Effects of covalently linked insulin dimers on receptor kinase activity and receptor down regulation

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Certain covalently linked insulin dimers have previously been found to have a greater ability to bind to the insulin receptor than to stimulate lipogenesis in adipocytes. The present report presents data indicating that the same insulin dimers also have a greater ability to bind to the receptor than to stimulate the kinase activity of the insulin receptor. In particular, one such covalently linked insulin dimer had less than 1% the potency of native insulin in stimulating the receptor kinase although it could bind to the solubilized receptor with 30% the potency of native insulin. In contrast, this dimer could down regulate the insulin receptor with approximately 30% the potency of native insulin. These results suggest that stimulation of the receptor kinase may require more than simple occupancy of the receptor binding site whereas down regulation of the receptor may require only the binding of ligand to the receptor.

Hormone receptor *Phosphorylation* *Insulin action*

1. INTRODUCTION

Insulin binds to a specific receptor protein on the surface of target cells and initiates a variety of biological responses [1,2]. Recently, authors in [3] reported that the binding of insulin to the receptor stimulates the extent of phosphorylation of the receptor. Furthermore, several studies have indicated that the insulin receptor is itself a protein kinase and that insulin stimulates this kinase activity of the receptor [4–8]. Since several effects of insulin are mediated via phosphorylation-dephosphorylation reactions [9–12], it has been hypothesized that the activation of the kinase activity of the receptor is the first step in initiating the response of the cell to the hormone. Alternatively, it has been hypothesized that the kinase activity of the receptor is important in mediating the insulin-induced decrease in hormone receptor binding, the phenomenon termed receptor down regulation [13–15].

To test these hypotheses, we have examined the ability of several covalently linked insulin dimers to regulate the kinase activity of purified insulin receptor and to down regulate the receptor. These dimers were prepared by crosslinking insulin monomers with a suberoyl chain at either the B1 Phe or B29 Lys [16]. The resulting 3 dimers (B1-B'1D, B1-B'29D, and B29-B'29D) are of interest because of their unusual properties; the ability of these dimers to inhibit the binding of [^{125}I]insulin to its receptor on either rat liver plasma membranes or adipocytes is 2.5-fold (for B1-B'29D) to 7-fold (for B29-B'29D) greater than their abilities to stimulate lipogenesis in adipocytes [16–18]. These discrepancies in binding and biological potencies of the covalent insulin dimers make them useful tools for studying the relationship between

Abbreviations: B1-B'29D, N^{B1},$N^{B'29}$-suberoyl-insulin dimer; B1-B'1D, $N^{B'}$,$N^{B'1}$-suberoyl-insulin dimer; B29-B'29D, N^{B29},$N^{B'29}$-suberoyl-insulin dimer

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the binding of insulin to its receptor and the subsequent events in insulin action.

2. MATERIALS AND METHODS

The following were purchased: porcine insulin (27.30 SP units per mg) from Elanco; wheat germ agglutinin coupled to agarose from Miles; *N*-acetyl-D-glucosamine, phenylmethylsulfonyl fluoride (PMSF) and bacitracin from Sigma; adenosine 5′-[γ-^{32}P]triphosphate (ATP) (16 Ci/mmol) from Amersham; and Affi-Gel 15 and gel reagents from Bio Rad.

The synthesis of the insulin dimers was as in [16]. The monoclonal antibody was prepared as in [19]. The [^{125}I]insulin was prepared by a stoichiometric chloramine T method [19] to a specific activity of 130 Ci/g.

2.1. *Purification of the insulin receptor*

Insulin receptors were purified from human placenta as follows: after removal of the amnion and chorion, the placenta was cut into small fragments and frozen in liquid nitrogen. Thirty gram portions were thawed and homogenized in a Brinkman Polytron in 60 ml of 1% Triton X-100 in 50 mM Hepes containing 1 mM PMSF and 1 mg/ml of bacitracin. The insoluble material was removed by successive centrifugations of 20 min at 20000 × *g* and 60 min at 100000 × *g*. The supernatant was passed over an 8 ml column of monoclonal anti-receptor antibody coupled to Affi-Gel 15 [14]. The column was washed with 50 ml of 50 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF and 1 mg/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 eluted receptor was immediately diluted 10-fold and further purified on a 5 ml wheat germ agglutinin affinity column [4]. For the experiments utilizing purified receptor, two separate receptor preparations were utilized.

