Receptor Cross-Linking Restores an Insulin Metabolic Effect Altered by Mutation on Tyrosine 1162 and Tyrosine 1163†

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ABSTRACT: The pivotal role that the tyrosine residues in positions 1162 and 1163 play in the control of the insulin action has been clearly established by substitution of these tyrosine residues for phenylalanine [Ellis, L. (1986) *Cell 45*, 721–732]. We have recently found that this type of mutation, which abolishes the effects of insulin on glucose metabolism, was without any effect on the mitogenic effect of the hormone [Debant, A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* (in press)]. Here, we provide evidence that a polyclonal antibody, raised against the human insulin receptor, can restore the receptor-mediated stimulation of glycogen synthesis that was abolished by the mutation. Stimulation of the biological effect by the anti-receptor antibody did not necessitate, whatsoever, the activation of the tyrosine kinase activity and/or receptor autophosphorylation. Furthermore, the antibody-induced reversal of the mutation was not observed when we used Fab fragments alone, but addition of anti-$(Fab')_2$ IgG in a second step resulted in a similar effect as that observed with intact IgG. We propose that Tyr 1162 and Tyr 1163 exert their control on the metabolic effects of insulin through the modulation of receptor aggregation.

The involvement of receptor tyrosyl kinase activation in the mediation of the biological effects of insulin is now well established (Chou et al., 1987). Ellis et al. have demonstrated that within the kinase domain of the insulin receptor, tyrosine residues in positions 1162–1163 play a most important role in the control of glucose uptake (Ellis et al., 1986). We have recently found that replacement of these residues by phenylalanine abolished the hypersensitivity to insulin that expression of a large number of human receptors conferred on CHO-transfected cells in the mediation of both glucose uptake and glycogen synthesis. Nevertheless, this type of mutation did not hinder the ability of insulin receptors to transduce the hormonal mitogenic effect (Debant et al., 1988). In order to further assess the role of Tyr 1162–1163 in the mediation of the action of insulin, we have studied the effect of a rabbit anti-insulin receptor (RAIR) antibody, raised against the human insulin receptor, on CHO cells expressing either the normal or a mutated form (Phe 1162–1163) of the human insulin receptor. Receptor aggregation has been postulated to play a key role in the mediation of both epidermal growth factor and insulin effects (Schreiber et al., 1983; Kahn et al., 1978). We provide evidence that the twin tyrosine residues could be involved in the control of receptor aggregation which seems to be, by its own, a sufficient signal to trigger the stimulation of glycogen synthesis.

Materials and Methods

Materials. [^{14}C]Glucose, [γ-^{32}P]ATP, and [^{32}P]inorganic phosphate were obtained from Amersham International, Amersham, U.K. Porcine monocomponent insulin was kindly provided by Novo Research Institute, Copenhagen, Denmark. Bovine serum albumin, bacitracin, phenylmethanesulfonyl fluoride, casein, and poly(Glu-Tyr, 4/1) were purchased from Sigma, St. Louis, MO. The IgG anti-$(Fab)_2$ was purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands.

Incorporation of Glucose into Glycogen on CHO Cells. CHO-transfected cells or parental CHO cells, grown to confluence in 10-mm dishes, were exposed for 60 min at 37 °C to the different effectors. Then, [^{14}C]glucose (5 mM, 4 μCi/mL) was added to the medium for 3 h. The cells were washed three times with ice-cold PBS and were lysed by the addition of 30% KOH. Incorporation of [^{14}C]glucose into glycogen was assayed as described previously (Chou et al., 1987). When the monovalent Fab fragments were used, CHO cells were exposed for 45 min with the different Fab, or intact IgG. The cells were washed once with PBS to remove Fab fragments present in the medium and then incubated with anti-$(Fab')_2$ IgG (1/10 dilution) for 45 min. The labeled glucose was added, and the experiment was pursued as described above.

