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Monoclonal antibodies to the human insulin receptor that activate glucose transport but not insulin receptor kinase activity

(tyrosine kinase)

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ABSTRACT **Three mouse monoclonal antibodies were produced that reacted with the α subunit of the human insulin receptor. All three both immunoprecipitated ^{125}I-labeled insulin receptors from IM-9 lymphocytes and competitively inhibited ^{125}I-labeled insulin binding to its receptor. Unlike insulin, the antibodies failed to stimulate receptor autophosphorylation in both intact IM-9 lymphocytes and purified human placental insulin receptors. Moreover, unlike insulin, the antibodies failed to stimulate receptor-mediated phosphorylation of exogenous substrates. However, like insulin, two of the three antibodies stimulated glucose transport in isolated human adipocytes. One antibody, on a molar basis, was as potent as insulin. These studies indicate, therefore, that monoclonal antibodies to the insulin receptor can mimic a major function of insulin without activating receptor kinase activity. They also raise the possibility that certain actions of insulin such as stimulation of glucose transport may not require the activation of receptor kinase activity.**

The binding of insulin to its receptor on the surface of target cells initiates the various actions of the hormone (1, 2). This receptor is a tetramer with two extracellular α subunits containing the hormone binding site and two transmembrane β subunits containing tyrosine kinase activity on their cytoplasmic domains (1, 2). These four subunits are linked by sulfhydryl and hydrophobic bonds. After insulin binding, receptor kinase is activated, followed rapidly by β-subunit autophosphorylation on tyrosine residues. This autophosphorylation process can be demonstrated in both purified receptor preparations and intact cells (1–3). With purified receptors, exogenous substrates are phosphorylated on tyrosine residues. With intact cells, autophosphorylation is also followed by tyrosine phosphorylation of cellular proteins. On the basis of these observations, it has been proposed that enhanced receptor kinase activity may mediate one or more of the actions of insulin (1–3).

Regulation of the transport of glucose into target cells is a major homeostatic action of insulin (4), but the relationship between glucose transport and insulin receptor kinase remains to be defined. It is possible to produce monoclonal antibodies that react with the α subunit of the human insulin receptor (5). We have now produced two monoclonal antibodies to the human insulin receptor that mimic the stimulation of glucose transport by insulin. Accordingly, we have examined the effects of these monoclonal antibodies on insulin receptor kinase activity to further understand the relationship between glucose transport and receptor kinase.

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MATERIALS AND METHODS

Monoclonal Antibody Production. Highly purified insulin receptors were prepared from fresh human term placentas by homogenization, solubilization, differential centrifugation, and chromatography on agarose coupled to a monoclonal antibody, followed by chromatography on agarose coupled to wheat germ agglutinin (6). Female BALB/c mice (6–8 weeks old) were then injected thrice at monthly intervals with 1–2 μg of receptor emulsified in Freund's complete adjuvant. After immunization, monoclonal antibodies were produced by fusing splenic lymphocytes to FO cells as described (5). Three monoclonal antibodies were produced (MA-5, MA-10, and MA-20) that inhibited the binding of insulin to its receptor. Antibody MA-5 was an IgG_1, and MA-10 and MA-20 were both IgG_{2b}s, as determined by specific antisera (Miles).

Preparation of Isolated Adipocytes. Nondiabetic subjects, after giving informed consent, underwent subcutaneous adipose tissue biopsy as described by Foley and co-workers (7). Isolated adipocytes were prepared by a modified collagenase digestion as described by Pederson and Gliemann (8).

^{125}I-Labeled Insulin (^{125}I-Insulin) Binding in Adipocytes. Freshly isolated human adipocytes at 20% lipocrit (±200,000 cells per ml) were incubated with ^{125}I-insulin (100 pM) in the absence and presence of either unlabeled insulin or monoclonal antibodies for 2 hr at 22°C in Hepes/Krebs–Ringer bicarbonate buffer (pH 7.4) containing bovine serum albumin (30 mg/ml) and glucose (2 mM). Aliquots of the incubation mixture were layered on silicone oil and bound and free hormone were separated by centrifugation. Nonspecific binding, determined in the presence of 1 μM insulin, was subtracted to determine specific ^{125}I-insulin binding. Degradation of ^{125}I-insulin as determined by the trichloroacetic acid method was <10% of total.

Measurement of Glucose Transport. The method of Foley and colleagues was used (7). Isolated adipocytes were incubated at 20% lipocrit in Hepes/Krebs–Ringer bicarbonate buffer containing bovine serum albumin (30 mg/ml) and trace D-[U-^{14}C]glucose (300 nM) for 1 hr at 37°C. The intraassay variation was <10% and data were expressed as fl per cell per s (7). Adipocyte-bound insulin was determined by centrifugation of the cells through silicone oil.