2.2. *Receptor kinase assays*

The purified receptor was incubated for 60 min at 24°C in 30 μl of a buffer containing 50 mM Hepes (pH 7.6), 0.1% Triton X-100, 150 mM NaCl, 2 mM $MnCl_2$ and the indicated insulin dimer. Then 5–8 μM [γ-^{32}P]ATP (16 Ci/mmol) was added and the reaction was allowed to proceed for 60 min at 24°C. Under these conditions, the phosphorylation of the receptor increased linearly for 60 min at 24°C and no significant ATP hydrolysis could be detected. The samples were then made 1% in SDS and 5% in mercaptoethanol, heated to 100°C for 1 min and electrophoresed on a 7.5% polyacrylamide gel [19]. The gel was stained, 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.

2.3. *Receptor binding assays*

Binding assays were performed with both purified receptor and intact IM-9 lymphocytes. For the purified receptor assay, the assay was performed exactly as described for the kinase assay except that 100 pM [^{125}I]insulin was included in the reaction in place of ATP. After 2 h at 24°C, the receptor was precipitated with 10.5% polyethylene glycol in the presence of carrier human γ globulin (1 mg/ml). The pellets (5 min at 10000 × *g* in a Beckman microfuge) were washed twice with 10.5% polyethylene glycol and counted. Non-specific binding was determined in the presence of 10^{-5} M insulin and subtracted.

The binding assay with IM-9 cells was performed essentially as in [19]. In brief, IM-9 cells (5×10^5) in 500 μl minimal Eagle's medium with 50 mM Hepes were incubated for 70 min at 15°C with the dimers and 40 pM [^{125}I]insulin. The cells were cooled to 4°C, centrifuged (200 × *g* for 10 min) and washed twice. The pellets were counted and non-specific binding (that bound in the presence of 10^{-5} M insulin) was subtracted.

2.4. *Receptor down regulation*

IM-9 cells (5×10^5 cells/ml) were incubated in normal growth media (minimal Eagle's medium with 10% fetal calf sera, 20 mM Hepes (pH 7.4) and non-essential amino acids) with the insulin dimers for 18 h at 37°C. The cells were harvested by centrifugation and extensively washed as in [14]. In brief, this wash procedure includes two incubations of 25 min at 30°C at pH 6.0 and three washing steps to allow the dissociation of bound ligands. The ability of the washed cells to bind [^{125}I]insulin was then measured by resuspending

the cells in binding buffer (100 mM Hepes (pH 7.8), 1.20 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose and 1% bovine serum albumin) and 70 pM [^{125}I]-insulin. After 70 min at 15°C, the cells were washed and the radioactivity in the cell pellet was measured. Non-specific binding, determined by including 10 μM unlabeled insulin, was subtracted to give specific binding.

3. RESULTS

3.1. *Receptor binding ability of the insulin dimers*

The insulin dimers were tested for their ability to inhibit the binding of [^{125}I]insulin to its receptor on IM-9 cells (fig.1). The concentrations of insulin and the dimers B1-B'29, B29-B'29, and B1-B'1 required to inhibit 50% of the binding (the I_{50}) were 400, 300, 400 and 1100 pM, respectively. These values for the dimers (in particular, B1-B'1) are less than those previously reported since the short incubation times which were chosen to mimic the kinase assay are not sufficient to reach equilibrium for the dimers [18].

The dimers were also tested for their ability to inhibit the binding of [^{125}I]insulin to purified, solubilized receptor under conditions identical to the kinase assays described below. In this assay, the concentrations of insulin and the dimers B1-B'29, B29-B'29 and B1-B'1 required to inhibit 50% of the binding were 2.7, 2.3, 7.3 and 17 nM, respectively.

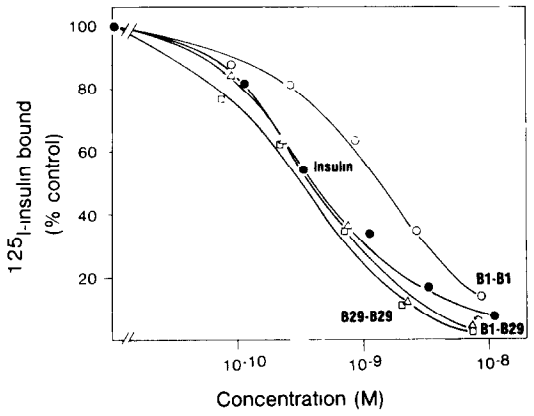

Fig.1. Receptor binding potencies of the insulin dimers. IM-9 cells were incubated with the indicated concentration of insulin dimers and 40 pM [^{125}I]insulin for 70 min at 15°C. The cells were then washed and the radioactivity bound was counted. Each point is the average of 6 determinations from 2 independent experiments and the standard errors for each value were less than 10% of the values.