Preparation of Monovalent Fab Fragments. Monovalent RAIR Fab or control Fab fragments were obtained according to others (Kahn et al., 1978). Intact IgG were submitted to proteolysis with pepsin (40 μg/mg of IgG), 1 h at 37 °C, to obtained bivalent $(Fab')_2$. The fragments Fc were eliminated by incubation with protein A–Sepharose for 1 h at 4 °C. The supernatant was applied on Ultrogel AcA 44 to purify the bivalent fragments. The column eluate was incubated for 1 h with 10 mM DTT at 20 °C. Iodoacetamide was added to give a final concentration of 10 mM for 30 min at 20 °C. The samples were finally dialyzed at 4 °C for 48 h against PBS containing 25 mM Hepes, pH 7.6. We checked by SDS–PAGE that Fab fragments were not contaminated by residual intact IgG.

Autophosphorylation of Human Insulin Receptor on Intact Cells. CHO-transfected cells grown to confluence in 35-mm dishes were loaded with [^{32}P]orthophosphate (1 mCi/mL) for 90 min at 37 °C. The effectors were then added for 20 min. The membrane proteins of labeled cells were solubilized in a

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Table I: Respective Sensitivities of the Three CHO Cell Lines toward Insulin To Stimulate Glycogen Synthesis

cell type	EC_{50} (nM)
CHO-R	0.2
CHO-Y2	>10
parental CHO	>10

buffer containing 1.2% Triton X-100 and phosphatase inhibitors to preserve the phosphorylated state of the insulin receptor (Ponzio et al., 1987). The labeled insulin receptor was isolated on wheat germ agglutinin–agarose and subjected to SDS–PAGE, as described elsewhere (Ponzio et al., 1987).

Kinase Activity toward the Copolymer Glutamate–Tyrosine (4/1) of Solubilized Insulin Receptors Previously Activated in Intact Cells. CHO cells, grown to confluence in 35-mm dishes, were incubated with the effectors for 20 min without preincubation with [^{32}P]orthophosphate. The membrane proteins were solubilized in the presence of phosphatase inhibitors, as described above. Cold phosphorylated insulin receptors were isolated on wheat germ agglutinin–agarose (WGA) without phosphatase inhibitors so as to avoid interference with the kinase assay. Kinase activity of normal and mutated insulin receptors was measured by incubating samples containing the WGA eluates with 8 mM $MgCl_2$, 4 mM $MnCl_2$, 15 μM ATP, and 1 μCi of [γ-^{32}P]ATP in the presence of 0.2 mg/mL copolymer glutamate–tyrosine (4/1) for 30 min at 20 °C. The incorporation of ^{32}P in copolymer glutamate–tyrosine was measured as described previously (Braun et al., 1984).

Results and Discussion

Three kinds of Chinese hamster ovary (CHO) cell lines were used in this study, which have been described elsewhere (Ellis et al., 1986; Debant et al., 1988), namely, parental CHO cell line, CHO cells transfected with a plasmid coding for the normal human receptor (CHO-R), and CHO cells transfected with a plasmid mutated on sites corresponding to the tyrosine residues 1162–1163 (CHO-Y2). This latter cell line has been shown to have lost the hormonal hypersensitivity for glycogen synthesis that CHO cells acquire when they express large numbers of normal insulin receptors (Debant et al., 1988), which is confirmed by the data presented in Table I. In order to better define the postbinding events, impaired by this type of mutation, we made use of a rabbit anti-insulin receptor (RAIR) antibody which has the ability to mimic the effects of insulin by interacting with epitopes on insulin receptors that are exclusive of the insulin binding domain (Ponzio et al., 1987). This feature suggests that the RAIR antibody is unlikely to transduce its effect through insulin-like-induced conformational changes. As shown in Figure 1, when incubated with CHO-R cells, RAIR IgG did stimulate the synthesis of glycogen up to a value that corresponded to 40–50% of the maximal value that we obtained in the presence of 1 nM insulin. This stimulatory effect was concentration dependent, with an EC_{50} = 5 μg/mL. Most surprising, the RAIR IgG was able to reverse, at least partially, the defect that the twin tyrosine substitution had caused in the stimulation of the glycogen synthesis in CHO-Y2 cells, which presented a similar concentration–response curve. In that case, the maximal effect induced by RAIR IgG represented 30–40% of the value elicited by insulin on CHO-R cells. We have verified that nonimmune IgG did not modify either the basal or the insulin-stimulated levels of glycogen synthesis. The question that arises thus is whether the effect induced by the antibody in both systems was mediated by the exogenous human insulin receptors. The fact that our antibody was

