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MONOCLONAL ANTIBODIES TO THE HUMAN INSULIN RECEPTOR MIMIC A SPECTRUM OF BIOLOGICAL EFFECTS IN TRANSFECTED 3T3/HIR FIBROBLASTS WITHOUT ACTIVATING RECEPTOR KINASE

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The effects of four monoclonal antibodies to the alpha subunit of the human insulin receptor were studied in transfected mouse 3T3 fibroblasts expressing human insulin receptors (3T3/HIR). Three antibodies, MA-5, MA-20, and MA-51, mimicked insulin stimulation of the uptake of both 2-deoxy-D-glucose and α-aminoisobutyric acid, and S6 kinase activity. Antibody MA-5 also mimicked insulin stimulation of [^{3}H]thymidine incorporation and cell growth. Although these antibodies mimicked insulin stimulation of biological effects, they failed to significantly activate insulin receptor tyrosine kinase activity. These studies suggest, therefore, that the insulin receptor can signal a variety of cellular functions without stimulation of receptor kinase activity.

The interaction of insulin with target cells is mediated by a specific tetrameric glycoprotein receptor located in the plasma membrane (1). This receptor consists of two identical extracellular α-subunits (Mr 130,000 each) that bind the hormone and two identical transmembrane β-subunits (Mr 95,000 each) that contain tyrosine kinase on their cytoplasmic domains. After insulin binds to its receptor α-subunit, tyrosine kinase activity increases, followed by both β-subunit autophosphorylation on specific tyrosine residues and tyrosine phosphorylation of several cellular proteins. Although insulin acts via its receptor to produce a wide range of effects in many cell types, the mechanism(s) whereby the receptor generates transmembrane signals are unknown.

Several lines of evidence with mutant insulin receptors (2-5) and antibodies directed to the tyrosine kinase domain (6) suggest that insulin receptor tyrosine kinase activity may be involved in insulin action. In order to study the insulin receptor and insulin

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receptor tyrosine kinase, we have generated species specific monoclonal antibodies which bind to the α-subunit of the human insulin receptor (7,8). These antibodies interact with the human but not the rodent receptor (7). We have recently reported that certain of these antibodies mimic insulin stimulation of amino acid uptake in rat cultured hepatoma cells transfected with and expressing human insulin receptors (9). However, unlike insulin, in these and other cell types these antibodies failed to activate receptor kinase activity (7-9).

Since these monoclonal antibodies stimulate certain actions of insulin, it would be important to understand therefore whether in such cultured cells these antibodies mimic a wide spectrum of insulin effects. Mouse NIH 3T3 fibroblasts have few or no insulin receptors and are unresponsive to insulin (10). When human receptor cDNA is expressed in these cells (3T3/HIR cells) a variety of cellular functions become insulin sensitive (10,11). Since 3T3/HIR cells are an important new cell line for studies of insulin action, we have now used these cells as a model to investigate the spectrum of biological effects regulated by monoclonal antibodies, and their relationship to receptor kinase.

METHODS AND MATERIALS

2-deoxy-D-[1,2-^{3}H]glucose (30 Ci/mmol), [^{3}H]α-aminoisobutyric acid (33 Ci/mmol), [^{3}H]thymidine, (20 Ci/mmol) and [γ-^{32}P]ATP, (1,000-3,000 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Monoclonal antibodies to the human insulin receptor (MA-5, MA-10, MA-20, MA-51) were prepared as described by Forsayeth, et al. (7). NIH 3T3 mouse fibroblasts were transfected with a bovine papilloma virus/human insulin receptor cDNA construct (3T3/HIR cells) and expressed high levels of human insulin receptors (10^6/cell) (10). Cells were maintained in DME H-21 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator gassed with 5% CO_2.

To measure stimulation of [^{3}H]α-aminoisobutyric acid and [^{3}H]2-deoxy-D-glucose uptake, cells were grown to confluence in 16 mm multiwell plates. Eighteen hours prior to assay, the medium was changed to DME H-21 containing 0.5% BSA, 15 mM HEPES, pH 7.4, and 1 μM dexamethasone. At the time of assay the medium was changed and the cells were incubated with either insulin or monoclonal antibodies for 2 h and uptake measured as previously described (9).

To assay S6 kinase, cells were scraped off the plates and then sonicated. After centrifugation the supernatants were collected and cellular protein quantitated. The S6 kinase activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into rat pancreatic ribosomes prepared as described by Sung and Williams (12).

