Anti-(insulin receptor) monoclonal antibody-stimulated tyrosine phosphorylation in cells transfected with human insulin receptor cDNA

Nicholas P. J. BRINDLE,* Jeremy M. TAVARE,† Martin DICKENS,† Jonathan WHITTAKER‡ and Kenneth SIDDLE*§

*Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K., †Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K., and ‡Division of Endocrinology, Department of Medicine, Health Science Center, State University of New York at Stony Brook, Stony Brook, NY 11794-8154, U.S.A.

The effects of insulin and anti-(insulin receptor) monoclonal antibodies on tyrosine phosphorylation were investigated in fibroblasts transfected with human insulin receptor cDNA (NIH 3T3HIR3.5 cells) using anti-phosphotyrosine immunoblotting. Insulin increased levels of tyrosine phosphorylation in two major proteins of molecular mass 97 kDa (pp97, assumed to be the insulin receptor β -subunit) and 185 kDa (pp185). Insulin-mimetic anti-receptor antibodies also stimulated tyrosine phosphorylation of both pp97 and pp185. The observation of antibody-stimulated pp97 phosphorylation, as detected by immunoblotting, is in contrast with previous data which failed to show receptor autophosphorylation in NIH 3T3HIR3.5 cells labelled with [32 P]P_i. The effect of insulin on pp97 was maximal within 1 min, but the response to antibody was apparent only after a lag of 1–2 min and rose steadily over 20 min. The absolute level of antibody-stimulated phosphorylation of both pp97 and pp185 after 20 min was only about 20 % of the maximum level induced by equivalent concentrations of insulin, even at concentrations of antibody sufficient for full occupancy of receptors. Another insulin-mimetic agent, wheat-germ agglutinin, stimulated receptor autophosphorylation with kinetics similar to those produced by the antibody. It is suggested that the relatively slow responses to both agents may be a function of the dependence on receptor cross-linking. These data are consistent with a role for the insulin receptor tyrosine kinase activity in the mechanism of action of insulin-mimetic anti-receptor antibodies.

INTRODUCTION

Binding of insulin to its cell surface receptor initiates a diverse range of metabolic effects. The molecular mechanisms involved in transducing the insulin signal, however, remain obscure, although much evidence implicates the intrinsic insulin-stimulated protein tyrosine kinase activity of the receptor (see [1–3] for reviews). On activation, the receptor undergoes both autophosphorylation of its β -subunit and stimulation of its kinase activity towards other, exogenous, substrate proteins. Thus insulin may initiate a phosphorylation cascade resulting in modulation of activities of key regulatory enzymes. Several cellular proteins have been shown to exhibit increased levels of tyrosine phosphorylation in response to insulin (e.g. [4–7]). However, the identities and roles, if any, of these proteins in insulin action have yet to be determined. It is also possible that autophosphorylation itself is the key reaction, perhaps inducing a conformational change in the receptor which influences its interaction with other proteins involved in the signalling pathway. However, stimulation by insulin of receptor kinase activity is normally dependent on autophosphorylation and vice-versa [1,2], so that it is difficult to separate the role of the former from that of the latter.

We have been using monoclonal antibodies to a number of distinct epitopes on the human insulin receptor [8] to probe receptor structure and mechanisms of insulin action. Several of these monoclonal antibodies mimic the metabolic effects of insulin on human adipocytes [9] and fibroblasts transfected with cDNA for the human insulin receptor (NIH 3T3HIR3.5 cells)

[10]. Studies with [32 P]P_i-labelled NIH 3T3HIR3.5 cells demonstrated receptor-mediated metabolic effects of these antibodies in the apparent absence of receptor autophosphorylation [10]. Other groups have reported polyclonal and monoclonal anti-receptor antibodies which have insulin-like metabolic effects [11–20]. Some of the polyclonal antibodies stimulated receptor autophosphorylation [12–15,19], although this was not always reported at first [11,12], whereas the monoclonal antibodies apparently did not induce autophosphorylation [16,17,20]. However, anti-receptor monoclonal antibodies failed to exhibit insulin-like effects in cells expressing mutant insulin receptors which lack tyrosine kinase activity [21]. One interpretation of these data is that antibodies are able to stimulate tyrosine kinase activity towards exogenous substrates independently of receptor autophosphorylation. It was the purpose of the present work, therefore, to investigate the effects of insulin-mimetic monoclonal antibodies on receptor kinase activity towards cellular substrates.