Immunoprecipitation of ^{125}I-Insulin Receptors. IM-9 cells (10^8) were washed thrice and then resuspended in 20 ml of 20 mM phosphate buffer (pH 7.4) containing 154 mM NaCl. The following reagents were then added: 0.2 ml of D-glucose (1 M), 0.1 ml of glucose oxidase (8 mg/ml), 0.5 ml of lactoper-

Abbreviation: ^{125}I-insulin, ^{125}I-labeled insulin.

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oxidase (5 mg/ml), and 5 mCi of $Na^{125}I$ (1 Ci = 37 GBq). The cells were then shaken for 30 min at 22°C. Next the cells were washed thrice, solubilized, and the receptors were immunoprecipitated with monoclonal antibodies (6). The immunoprecipitates were washed, solubilized, and analyzed by gel electrophoresis under reducing conditions followed by autoradiography (6).

Insulin Receptor Autophosphorylation. Wheat germ agglutinin-purified adipocyte receptors were prepared by the method of Sinha *et al.* (9). Solubilized receptors (5 μg of protein) were incubated for 16 hr at 4°C in the absence and presence of insulin and monoclonal antibodies (100 pM to 100 nM) in a volume of 75 μl. Then, [γ-^{32}P]ATP (100 μM) was added in the presence of Glu^{80}–Tyr^{20} (2.5 mg/ml) (Sigma)/10 mM $MgCl_2$/0.5 mM $MnCl_2$. After 30 min at 22°C, the reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid containing 10 mM pyrophosphate, and then 100 μl of bovine serum albumin (30 mg/ml). The precipitated proteins were washed four times with 10% trichloroacetic acid, and the final pellet was dissolved in 1 ml of 0.5 M NaOH and counted in a liquid scintillation counter. Blanks run without Glu^{80}–Tyr^{20} gave negligible amounts of acid-precipitable radioactivity.

Purified human placental insulin receptors (50 ng) were incubated in 50 mM Hepes buffer (pH 7.5) containing 0.1% (vol/vol) Triton X-100, 150 mM NaCl, bacitracin (2 mg/ml), 1 mM phenylmethylsulfonyl fluoride, 2 mM $MnCl_2$, and either insulin or monoclonal antibodies (100 nM) in a volume of 20 μl. For histone 2B studies, histone 2B (Sigma) was added to a concentration of 0.5 mg/ml. This mixture was incubated for 60 min at 24°C. Next, [γ-^{32}P]ATP (New England Nuclear; 40 Ci/mmol) was added to a final concentration of 5 μM and the incubation was continued for a further 60 min. The phosphorylation reaction was terminated by the addition of a mixture of $NaDodSO_4$ (20 mg/ml), 2% (vol/vol) glycerol, bromophenol blue (2 mg/ml), and 2.5 M 2-mercaptoethanol. Samples were then boiled for 3 min and electrophoresed on a 7.5% polyacrylamide gel; the radioactive bands were then localized by autoradiography.

[^{32}P]Orthophosphate Labeling of IM-9 Lymphocytes. [^{32}P]Orthophosphate labeling of IM-9 cells was accomplished by resuspending cells at 10^6 cells per ml in phosphate-free culture medium supplemented with 25 mM Hepes buffer (pH 7.4). Next, [^{32}P]orthophosphate was added to 0.4 mCi/ml, and the cells were incubated for 60 min at 37°C. Then, either insulin or monoclonal antibodies were added (all at 100 nM) for a further 15 min. After incubation and washing, the cells were solubilized and the solubilized receptors were purified by means of wheat germ agglutin/agarose chromatography (6). The receptors were precipitated with a rabbit polyclonal antibody (6) and the immunoprecipitates were analyzed by gel electrophoresis and autoradiography.

RESULTS

Monoclonal Antibodies Are Directed Against the α Subunit of the Human Insulin Receptor. The three monoclonal antibodies used in this study (MA-5, MA-10, and MA-20) were directed against sites on the α subunit of the human insulin receptor. All the antibodies precipitated ^{125}I-insulin receptors from IM-9 lymphocytes (Fig. 1). Furthermore, the three antibodies competitively inhibited ^{125}I-insulin binding to its receptor in both isolated human adipocytes (Fig. 2), and in cultured IM-9 lymphocytes and HEP-G2 hepatoma cells (19). Antibody MA-10 was equipotent to insulin, whereas antibodies MA-5 and MA-20 were 1/10th as potent. The same potency ratios were also seen in IM-9 and HEP-G2 cells. Although the antibodies readily immunoprecipitated insulin receptors from human cells, they did not react with receptors from rat and mouse cells.