To measure stimulation of [^{3}H]thymidine incorporation, subconfluent 3T3/HIR cultures were shifted to serum-free medium containing 0.5% bovine serum albumin for 24 h to reduce basal DNA synthesis and insulin and monoclonal antibodies were then added to the serum free medium for 16 h. Next, [^{3}H]thymidine was added to the medium to yield a final concentration of 0.5 μCi/ml (0.075 μM) at 37°C for 2 h and [^{3}H]thymidine incorporation measured (13).

Two receptor kinase assays were employed. First, insulin receptors from 3T3/HIR cells were solubilized in 1% Triton X-100 containing 50 mM HEPES (pH 7.6), 1 mM PMSF, 2 mM Na-orthovanadate and 0.1 mg/ml aprotinin. Next, the solubilized cells were centrifuged at 100,000 xg and purifed on wheat germ agglutinin-agarose as previously described. The phosphorylation of histone 2B was carried out as described previously (8). Second, the intact cell method of Gherzi et al. was used (14). In brief, cells were incubated in phosphate-free minimal essential medium and [^{32}P]orthophosphate (0.25 mCi/ml) was added. After 90 min at 37°C, either 100 nM insulin or monoclonal antibodies was added and the incubations continued for 60 min. Cells were then cooled on ice, solubilized cell extracts centrifuged, and the receptors in the supernatants purified on wheat germ agglutinin-agarose. The receptors were then immunoprecipitated and subjected to SDS-PAGE, KOH treatment, and autoradiography.

RESULTS

Insulin stimulated 2-deoxy-D-glucose uptake (Figure 1a). A one half maximal effect was observed at approximately 100 pM and a maximal effect was seen between 1 nM and 10 nM. Three monoclonal antibodies (MA-5, MA-20, MA-51) also stimulated this function; antibody MA-10, and nonimmune IgG had no effect. MA-5, the antibody that was most

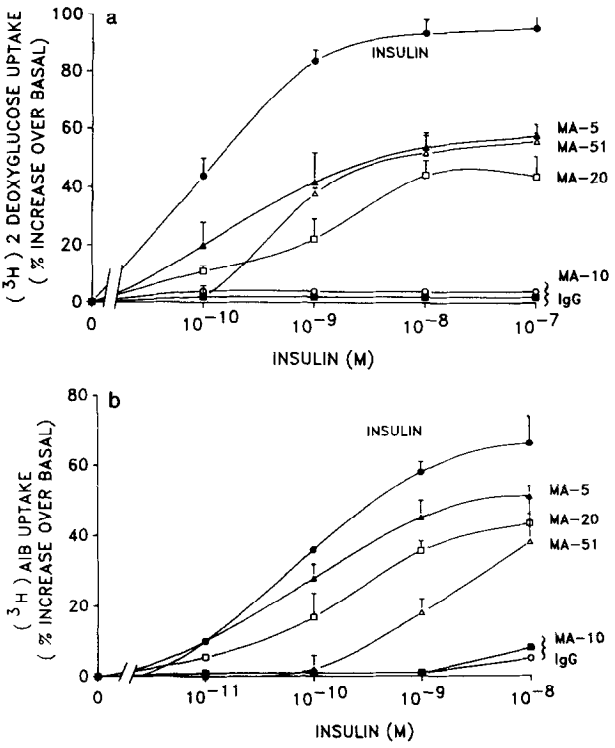

Figure 1. Stimulation of [^{3}H]2-deoxy-D-glucose uptake and [^{3}H]α-amino-butyric acid by insulin and monoclonal antibodies. Dose response curves are shown comparing stimulation of [^{3}H]2-deoxy-D-glucose uptake (a) and [^{3}H]α-aminoisobutyric acid uptake (b) in 3T3/HIR cells by insulin and monoclonal antibodies. Each point is the mean ± SEM of three separate experiments.

potent, had a sensitivity that was similar to that of insulin and its responsiveness was approximately 60% that of insulin.

Insulin also stimulated α-aminoisobutyric acid uptake (Figure 1b). A one half maximal effect was observed at a concentration of 100 pM and a maximal effect was seen at 10 nM. Uptake was also stimulated by monoclonal antibodies MA-5, MA-20 and MA-51. Antibody MA-5 had a sensitivity that was similar to that of insulin and a responsiveness that was 80% that of insulin. In contrast antibody MA-10 and IgG had no effects up to 1 nM, but both showed a small effect at 10 nM.