EXPERIMENTAL

Materials

Nitrocellulose immunoblotting filters (Schleicher and Schuell) were obtained from Millipore (Watford, Herts., U.K.). Tissue culture dishes (Falcon) were from Becton Dickinson (Cowley, Oxford, U.K.) and medium was from Gibco BRL (Uxbridge, Middlesex, U.K.). 125 I-labelled Protein A was from Amersham International (Aylesbury, Bucks., U.K.). The sources of all other materials were as previously described [11,22].

Abbreviations used: WGA, wheat-germ agglutinin; DMEM, Dulbecco's modified Eagle's medium.

§ To whom correspondence should be addressed.

Antibodies

Monoclonal antibodies to the human insulin receptor [8] were purified from ascites fluids by precipitation with ammonium sulphate followed by chromatography on hydroxyapatite [23]. Polyclonal anti-(insulin receptor) antibodies were raised in rabbits using purified placental receptor as the immunogen. Polyclonal anti-phosphotyrosine antibodies were raised in rabbits using keyhole-limpet-haemocyanin-conjugated phosphotyramine as immunogen [24], and were affinity purified on phosphotyramine-Sepharose.

Cell incubations

NIH 3T3HIR3.5 cells [25] were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% foetal calf serum and 400 µg of G418/ml for 2–3 days after passage, in either 10 cm² tissue culture plates or 75 cm² flasks. On reaching near confluence, cells were incubated for 16 h in Hepes-buffered DMEM containing 1 mg of BSA/ml in the absence of foetal calf serum. Agonist was added to the medium, mixed, and after the appropriate time, incubations were terminated by rapidly aspirating medium and adding boiling Laemmli [26] sample buffer containing 100 mM-dithiothreitol, 1 mM-sodium orthovanadate, 10 mM-NaF, 30 mM-sodium pyrophosphate and 10 mM-sodium EDTA. Cell extracts were scraped from tissue culture plates, boiled for 5 min and sonicated for 20 s. In experiments in which proteins were adsorbed to lectin, incubations were terminated by placing cells on ice, rapidly aspirating medium and solubilizing in ice-cold lysis buffer [50 mM-Hepes, pH 7.4, 1 mM-sodium orthovanadate, 10 mM-NaF, 1 mM-sodium EDTA, 1% (w/v) Triton X-100, 1 mg of bacitracin/ml and 1 mM-phenylmethanesulphonyl fluoride]. Following centrifugation (10000 g, 10 min, 4 °C), lysate was incubated with approx. 50 mg of wheat-germ-agglutinin (WGA)-Sepharose or hydroxyapatite on a rotator at 4 °C for 2 h. Proteins were eluted by boiling in Laemmli sample buffer (reducing).

Immunoblotting

Proteins in cell extracts were subjected to SDS/PAGE in 7.5% acrylamide gels [26]. Proteins were transferred to 0.2 µm-pore-size nitrocellulose filters in Towbin [27] buffer containing 2.6 mM-SDS and 0.5 mM-sodium orthovanadate [28] at 380 mA for 5 h at 4 °C. Filters were stained with Ponceau S and blocked by incubating at room temperature for 2 h in blocking buffer [10 mM-Tris/HCl, pH 7.5, 150 mM-NaCl, 1% (w/v) Triton X-100, 1 mM-sodium EDTA and 3% BSA]. Filters were incubated in blocking buffer containing polyclonal antibody [anti-phosphotyrosine, 0.3 µg/ml; anti-(insulin receptor) serum diluted 1:500] for 16 h and washed four times for 10 min in blocking buffer. Bound antibodies were detected by incubating filters for 4 h with ¹²⁵I-labelled Protein A (30 mCi/mg) at 0.1 µCi/ml in blocking buffer. Filters were washed extensively, dried and subjected to autoradiography at -70 °C. Blots were quantified by both densitometric scanning (Joyce-Loebl, Gateshead, Tyne and Wear, U.K.) and counting of bands excised from the filter for radioactivity in an NE 1600 gamma counter. When quantifying blots by scanning, any differences in background between tracks was corrected for by scanning an equivalent sized window immediately below the band in question.

There was a linear relationship between autoradiographic density and counts of ¹²⁵I-labelled Protein A bound. Over the range seen in these experiments there was also a direct proportionality between amount of phosphotyrosine-containing protein and the immunoblot signal, determined by applying different volumes of cell extract to the electrophoresis and immunoblotting procedure.