3.2. *Stimulation of receptor kinase activity by the insulin dimers*

The insulin dimers were then tested for their ability to stimulate the phosphorylation of the β-subunit of the purified receptor (fig.2). The B1-B'29 dimer had the greatest ability to stimulate the receptor kinase activity (approximately half that of insulin), B1-B'1 dimer was next and B29-B'29 dimer had the weakest ability to stimulate the receptor kinase (less than 1% that of native insulin).

3.3. *Down regulation of the insulin receptor by the dimers*

The dimers were then tested for their ability to regulate the expression of the insulin receptor. Prior studies have shown that preincubation of IM-9 cells with insulin causes a dose-dependent

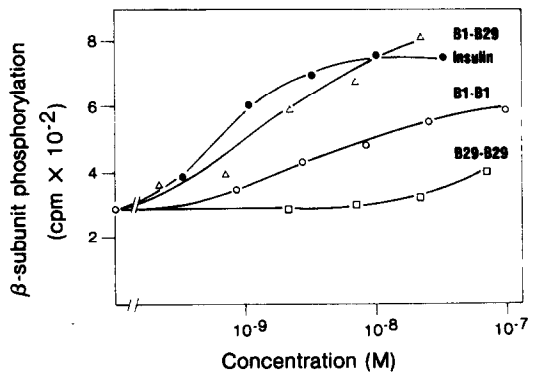

Fig.2. Stimulation of receptor kinase by insulin dimers. Purified insulin receptor was preincubated with the indicated concentrations of dimers or insulin for 60 min at 24°C, and then the reaction was started by the addition of 10 μM [γ-^{32}P]ATP. After 60 min at 24°C, the reaction was stopped by heating the samples at 100°C with 1% SDS. The samples were electrophoresed and the gels were processed as in [4]. The β-subunit band was excised and the radioactivity in this band determined and shown in the figure. Two additional experiments utilizing different receptor preparations gave comparable results. The average relative potency for the dimers B1-B'29, B1-B'1 and B29-B'29 were 30%, 10% and less than 1% that of insulin, respectively.

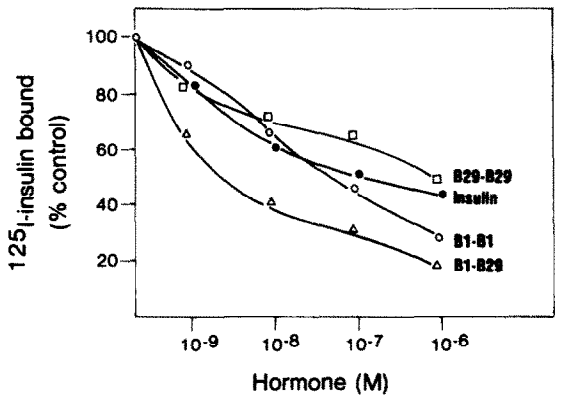

Fig.3. Down regulation of the insulin receptor of IM-9 cells by insulin dimers. IM-9 cells were incubated for 18 h at 37°C with the indicated concentrations of insulin dimers. The cells were then extensively washed and tested for their ability to bind [^{125}I]insulin. The results shown are averages of 6 determinations from 2 independent experiments and the standard errors for each value were less than 10% of the values.

decrease in the subsequent ability of these cells to bind insulin, the phenomenon termed down regulation [13–15]. The B1-B'29 dimer was found to be the best at down regulating the insulin receptor, B1-B'1 was next and B29-B'29 had the weakest ability (approximately 1/3 that of insulin) (fig.3).

4. DISCUSSION

Prior studies have indicated a discrepancy between the ability of covalently linked insulin dimers to bind to the insulin receptor and to stimulate lipogenesis [16–18]. This discrepancy was most pronounced with the dimer B29-B'29. The present data indicate that this dimer also exhibited the greatest discrepancy between its ability to bind to the receptor and its ability to stimulate the receptor kinase. Although it inhibited [^{125}I]insulin binding to IM-9 cells with approximately the same potency as native insulin, it had less than 1% the potency of native insulin in stimulating the receptor kinase. This discrepancy was observed even when binding ability was assessed using the same purified receptor system as used for the kinase assays. These results indicate that the binding of this molecule to the receptor does not induce the necessary change in the state of the receptor which is needed for the activation of the receptor kinase. This change in the state of the receptor could be a conformational change [20] or a change in the sulfhydryl status of the receptor [21].

In contrast to the weak potency of the B29-B'29 dimer in stimulating the receptor kinase, this dimer was approximately 30% as potent as native insulin at down regulating the insulin receptor of IM-9 cells. This result is consistent with the hypothesis that receptor occupancy is sufficient for down regulation of the receptor. This conclusion is also supported by the finding that the insulin receptor can be down regulated by an antagonist of insulin, a monoclonal anti-receptor antibody [22].

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