Insulin at 100 nM increased S6 kinase activity serveral fold (Figure 2). Three of the monoclonal antibodies MA-5, MA-20, and MA-51 at 100 nM, also increased S6 kinase

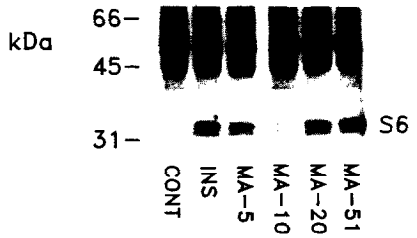

Figure 2. Autoradiograph of protein S6 phosphorylation by insulin, monoclonal antibodies and normal mouse IgG, all at 100 nM.

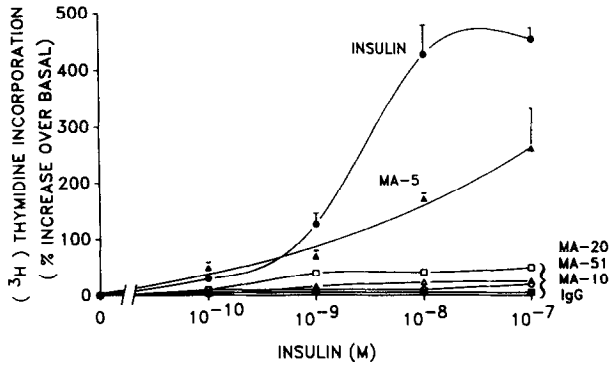

Figure 3. Insulin and monoclonal antibody stimulation of [^{3}H]thymidine incorporation into DNA. Dose response curves are shown for stimulation of [^{3}H]thymidine incorporation by insulin, and monoclonal antibodies. Each value is the mean ± SEM of 3 separate experiments.

activity to values approaching that of insulin. When cells were treated with either MA-10 or normal mouse IgG there was little or no stimulation of S6 kinase activity.

Insulin stimulated [^{3}H]thymidine incorporation into 3T3/HIR cells (Figure 3). An effect was detectable between 100 pM and 1 nM and a maximal effect was observed between 10 nM and 100 nM. Antibody MA-5 but not the other antibodies, also stimulated this function but to a lesser extent than insulin. After 24 h, both insulin and antibody MA-5 increased cell division as measured by an increase in cell number (insulin 40±13% increase, MA-5 60±9% increase, mouse IgG 3±1.4% increase, mean ± SEM, n=3). In 3T3 cells not transfected with human insulin receptor, insulin and monoclonal antibodies had little or no ability to stimulate the uptake of 2-deoxy-D-glucose and α-aminoisobutyric acid uptake, and [^{3}H]thymidine incorporation and S6 kinase activity (data not shown).

With solubilized insulin receptors, insulin induced a 7 fold stimulation of the *in vitro* phosphorylation of the exogenous substrate histone 2B (Figure 4a). A small effect was observed at 100 pM and a maximal effect was observed at 10 nM. In contrast, neither IgG nor monoclonal antibodies MA-10, MA-20, and MA-51 stimulated this function. At 10 nM, MA-5 had a small but significant effect on histone 2B phosphorylation. Employing another artificial substrate, poly (Glu,Tyr) none of the antibodies including MA-5 increased its phosphorylation (data not shown). When intact cells were labeled with [^{32}P]orthophosphate and treated with 100 nM insulin, there was a 20-50 fold increase in receptor β-subunit autophosphorylation (Figure 4b). The antibodies at 100 nM did not increase tyrosine phosphorylation of the β-subunit. A small amount, β-subunit autophosphorylation was seen with antibodies MA-5, MA-20 and MA-51. However phosphoaminoacid analysis of the phosphorylated amino acids indicated that this increase was due to an increase in phosphoserine content and not in phosphotyrosine content.

DISCUSSION

The present studies demonstrate that three species-specific monoclonal antibodies to the human insulin receptor mimic insulin stimulation of a spectrum of biological functions in 3T3/HIR cells including 2-deoxy-D-glucose and α-aminoisobutyric acid