RESULTS

Immunoblotting of cellular proteins

In previous work we have analysed insulin-receptor phosphorylation by specific immunoprecipitation using anti-receptor antibodies from detergent lysates of cells incubated with [³²P]P_i [10]. In the present work we instead used immunoblotting with anti-phosphotyrosine antibodies. This has the advantage that incubations could be terminated by extracting cells directly into electrophoresis sample buffer, thereby avoiding potential problems of dephosphorylation during extraction and immunoprecipitation. Immunoblots with anti-(insulin receptor) antibodies revealed three major immunoreactive proteins at 205, 135 and 97 kDa (Fig. 1, track 2), representing insulin receptor precursor, α -subunit and β -subunit respectively.

Phosphotyrosine-containing proteins in cell extracts were detected by immunoblotting with anti-phosphotyrosine antibodies (Fig. 1, tracks 3 and 4). Addition of insulin to cells caused a dramatic stimulation of tyrosine phosphorylation in two major proteins, one of 185 kDa and one of 97 kDa. This latter protein was assumed to be the insulin receptor β -subunit, as it co-migrated with the 97 kDa protein identified by anti-(insulin receptor) antibodies (Fig. 1), and was immunoprecipitated by anti-(insulin receptor) monoclonal antibodies (results not shown). The insulin-stimulated 185 kDa protein did not migrate with proteins identified by anti-(insulin receptor) immunoblotting and was not precipitated by anti-(insulin receptor) antibodies

Fig. 1. Immunoblotting of cellular proteins from NIH 3T3HIR3.5 cells with anti-(insulin receptor) and anti-phosphotyrosine antibodies

NIH 3T3HIR3.5 cells were serum-starved for 16 h and then incubated with (lane 4) or without (lanes 1–3) 10⁻⁷ M-insulin for 5 min. Incubations were terminated by rapidly aspirating the medium and adding boiling electrophoresis sample buffer containing phosphatase inhibitors. Proteins were separated by SDS/PAGE and immunoblotted as described in the Experimental section. Blots were probed with a 1:500 dilution of rabbit control serum (lane 1) or anti-(insulin-receptor) antiserum (lane 2), or 0.3 µg of anti-phosphotyrosine antibody/ml (lanes 3 and 4), and ¹²⁵I-Protein A. Molecular masses were assigned by comparison with migration of standards: carbonic anhydrase, ovalbumin, BSA, phosphorylase *b* and β -galactosidase.

Fig. 2. Binding of phosphotyrosine-containing cellular proteins to WGA or hydroxyapatite

NIH 3T3HIR3.5 cells in 75 cm² flasks were serum-starved for 16 h and then incubated with (lanes 2 and 4) or without (lanes 1 and 3) 10⁻⁷ M-insulin for 5 min. Cells were then lysed and supernatants from centrifuged lysates were incubated with 50 mg of WGA-Sepharose (lanes 1 and 2) or hydroxyapatite (lanes 3 and 4) for 2 h at 4 °C as described in the Experimental section. Adsorbed proteins were collected by centrifugation, eluted in electrophoresis sample buffer, subjected to SDS/PAGE, immunoblotted and probed with anti-phosphotyrosine antibodies and ¹²⁵I-Protein A as described in the Experimental section.

(results not shown), suggesting that it is distinct from the insulin receptor. Both basal and stimulated cells contain a major phosphotyrosine-containing protein of approx. 120 kDa. In a number of experiments there was a tendency for insulin to increase phosphorylation of this protein. Reproducibility of this effect, however, was poor (maximal stimulation of 161 ± 24 % of control values). Control (non-immune) serum was not reactive with any of the proteins recognized by the anti-receptor or anti-phosphotyrosine antibodies.

The two proteins showing the largest response to insulin, pp97 (97 kDa) and pp185 (185 kDa), were used as an easily detectable index of tyrosine phosphorylation in these cells. Other more minor phosphotyrosine bands were also seen in several experiments, including one of about 42 kDa which was responsive to insulin (see Figs. 1 and 3).