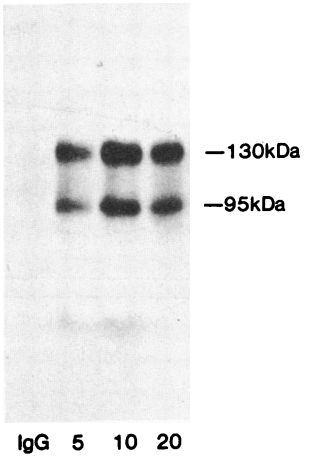

FIG. 1. Immunoprecipitation of ^{125}I-insulin receptors from IM-9 lymphocytes. Intact IM-9 cells were labeled with ^{125}I as described. The cells were then washed and solubilized, and the solubilized receptors were immunoprecipitated with either normal IgG or the three monoclonal antibodies (MA-5, MA-10, and MA-20).

Monoclonal Antibodies and Receptor Kinase Activity. With semipurified adipocyte insulin receptors, insulin induced a dose-dependent stimulation of the phosphorylation of the synthetic substrate Glu^{80}–Tyr^{20} (Fig. 3*a*). In contrast, none of the monoclonal antibodies influenced this parameter. Because of the limited availability of human adipocytes, additional studies were carried out with IM-9 cells and human placenta particles.

We next examined whether the antibodies influenced the autophosphorylation of highly purified human placental insulin receptors (Fig. 3*b*). In contrast to the stimulatory effect of insulin, the antibodies did not increase β-subunit autophosphorylation. In addition, we examined whether the antibodies influenced the ability of the placental insulin receptors to phosphorylate an exogenous substrate. When purified placental insulin receptors were incubated in the presence of insulin, the degree of phosphorylation of histone 2B was markedly enhanced (Fig. 3*c*). However, none of the antibodies increased histone 2B phosphorylation. Then, we examined whether these antibodies influenced insulin receptor autophosphorylation in intact cells. Insulin induced a severalfold increase in the autophosphorylation of the insulin receptor β subunit in IM-9 lymphocytes (Fig. 4), a cell type that is relatively abundant in insulin receptors (10). However, when IM-9 cells were incubated in the presence of the monoclonal antibodies, there was no stimulation of receptor kinase activity.

Monoclonal Antibodies and Glucose Transport. Next, we investigated whether these antibodies mimicked the effect of

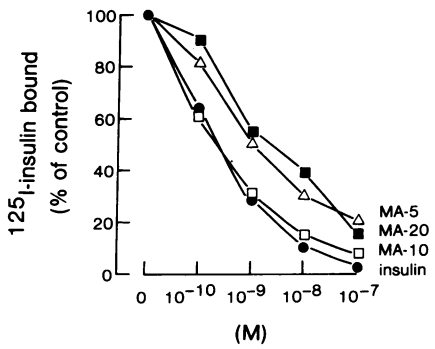

FIG. 2. Effect of monoclonal antibodies (MA-5, MA-10, and MA-20) and insulin to competitively inhibit ^{125}I-insulin binding in human adipocytes. A representative of three experiments is shown.