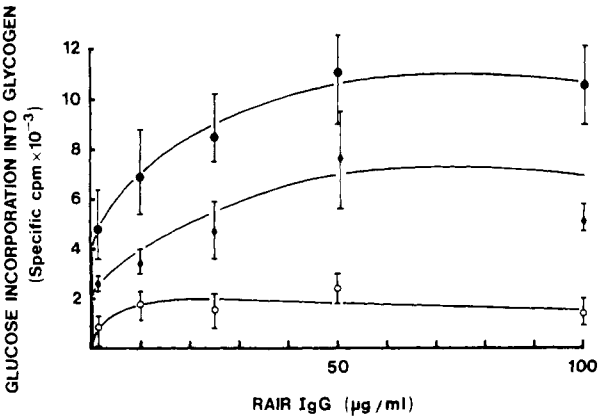

FIGURE 1: Stimulation of glycogen synthesis by RAIR IgG. CHO-R (●), CHO-Y2 (♦), and parental CHO (○) cells were exposed for 60 min at 37 °C to various concentrations of RAIR IgG or control IgG. Then [^{14}C]glucose was added to the medium, and the incorporation of [^{14}C]glucose into glycogen was assayed as described under Materials and Methods. The RAIR IgG effect was expressed as specific cpm incorporated: for example, on CHO-R cells, 50 μg/mL RAIR IgG (15 000 cpm) − 50 μg/mL control IgG (3950 cpm) = 11 050 cpm. For the other types of cells the values obtained were as follows: for CHO-Y2 cells, 50 μg/mL RAIR IgG (12 150 cpm) − 50 μg/mL control IgG (4500 cpm) = 7650 cpm; for parental CHO cells, 50 μg/mL RAIR IgG (5400 cpm) − 50 μg/mL control IgG (4000 cpm) = 1400 cpm. For reference, insulin (1 nM) was also used on CHO-R cells to determine the maximal effect expected: control IgG supplemented with 1 nM insulin (30 770 cpm) − control IgG (3960 cpm) = 26 810 cpm. The extent of radioactivity incorporated in the absence of control IgG was 3850 cpm and 30 500 cpm, respectively, for basal and insulin-stimulated (1 nM) levels.

unable to produce any effect on parental CHO cells indicates that the RAIR effect on CHO-R and CHO-Y2 cells cannot be accounted for by the interaction of RAIR antibody with endogenous insulin receptors and/or IGF-1 receptors of rodent origin. Since it has been described for other antibodies (Kahn et al., 1978; O'Brien et al., 1987) that their insulin-mimetic activity was due to their capacity to aggregate the insulin receptors at the membrane surface, we explored the incidence of RAIR valency in the induction of the biological effect. We thus tried to mimic the insulin effect by exposing both kinds of CHO-transfected cells with monovalent Fab fragments, unable to induce receptor aggregation. We first verified that monovalent nonimmune Fab fragments had no effect on both the basal and the insulin-stimulated levels of glycogen synthesis. The data presented in Figure 2 show that Fab fragments alone slightly stimulated glycogen synthesis in CHO-R and CHO-Y2 cells. However, the second-step addition of anti-$(Fab')_2$ IgG markedly stimulated glycogen synthesis in CHO-R as well as in CHO-Y2 cells, up to an extent comparable to that observed with intact RAIR IgG. We verified that anti-$(Fab')_2$ alone did not induce any insulin-like effect. These data strongly suggest that RAIR IgG produced most of its insulin-like effect through aggregation of insulin receptor molecules, as it has been previously proposed (Kahn et al., 1978).