Basal and insulin-treated cells were lysed and treated with WGA or hydroxyapatite (Fig. 2). All three major phosphotyrosine proteins were solubilized in the Triton-containing lysis buffer, but only pp97 (insulin receptor β -subunit) bound to the lectin. It appears, therefore, that, in contrast with the insulin receptor, pp185 and pp120 are not glycoproteins. This also indicates that pp185 and pp120 are not related to epidermal growth factor or platelet-derived growth factor receptors, both of which have been shown to adhere to WGA [29,30].

Effects of insulin-mimetic anti-(insulin receptor) monoclonal antibodies on tyrosine phosphorylation of cellular proteins

Cells were stimulated with antibody at a concentration previously shown to elicit maximal metabolic effects [9,10]. Cell extracts were prepared and probed by anti-phosphotyrosine immunoblotting (Fig. 3). Antibody 25-49 stimulated tyrosine phosphorylation of pp97 and pp185. As with insulin, some minor phosphotyrosine-containing bands were also seen after

Fig. 3. Insulin- and anti-receptor-antibody-stimulated tyrosine phosphorylation in NIH 3T3HIR3.5 cells

NIH 3T3HIR3.5 cells were serum-starved for 16 h and then incubated with 10⁻⁷ M-insulin (a) or 10⁻⁷ M-antibody 25-49 (b) for the times indicated. Incubations were terminated by rapidly aspirating the medium and adding boiling electrophoresis sample buffer containing phosphatase inhibitors. Proteins were separated by SDS/PAGE, immunoblotted and probed with anti-phosphotyrosine antibodies and ¹²⁵I-Protein A as described in the Experimental section.

antibody stimulation. The responses of pp97 and pp185 to antibody were markedly different from those induced by insulin. In contrast with the rapid effects of insulin on these proteins, antibody-stimulated phosphorylation was slow to develop. The level of tyrosine phosphorylation induced by antibody after 20 min was considerably less than that seen in response to equivalent concentrations of insulin. Antibody did tend to increase pp120 phosphorylation but, as with insulin, there was great variability between experiments (maximal stimulation to 222 ± 90 % of control value).

At the relatively low levels of phosphorylation induced by antibody, pp185 appears as a doublet. This is also seen with lower (10⁻¹⁰ M) concentrations of insulin and suggests that pp185 may in fact be two proteins running close together on SDS/PAGE. This possibility has also been raised by other workers [31]. The data presented are for antibody 25-49, but similar results were obtained with another insulin-mimetic anti-receptor antibody, 83-14, which recognizes a distinct epitope.

Kinetics of insulin- and antibody-stimulated tyrosine phosphorylation

Fig. 4 shows the effect of incubating cells, for different times, with various concentrations of insulin or antibody on levels of insulin receptor (pp97) autophosphorylation, as determined by immunoblotting. Regardless of concentration, insulin stimulated a rapid elevation of receptor autophosphorylation (earliest time point shown is 1 min). This was maintained over the 20 min period of the experiment. The level of phosphorylation was concentration-dependent, an effect being just detectable at 10⁻¹⁰ M-insulin.

Antibody at concentrations of 10⁻⁷ and 10⁻⁸ M stimulated autophosphorylation (Fig. 4). Antibody-induced phosphorylation occurred at a lower rate than that induced by insulin. The rate of antibody-stimulated autophosphorylation was concentration-dependent, the lower concentration inducing a slower rise. Levels of antibody-stimulated autophosphorylation were only approx. 20 % of those induced by equimolar concentrations of insulin.

The time course of insulin-stimulated pp185 phosphorylation

Fig. 4. Kinetics of insulin- and antibody-stimulated insulin receptor autophosphorylation in NIH 3T3HIR3.5 cells

NIH 3T3HIR3.5 cells were serum-starved for 16 h and then incubated with insulin (a) or antibody 25-49 (b) at 10^{-7} M (○), 10^{-8} M (△), 10^{-9} M (□) or 10^{-10} M (●) for the times indicated. Incubations were terminated by rapidly aspirating medium and adding boiling electrophoresis sample buffer containing phosphatase inhibitors. Proteins were separated by SDS/PAGE, immunoblotted and probed with anti-phosphotyrosine antibodies and ¹²⁵I-Protein A as described in the Experimental section. Phosphorylation of the insulin receptor β -subunit (pp97) was quantified by scanning of the resulting autoradiogram. Results are means $\pm$ S.E.M. for three independent experiments.