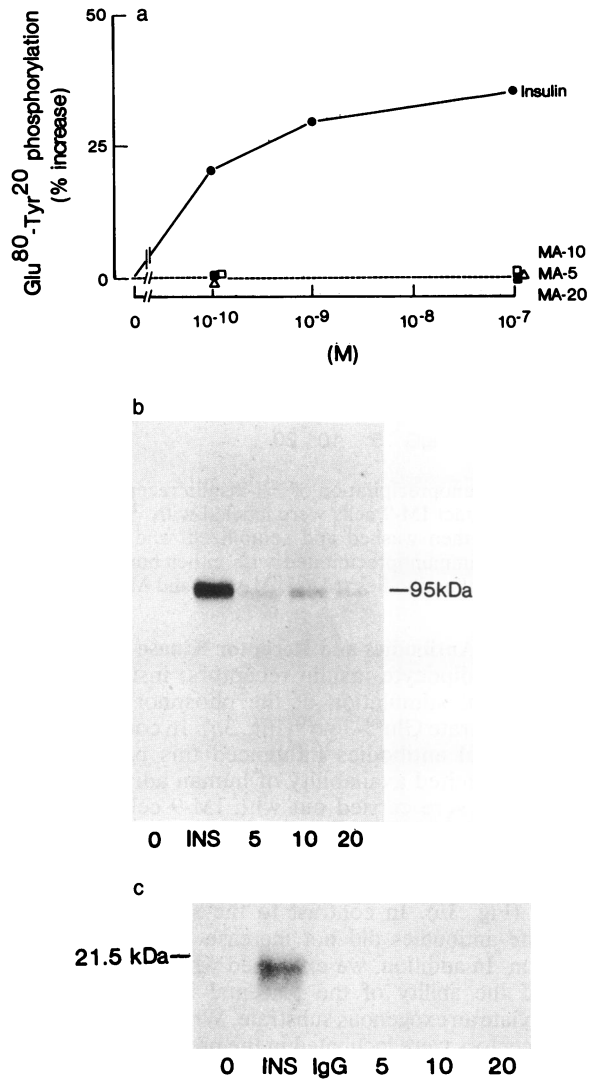

FIG. 3. Effect of insulin and monoclonal antibodies (MA-5, MA-10, and MA-20) on receptor kinase activity. (*a*) Adipocyte receptor phosphorylation of Glu^{80}–Tyr^{20}. (*b*) Placental receptor autophosphorylation. (*c*) Placental receptor phosphorylation of histone 2B.

insulin on glucose transport in human adipocytes. Insulin induced a detectable effect on glucose transport at 10 pM and

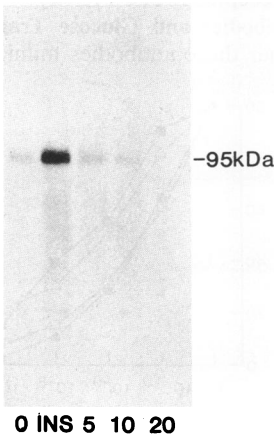

FIG. 4. Effect of insulin and monoclonal antibodies (MA-5, MA-10, and MA-20) on insulin receptor autophosphorylation in intact IM-9 lymphocytes.

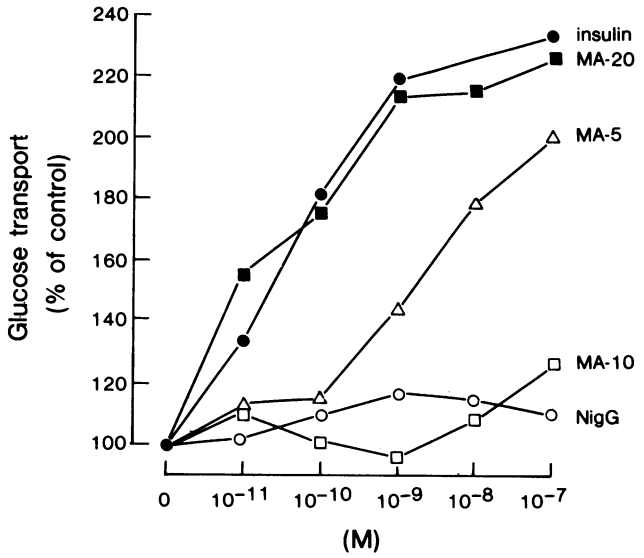

FIG. 5. Stimulation of glucose transport in human adipocytes by insulin and monoclonal antibodies (MA-5, MA-10, and MA-20). A representative of three experiments is shown.

a maximal effect at 100 nM (Fig. 5). Both nonimmune mouse IgG and MA-10 had no effect on this function. In contrast, both antibodies MA-5 and MA-20 stimulated glucose transport in human adipocytes. On a molar basis, MA-20 was as potent as insulin. MA-5 was less potent than MA-20, but at 100 nM the effect of MA-5 approached that of insulin and MA-20.

In the presence of 100 pM insulin, glucose transport was stimulated by 54.4% over basal (Table 1). MA-10 at 100 nM did not stimulate glucose transport, whereas 100 nM MA-5 and MA-20 both stimulated glucose transport. When adipocytes were incubated in the presence of insulin and antibodies, MA-10 abolished the effect of insulin on glucose transport, whereas with MA-5 and MA-20 no additional effect was observed above the effects of the antibodies themselves.

DISCUSSION

In the present study, we used three monoclonal antibodies to the human insulin receptor to investigate the relationship between the binding of insulin to its receptor, the subsequent activation of the β-subunit kinase activity, and stimulation of glucose transport. Several lines of evidence indicated that the three antibodies were specific for the α subunit of the insulin receptor. First, all antibodies inhibited ^{125}I-insulin binding to human adipocytes and other human cells. Second, they immunoprecipitated insulin receptors from ^{125}I-labeled cells.