At this point it was of interest to know what the effects of RAIR antibodies were at the molecular level. We were particularly interested to know if, in our system, RAIR IgG at a concentration giving the maximal biological effect was able to stimulate the autophosphorylation of the exogenous human receptors in CHO cells, the earliest postbinding event characterized thus far. Insulin stimulated dramatically the incorporation of radioactivity in the β subunit of normal receptors from CHO-R cells previously loaded with [^{32}P]-orthophosphate (Figure 3, panel A, lane 3). Under similar

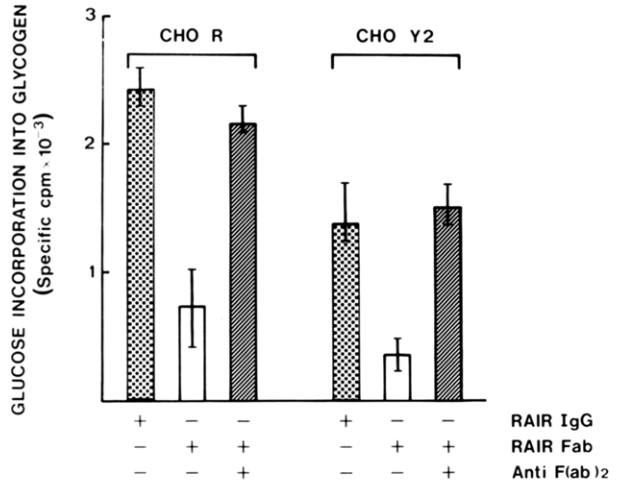

FIGURE 2: Induction of glycogen synthesis by monovalent RAIR Fab cross-linked by the use of anti-$(Fab')_2$ IgG. CHO-R and CHO-Y2 cells were incubated for 45 min with 40 μg/mL RAIR Fab or 40 μg/mL control Fab (corresponding to the same molar concentration of intact IgG as used in Figure 1) or with 100 μg/mL intact RAIR IgG or 100 μg/mL control IgG. The anti-$(Fab')_2$ IgG was then added to the medium as described under Materials and Methods. The results are represented as specific cpm incorporated into glycogen upon stimulation by RAIR IgG, RAIR Fab, or RAIR Fab cross-linked with anti-$(Fab')_2$ IgG. Specific cpm were calculated by subtracting the number of counts obtained in the presence of nonimmune IgG or Fab or Fab supplemented with anti-$(Fab')_2$ IgG from the counts incorporated in the presence of the corresponding RAIR components. For example, in the present experiment the values obtained with CHO-R cells were as follows: RAIR IgG (6700 cpm) – control IgG (4300 cpm) = 2400 cpm; RAIR Fab (4750 cpm) – control Fab (4000 cpm) = 750 cpm; RAIR Fab plus anti-$(Fab')_2$ IgG (6050 cpm) – control Fab plus anti-$(Fab')_2$ IgG (3850 cpm) = 2200 cpm. For reference, the specific value obtained in the presence of 1 nM insulin in that experiment was control IgG plus 1 nM insulin (11 000 cpm) – control IgG (4300 cpm) = 6700 cpm and control Fab plus 1 nM insulin (10 500 cpm) – control Fab (4350 cpm) = 6150 cpm. The anti-$(Fab')_2$ IgG alone had no effect (4150 cpm).