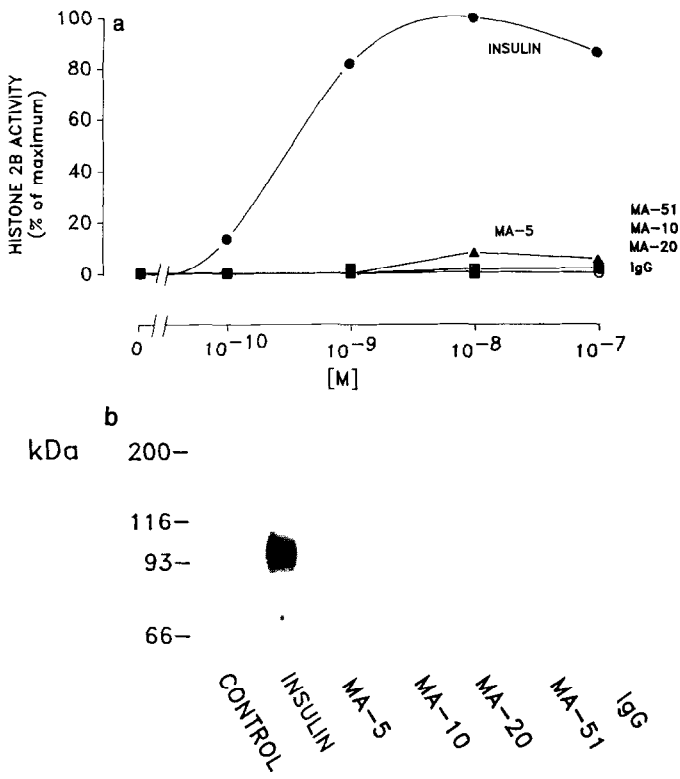

Figure 4. (a). Activation of 3T3/HIR insulin receptor tyrosine phosphorylation of histone 2B by insulin, monoclonal antibodies and normal mouse IgG. (b). Autophosphorylation of [^{32}P]orthophosphate-labeled 3T3/HIR cells by insulin, monoclonal antibodies and normal mouse IgG, all at 100 nM.

uptake, S6 kinase activity and thymidine incorporation. Two of these antibodies (MA-20, and MA-51) stimulated these functions without significantly increasing receptor kinase activity. The third antibody (MA-5), showed a slight stimulation of the phosphorylation of the exogenous substrate histone 2B (but not poly(Glu, Tyr) when solubilized receptors were employed. Since all three of these antibodies increased 2-deoxy-D-glucose and AIB uptake, and S6 kinase acitivity, it is likely that the stimulation of these 3 functions occurred without activation of receptor kinase.

In our studies MA-5 but not the other antibodies significantly increased [^{3}H]thymidine incorporation. Since MA-5 had a small effect in in vitro on histone 2B phosphorylation, in the present studies it could not be ruled that on this function MA-5 was mimicking insulin action by a small activation of receptor kinase in intact cells. However, Siddle and colleagues have recently reported in 3T3/HIR cells that several other monoclonal antibodies to the human insulin receptor can increase [^{3}H]thymidine incorporation without activation of receptor kinase (15). Therefore it is likely that, for this function as well as other functions, tyrosine kinase activation is not a necessary step in insulin receptor signalling.

Similar data concerning our monoclonal antibodies and receptor kinase activity were previously observed in human adipocytes, IM-9 lymphocytes, HEP-G2 hepatoma cells and HTC hepatoma cells expressing human insulin receptors (7-9). In all these cell types, the

antibodies stimulated these functions without significantly increasing receptor tyrosine kinase activity. Others have reported that antibodies to the IGF-I receptor can mimic a variety of biological functions without activation of receptor kinase including [^{3}H]thymidine incorporation (17). All of these data suggest therefore that activation of receptor kinase activity may not be a prerequisite for transmembrane signalling by insulin and related receptors.

In contrast to 3T3/HIR cells, the antibodies failed to mimic insulin action in nontransfected 3T3 cells, indicating that they are acting via the transfected human receptor and not via either the mouse insulin receptor or other 3T3 cell surface proteins. Moreover, stimulatory effects were not seen with either non-immune mouse IgG or with monoclonal antibody MA-10. MA-10 is an insulin antagonist in 3T3/HIR and other cells (13,18). This latter observation with MA-10 indicates therefore that the binding of a monoclonal antibody with the insulin receptor per se is not sufficient to generate biological signals.

These studies, however, do not suggest that insulin receptor kinase activity is unnecessary for insulin action. By employing antibodies directed against specific regions of the insulin receptor β-subunit, it has been shown that autophosphorylation of the insulin receptor β-subunit induces a conformational change (19) which may be the mechanism whereby the insulin-activated receptor influences the function of other cellular elements. We have previously reported that the interaction of monoclonal antibodies with the insulin receptor alters the conformation of its α-subunit (20). It is possible, therefore, that these antibodies also alter the structure of the β-subunit, inducing in it a conformation which is similar to the conformation induced by insulin mediated autophosphorylation.

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