Table 1. Combined effects of insulin and monoclonal antibodies (MA-5, MA-10, and MA-20) on glucose transport

	Glucose transport, fl per cell per s	% increase
Control	45.8 ± 6.1	
Insulin (100 pM)	70.7 ± 14.9	54
Antibody MA-5 (100 nM)	86.6 ± 17.1	89
Antibody MA-10 (100 nM)	53.4 ± 7.7	17
Antibody MA-20 (100 nM)	91.4 ± 20.3	100
Insulin (100 pM) + MA-5 (100 nM)	84.7 ± 21.0	85
Insulin (100 pM) + MA-10 (100 nM)	50.9 ± 5.8	11
Insulin (100 pM) + MA-20 (100 nM)	90.3 ± 17.5	97

Human adipocytes were incubated for 60 min at 37°C with 500 nM D-[U-^{14}C]glucose in the absence and presence of insulin (100 pM) and monoclonal antibodies (100 nM). Means ± SEM are given from three separate experiments.

Third, when the β subunit of the receptor was destroyed with collagenase (11), the antibodies still precipitated the isolated α subunit (19).

Next, we investigated whether the antibodies could activate receptor kinase activity. In contrast to insulin, the antibodies did not activate insulin receptor kinase activity in intact IM-9 cells and with purified placental and semipurified adipocyte insulin receptors. These results indicated, therefore, that antibodies may directly interact with the α subunit of the insulin receptor without influencing the kinase activity of the β subunit.

Then we investigated whether the antibodies could influence a rapid well-characterized effect of insulin, stimulation of glucose transport. Two of the antibodies, MA-5 and MA-20, mimicked the effect of insulin on this function. Since the antibodies did not activate receptor kinase activity, we conclude that antibodies are able to stimulate glucose transport without affecting this function. Moreover, since the antibodies interact with the binding subunit of the insulin receptor, it is also possible that insulin activates glucose transport independently of its effects on receptor kinase activity.

In a study of intact rat adipocytes, a polyclonal antiserum to the insulin receptor from a patient with extreme insulin resistance mimicked insulin stimulation of glucose transport, but it did not increase insulin-induced β-subunit autophosphorylation (12). In another study with semipurified rat liver insulin receptors, two other such antisera stimulated lipogenesis in rat adipocytes but failed to increase β-subunit autophosphorylation and phosphorylation of an exogenous substrate (13). Thus, these studies suggested that receptor kinase activity may not mediate all the actions of insulin. However, the interpretation of these data with polyclonal antisera was complicated by the observations that antisera from patients contain multiple populations of antibodies that have differential effects on the insulin receptor (14). However, in view of the present studies with monoclonal antibodies, the data indicate that various types of antibodies can mimic insulin action without stimulating receptor kinase activity.

Recently, Ellis *et al.* (15) performed site-specific mutagenesis of human insulin receptor cDNA and transfected the mutated receptors into Chinese hamster ovary cells. One or both of the tyrosine residues 1162 and 1163, which are potential autophosphorylation sites, were converted to phenylalanines and the ability of insulin to stimulate the uptake of the glucose analogue, 2-deoxy-D-glucose, was studied. Both of these mutations reduced the ability of insulin to stimulate this function. The authors concluded, therefore, that insulin receptor kinase activity and autophosphorylation are important for the stimulation of glucose uptake by insulin. These data, however, may not be in conflict with the present study. First, the substitution of phenylalanines for tyrosines in the receptor β subunit may induce structural alterations in the receptor that could block transmembrane signaling by a mechanism other than by inhibition of autophosphorylation. Second, these authors did not study the transport of glucose but rather the uptake of 2-deoxy-D-glucose, a function that requires both the transport and ATP-dependent phosphorylation of this sugar. Moreover, the cells in this study had been pretreated for a prolonged period with unlabeled 2-deoxy-D-glucose, a procedure known to lower ATP levels. Thus, the authors may have been studying the effect of insulin on biological parameters other than glucose transport.

In addition to stimulation of glucose transport, insulin has effects on other functions such as enzyme activation, protein and RNA synthesis, and mitogenesis. Studies with various oncogenes have indicated that tyrosine kinase activity is important for their oncogenic activity (16, 17). Moreover, a recent study has suggested that the injection of monoclonal antibodies to the β subunit of the insulin receptor may inhibit subsequent stimulation of amphibian oocyte maturation (18). It is possible, therefore, that receptor tyrosine kinase activity may mediate certain actions of insulin.

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