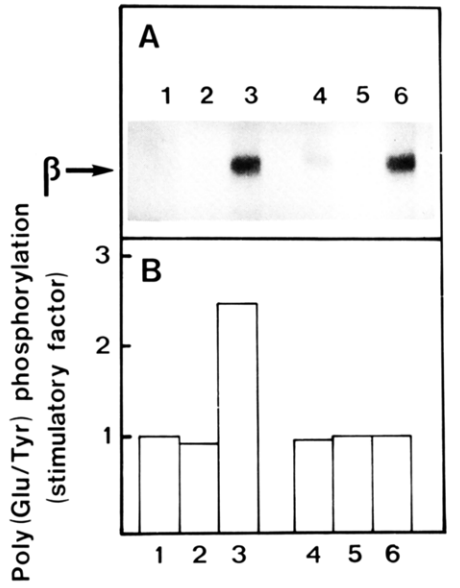

FIGURE 3: Effect of RAIR IgG on human insulin receptor autophosphorylation and kinase activity. Panel A: Autophosphorylation of the insulin receptor on ^{32}P-labeled cells. CHO-R cells (lanes 1–3) and CHO-Y2 cells (lanes 4–6) were loaded with [^{32}P]orthophosphate (1 mCi/mL) for 90 min at 37 °C. The effectors were then added as follows for 20 min: 100 μg/mL control IgG (lanes 1 and 4), 100 μg/mL RAIR IgG (lanes 2 and 5), or 100 μg/mL control IgG with 100 nM insulin (lanes 3 and 6). The autoradiogram represents the labeled β subunit of human insulin receptor isolated as described under Materials and Methods. Panel B: Kinase activity toward copolymer glutamate–tyrosine of solubilized insulin receptors previously activated in intact cells. CHO-R cells (lanes 1–3) and CHO-Y2 cells (lanes 4–6) were incubated with the same effectors as described in panel A, without preincubation with [^{32}P]orthophosphate. The isolation of the insulin receptors and the kinase assay toward copolymer glutamate–tyrosine (4/1) are described under Materials and Methods. Panel B represents the stimulatory factor of the hormone-stimulated [^{32}P]phosphate incorporated in poly(Glu-Tyr).

conditions on CHO-Y2 cells, we observed that replacement of the twin tyrosines with phenylalanine affected moderately autophosphorylation of the mutated receptor (Figure 3, panel A, lane 6) in accord with our previous report (Debant et al., 1988; Ellis et al., 1986). In contrast, RAIR IgG at a concentration which induced a maximal response on glycogen synthesis (100 μg/mL) was without any effect on the autocatalytic phosphorylation of both kinds of receptors (Figure 3, panel A, lanes 2 and 5). These data are in accord with previous reports that monoclonal (O'Brien et al., 1987; Forsayeth et al., 1987) and polyclonal (Simpson et al., 1984) antibodies can mimic the effect of insulin, without autophosphorylation of the receptor, even though these data are still considered controversial (Gherzi et al., 1987). In a second step we investigated the respective effects of insulin and RAIR IgG on the tyrosine-specific protein kinase activity elicited by the normal and mutated forms of the insulin receptors. The two transfected CHO cell lines were exposed to the two effectors under conditions identical with those used for the measurement of glycogen synthesis. Insulin receptors were then extracted and isolated under conditions where their activated state could be preserved by the use of phosphatase inhibitors. As presented in Figure 3, insulin markedly stimulated the tyrosine-specific protein kinase activity catalyzed by normal receptors, with poly(Glu-Tyr) (panel B, lane 3) as substrate. As expected, the hormone failed to stimulate tyrosine kinase activity in the mutated form of the receptor (Figure 3, panel B, lane 6). Interestingly, RAIR antibody at 100 μg/mL, corresponding to the maximal effect for glycogen synthesis, failed to stimulate tyrosine kinase activity toward the synthetic substrate, regardless of the nature of the receptor studied (Figure 3, panel B, lanes 2 and 5).

In conclusion, receptor cross-linking appears to be a sufficient signal to trigger the cascade of events mediating metabolic effects such as glycogen synthesis. This process, when imposed by bivalent antibodies, neither needs nor activates autophosphorylation or kinase activity of the insulin receptors as measured by the use of exogenous substrates. The lack of involvement of tyrosine kinase activity in the antibody-induced aggregation process was particularly clear on CHO-Y2 cells, because RAIR IgG stimulated glycogen synthesis in these cells despite the fact that they express insulin receptors which are devoid of protein kinase activity (Debant et al., 1988). These data provide the first evidence that physical receptor aggregation may play by itself a crucial role in the mediation of insulin-stimulated glycogen synthesis.