Fig. 5. Kinetics of insulin- and antibody-stimulated pp185 phosphorylation in NIH 3T3HIR3.5 cells

NIH 3T3HIR3.5 cells were serum-starved for 16 h and then incubated with insulin (a) or antibody 25-49 (b) at 10^{-7} M (○), 10^{-8} M (△), 10^{-9} M (□) or 10^{-10} M (●) for the times indicated. Cell extracts were prepared and analysed by SDS/PAGE and anti-phosphotyrosine immunoblotting as described in the Experimental section and legend to Fig. 3. Phosphorylation of pp185 was quantified by scanning of the resulting autoradiogram. Results are means $\pm$ S.E.M. for three independent experiments.

is shown in Fig. 5(a). In parallel with autophosphorylation, insulin-stimulated pp185 phosphorylation was rapid, and was characterized by an initial transient peak. There was some indication of 'spare receptors' for pp185 phosphorylation in that the response, as a percentage of maximum, was higher than that for pp97 phosphorylation at a given insulin concentration. Maximal levels of pp185 phosphorylation were similar for 10^{-7} and 10^{-8} M-insulin, despite differences in levels of receptor autophosphorylation induced by these two hormone concentrations.

As for receptor autophosphorylation, antibody-stimulated pp185 phosphorylation was slower than the insulin-stimulated event, and the maximum effect was much less (Fig. 5b). The rate

of antibody-stimulated pp185 phosphorylation, however, was slightly faster than antibody-stimulated autophosphorylation.

WGA-stimulated autophosphorylation

Addition of WGA to cells resulted in a relatively slow activation of insulin receptor autophosphorylation (Fig. 6). As with anti-(insulin receptor) antibody, decreasing the lectin concentration decreased the rate of autophosphorylation. Again, like antibody, levels of lectin-stimulated phosphorylation were appreciably lower than those seen at concentrations of insulin causing maximal effects. WGA also stimulated pp185 phosphorylation (results not shown).

Fig. 6. Kinetics of WGA-stimulated insulin receptor autophosphorylation in NIH 3T3HIR3.5 cells

NIH 3T3HIR3.5 cells were serum-starved for 16 h and then incubated with WGA at 50 µg/ml (○) or 5 µg/ml (△) for the times indicated. Cell extracts were prepared and analysed by SDS/PAGE and anti-phosphotyrosine immunoblotting as described in the Experimental section and the legend to Fig. 3. Phosphorylation of pp97 was quantified by scanning of the resulting autoradiogram. Results are shown for a representative experiment.

DISCUSSION

We have previously shown that certain anti-(insulin receptor) monoclonal antibodies exert insulin-like metabolic effects in both adipocytes [9] and cells transfected with human insulin receptor cDNA [10], with a concentration-dependence very similar to that of insulin. Some, but not all, of these antibodies stimulated the autophosphorylation and tyrosine kinase activity of solubilized receptors *in vitro* [22]. The metabolic effects of the antibodies occurred without detectable receptor autophosphorylation *in situ*, suggesting that this may not be a necessary step in receptor-mediated signal transduction [10,20]. In the present investigation we have shown that both antibodies and insulin stimulate receptor tyrosine kinase activity towards endogenous substrates in intact cells.

Anti-phosphotyrosine immunoblotting of cell extracts confirmed the presence of three major phosphotyrosine-containing proteins in insulin-treated cells [32], designated pp97, pp120 and pp185. The 97 kDa protein is clearly the insulin receptor β -subunit, as judged by its co-migration with β -subunit on SDS/PAGE, adsorption to WGA and immunoprecipitation by anti-(insulin receptor) antibodies. Insulin elicited a rapid increase, of about 20-fold, in tyrosine phosphorylation of this protein (Fig. 4), as in experiments using [32 P]P_i-prelabelled cells [10]. As described, insulin did not consistently affect tyrosine phosphorylation of pp120. Other groups have reported a phosphotyrosine-containing protein of approx. 116–120 kDa present in several cell lines under basal conditions [4,6], which is distinct from an insulin-sensitive liver-specific 120 kDa phosphotyrosyl glycoprotein [7].

Insulin caused a rapid, more than 10-fold, stimulation of tyrosine phosphorylation of pp185 (Fig. 5). A protein of similar molecular mass has been shown to undergo tyrosine phosphorylation in response to insulin and insulin-like growth factor I in other cells [4–6,31,33–35]. The pp185 protein identified in the present study is also similar to that previously described in

its kinetics of phosphorylation and its inability to bind to WGA. This contrasts with two other high molecular mass endogenous substrates for insulin receptor kinase which bind to the lectin [36,37]. The widespread occurrence of pp185 suggests it may have an important role in insulin action.

