antibody.

Next, the effects of insulin mimickers on the kinase activity of purified insulin receptors were studied. Polyclonal antibodies to the insulin receptor, which have insulin-like actions in intact cells (20), mimicked the effects of insulin on stimulating receptor kinase activity (Fig. 2); 300 nl antisera B-2 (a 1:100 dilution) caused a 203±23% (n=3)



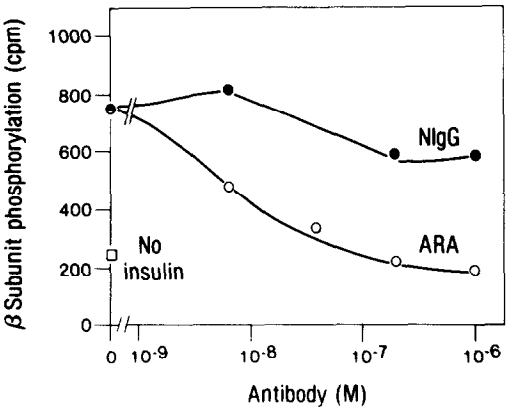


Figure 3 Top. Effect of monoclonal anti-receptor antibody on purified receptor kinase activity. Purified insulin receptor from IM-9 cells was incubated with either buffer (A), 1, 10 or 100 nM insulin (B,C or D, respectively), monoclonal anti-receptor antibody (1 μM) plus 1, 10 or 100 nM insulin (E,F or G, respectively), control antibody (1 μM) plus 1, 10 or 100 nM insulin (H,I or J, respectively), or either 1, 10, 100 or 1000 nM anti-receptor antibody (K,L,M or N, respectively). An autoradiogram of the gel is shown.

Figure 3 Bottom. Monoclonal antibody inhibits the insulin stimulated receptor kinase activity. Purified placental insulin receptor was incubated with 10 nM insulin in the presence of various concentrations of either monoclonal anti-receptor antibody (ARA) or normal antibody (NIgG). A control without insulin is also shown.

increase in β subunit phosphorylation. In contrast, normal human serum at the same concentrations was without effect. Plant lectins, another type of insulin mimicker (16-19), were also studied. Like insulin, both concanavalin A and wheat germ agglutinin at concentrations of 50 to 200 μg/ml stimulated receptor kinase activity from 2 to 5-fold (Table I).

Table I.

Effect of plant lectins on kinase activity of purified placental receptor

Additions (μg/ml)	β subunit phosphorylation (cpm incorporated)
None	72
Concanavalin A (50)	170
(100)	212
(200)	250
Wheat germ agglutinin (50)	190
(100)	236
(200)	396
Insulin (0.57)	320

DISCUSSION

Since the initial report of Kasuga et al demonstrating that insulin enhances the phosphorylation of its receptor (2), subsequent data have indicated that the insulin receptor is itself a tyrosine specific protein kinase (3-13). The question arises, therefore, as to the physiological role of the kinase activity of the receptor. One possibility is that insulin-induced phosphorylation of the receptor is related to the ability of insulin to regulate the expression of its receptor, the phenomenon termed down regulation (22,23). However, recently we have found that the monoclonal insulin receptor antibody can also down regulate the receptor (24). Since this antibody cannot enhance the phosphorylation of the receptor (this report), these results suggest that the increased phosphorylation of the receptor is not necessary for down regulation of the receptor.

An alternate hypothesis is that the kinase activity of the insulin receptor is important in mediating the biological effects of insulin. This hypothesis is attractive since in several instances insulin's effects are associated with changes in the state of phosphorylation of various proteins (25-27). The present data are consistent with this hypothesis because an

antagonist of insulin action was found not to stimulate the phophorylation of the receptor but instead antagonized insulin's ability to stimulate the kinase activity of the receptor in both intact cells and purified receptor preparations. Moroever, three agents which mimic insulin's effects on intact cells were found to mimic insulin's ability to stimulate the kinase activity of purified insulin receptors. These three agents (polyclonal antibody to the receptor, concanavalin A and wheat germ agglutinin) are all believed to act through the insulin receptor (16-18,20). In contrast, two agents (spermine and vitamin K_5) which mimic insulin effects on intact cells but are believed not to act through the receptor (28-31), have recently been reported not to stimulate the phosphorylation of the receptor in a cell-free system (8). Finally, vanadate, another mimicker of insulin action in the adipocyte (32,33), has recently been reported to enhance the degree of phosphorylation of the receptor in a cell-free system, possibly by inhibiting its dephosphorylation (34). Thus, it may be possible to further clarify the mechanism of action of the various mimickers of insulin action by their effects on the phosphorylation of the insulin receptor.

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