In an attempt to interpret our results, in view of all the available data, we propose that the major effect caused by mutation on Tyr 1162–1163 is to impair insulin receptor aggregation which normally occurs upon insulin stimulation (Kahn et al., 1978). Reversal of the defect, caused by substitution of the twin tyrosines, would result from the cross-linking property of RAIR IgG, without activating tyrosine kinase activity. In this formulation, the pivotal role that the twin tyrosines have been shown to play in the mediation of the metabolic effects of insulin would only rely on the control it imposes on receptor aggregation. The fact that phosphorylation on the twin tyrosine residues can be bypassed by the use of antibodies prompts us to postulate that (i) under normal

insulin stimulation phosphorylation of Tyr 1162-1163 precedes receptor aggregation and (ii) no additional information which is mediated by the insulin receptor (related to tyrosine kinase activation) seems to be necessary for the propagation of the postreceptor signal leading to glycogen synthesis, once the physical receptor aggregation is achieved.

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Articles

Cholesterol Modifies the Short-Range Repulsive Interactions between Phosphatidylcholine Membranes†

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ABSTRACT: Pressure versus distance relationships have been obtained for egg phosphatidylcholine bilayers containing a range of cholesterol concentrations. Water was removed from between adjacent bilayers by the application of osmotic pressures in the range of 0.4–2600 atm (4×10^5–2.6×10^9 dyn/cm^2), and the distance between adjacent bilayers was obtained by Fourier analysis of X-ray diffraction data. For applied pressures up to about 50 atm and bilayer surface separations of 15–5 Å, the incorporation of up to equimolar cholesterol has little influence on plots of pressure versus bilayer separation. However, for the higher applied pressures, cholesterol reduces the interbilayer separation distance by an amount that depends on the cholesterol concentration in the bilayer. For example, the incorporation of equimolar cholesterol reduces the distance between bilayers by as much as 6 Å at an applied pressure of 2600 atm. At this applied pressure, electron density profiles show that the high-density head-group peaks from apposing bilayers have merged. This indicates that equimolar concentrations of cholesterol spread the lipid molecules apart in the plane of the bilayer enough to allow the phosphatidylcholine head groups from apposing bilayers to interpenetrate as the bilayers are squeezed together. All of these X-ray and pressure–distance data indicate that, by reducing the volume fraction of phospholipid head groups, cholesterol markedly reduces the steric repulsion between apposing bilayers but has a much smaller effect on the sum of the longer ranged repulsive hydration and fluctuation pressures. Increasing concentrations of cholesterol monotonically increase the dipole potential of egg phosphatidylcholine monolayers, from 415 mV with no cholesterol to 493 mV with equimolar cholesterol. These dipole measurements predict that cholesterol should increase slightly the magnitude of the hydration pressure, in qualitative agreement with the X-ray results. These observations are pertinent to cholesterol's role in vesicle adhesion and fusion and also imply that cholesterol can alter the membrane binding and permeability of ions and certain drugs and metabolites.

The close approach of uncharged bilayer membranes is thought to be resisted by three principal nonspecific repulsive interactions. The first of these, commonly called the solvation or hydration pressure, P_h, arises from the polarization of water molecules by the zwitterionic lipid head groups (Marcelja & Radic, 1976; LeNeveu et al., 1976, 1977; Israelachvili & Pashley, 1983). It has been found empirically that $P_h = P_0 \exp(-d_f/\lambda)$, where d_f is the distance between bilayers and the decay length λ is on the order of 1–3 Å (LeNeveu et al., 1977; Parsegian et al., 1979; Lis et al., 1982; McIntosh & Simon,

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