A major finding of the present study was that insulin-mimetic anti-(insulin receptor) monoclonal antibodies 25-49 and 83-14 stimulated the phosphorylation of pp185 (Fig. 5). This indicates that antibodies activate the receptor kinase *in situ*. A surprising finding was that antibodies also stimulated tyrosine phosphorylation of pp97. This was in contrast with previous work on the effects of these antibodies on receptor autophosphorylation in cells prelabelled with [32 P]P_i [10]. The reason for this discrepancy is not known, but several factors may contribute. Certainly, detection of tyrosine phosphorylation by immunoblotting gave a much stronger signal (10–20-fold higher c.p.m. associated with β -subunit phosphorylation detected by blotting compared with 32 P incorporation). Further, the immunoblotting technique will specifically detect tyrosine phosphorylation, which is reportedly very low in the basal state, whereas [32 P]P_i labelling will include a significant contribution to the basal state from serine/threonine phosphorylation [38]. Thus blotting may be able to reveal levels of tyrosine phosphorylation which would be difficult to detect against the basal phosphoserine/threonine seen using 32 P. Other methodological differences, such as the efficiency of immunoprecipitation in 32 P experiments, and the incubation times after which phosphorylation was determined, may contribute to the apparent discrepancy.

Levels of antibody-induced pp97 and pp185 phosphorylation as detected by blotting were considerably lower than those seen with equimolar concentrations of insulin, and similar magnitudes of phosphorylation required much higher concentrations of antibody than of insulin. However, it should be noted that autophosphorylation of receptor in response to insulin involves at least five tyrosine residues [39,40]. Steric considerations will almost certainly preclude the binding of multiple antibody molecules to phosphotyrosine residues which are very close together in the primary sequence. It is therefore not possible to compare directly the extent of kinase stimulation by antibodies and insulin from the relative intensities of pp97 immunoblotting.

The kinetics of antibody- and insulin-induced receptor autophosphorylation were very different. Insulin caused a concentration-dependent increase of receptor phosphorylation which was too rapid to permit assessment of initial rates. In contrast, antibody-stimulated autophosphorylation was relatively slow and initial velocity was dependent on ligand concentration. It has previously been shown that bivalency is required for insulin-like effects of anti-receptor antibodies ([41], M. A. Soos, R. Taylor & K. Siddle, unpublished work), indicating that cross-linking is important in antibody action. WGA has been shown to induce insulin-like metabolic effects in several cells and to stimulate the receptor kinase by a mechanism which depends on cross-linking [42]. Interestingly, antibody- and WGA-stimulated receptor autophosphorylation exhibited similar kinetics in NIH 3T3HIR3.5 cells. The relatively slow induction of receptor autophosphorylation by antibodies and lectin may reflect slower rates of binding compared with insulin or slower responses of the receptor to ligand binding, perhaps involving aggregation.

A crucial question is whether the extent of kinase activation by antibodies, however brought about, is sufficient to account for their metabolic effects compared with insulin. It may not be valid to compare too closely the responses to ligand concentrations which are supra-maximal for metabolic effects, particularly when phosphorylation stoichiometries are also uncertain. However,

there are clearly very substantial differences in the magnitude and time course of the effects of antibody and insulin on kinase activity (Figs. 4 and 5), which are not paralleled by differences in binding affinities or metabolic responses [9,10]. It is possible, therefore, that at least some aspects of signalling depend on receptor aggregation *per se*, which occurs secondarily to antibody-induced cross-linking or insulin-induced autophosphorylation [43]. However, it is now clear that insulin-mimetic anti-(insulin receptor) monoclonal antibodies do stimulate both autophosphorylation of the receptor and its tyrosine kinase activity. This, together with the previous demonstration that antibodies require a functional receptor tyrosine kinase to exert insulin-like metabolic effects [21], suggests strongly that the kinase plays an important role in signal transduction for antibodies, as for insulin.

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Stimulation of insulin receptor kinase by various anti-receptor antibodies, including those used in the present study, has recently been demonstrated in several cell lines [44]. These workers report similar findings with regard to the time course and relative magnitude of the response to antibodies compared with insulin.

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