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High Affinity Insulin Binding in the Human Placenta Insulin Receptor Requires $\alpha\beta$ Heterodimeric Subunit Interactions

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Summary. Insulin binding to human placenta membranes treated at pH 7.6 or 8.5 in the presence or absence of 2.0 mM DTT for 5 min, followed by the simultaneous removal of the DTT and pH adjustment to pH 7.6, displayed curvilinear (heterogeneous) insulin binding plots when analyzed by the method of Scatchard. However, Triton X-100 solubilization followed by Bio-Gel A-1.5m gel filtration chromatography of the placenta membranes previously treated with DTT at pH 8.5 generated a nearly straight line (homogeneous) Scatchard plot. ^{125}I-insulin affinity crosslinking studies coupled with Bio-Gel A-1.5m gel filtration chromatography demonstrated that the alkaline pH and DTT treatment of placenta membranes followed by detergent solubilization generated an $\alpha\beta$ heterodimeric insulin receptor complex from the $\alpha_2\beta_2$ heterotetrameric disulfide-linked state. The ability of alkaline pH and DTT to produce a functional $\alpha\beta$ heterodimeric insulin receptor complex was found to be time dependent with maximal formation and preservation of tracer insulin binding occurring at 5 min. These data demonstrate that (i) a combination of alkaline pH and DTT treatment of placenta membranes can result in the formation of a functional $\alpha\beta$ heterodimeric insulin receptor complex. (ii) the $\alpha\beta$ heterodimeric complex displays homogeneous insulin binding. (iii) the insulin receptor membrane environment maintains the $\alpha_2\beta_2$ association state, which displays heterogeneous insulin binding, despite reduction of the critical domains that are responsible for the covalent interaction between the $\alpha\beta$ heterodimers.

Key Words insulin binding · insulin receptor · subunit interaction

Introduction

The insulin receptor is generally accepted to minimally exist as a covalent heterotetrameric disulfide-linked complex composed of two identical M_r = 135,000 (α) and two identical M_r = 95,000 (β) subunits [12, 26]. The α subunit is thought to encompass the extracellular high affinity insulin binding site [13, 29, 30, 42–44], whereas the β subunit contains the intracellular ATP[1] binding and tyrosine-specific protein kinase domains [1, 15–18, 27, 28, 32, 35, 39, 41, 45]. The insulin receptor has been shown to be initially synthesized as an M_r = 155,000 polyprotein precursor [7, 40], which is further processed by acylation, glycosylation, proteolytic cleavage and disulfide bond assembly into the mature $\alpha_2\beta_2$ heterotetrameric complex [5, 10, 14].

The disulfide bonds responsible for maintaining the covalent linkages between the insulin receptor subunits have been operationally categorized into two different classes [8, 23, 31]. The Class I disulfides have been shown to maintain the covalent linkages between the $\alpha\beta$ heterodimers and can be readily reduced with low concentrations (1.0–2.0 mM) of DTT. In contrast, the Class II disulfides maintain the covalent linkages between the individual α and β subunits and require higher concentrations (10–50 mM) of DTT in order to become reduced. We have previously demonstrated that reduction of the Class I and/or the Class II disulfides does not result in subunit dissociation under normally used buffer conditions [38]. However, it has been observed that treatment of detergent soluble insulin receptor preparations with a combination of alkaline pH and low DTT concentrations can dissociate the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex into an $\alpha\beta$ heterodimeric state [2, 37]. Insulin binding to the isolated $\alpha\beta$ heterodimeric complex was observed to generate a linear Scatchard plot, whereas insulin binding to the $\alpha_2\beta_2$ heterotetrameric complex generated the typical curvilinear heterogeneous insulin binding curve [37]. Similarly, insulin binding to endogenously present $\alpha\beta$ heterodimeric insulin receptors isolated from rat liver membranes was also observed to display homogeneous insulin binding [19] without any detectable negative cooperative insulin binding interactions [6].

Recently it has been demonstrated that, in addition to the covalent disulfide linkages and pH-dependent subunit interaction [2, 37, 38], the membrane environment also plays a significant role in

[1] *Abbreviations used are:* ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; NEM, N-ethylmaleimide; IGF-I, insulin-like growth factor-I; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

maintaining the appropriate association state and high affinity curvilinear insulin binding properties of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex [3]. In this communication we have further examined the insulin binding properties of the $\alpha\beta$ heterodimeric insulin receptor complex isolated from alkaline pH and DTT-treated human placenta membranes.

Materials and Methods

Materials

Bovine serum albumin, bacitracin, protease inhibitors and DTT were purchased from Sigma. Triton X-100, Bio-Gel A-1.5m resin and electrophoresis reagents were obtained from Bio-Rad. DSS, XAR-5 film and Cronex lightning-plus intensifying screens were obtained through Pierce, Kodak and DuPont, respectively. Molecular weight SDS-polyacrylamide gel standards were obtained from Bethesda Research Laboratories. Recombinant IGF-1 was purchased from Toyobo. Porcine insulin was graciously provided by Dr. R. Chance, Eli Lilly, and monoiodinated-[^{125}I]A_{14}-insulin was furnished by the Diabetes and Endocrinology Research Center, The University of Iowa. Monoiodinated-[^{125}I]B_{26}-insulin was purchased from Amersham.

Methods

Membrane Preparation

Human placenta membranes were prepared from freshly obtained human placentas by the procedure of Harrison and Itin [9]. Placenta membranes (10 mg/ml) were stored at −70°C in 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM benzamidine-hydrochloride, 10 μM leupeptin, 50 trypsin inhibitor units of aprotinin, 1 mM 1,10-phenanthroline, and 1 μM pepstatin A. Protein concentration was determined by a modification of the method described by Lowry et al. [21].

Treatment of Membranes with DTT

Placenta membranes (10 mg/ml) were incubated at 23°C for 25 min at pH 7.6 or 8.5 by the addition of the appropriate amount of 1.0 M Tris. Membranes were then incubated for 5 min at 23°C in the presence or absence of freshly prepared 2.0 mM DTT in water. The placenta membranes were immediately diluted with a 10-fold excess of 50 mM Tris-HCl, pH 7.6 or 8.5, 150 mM NaCl, 2 mM EDTA and 0.02% NaN_3 (TEN buffer, pH 7.6 or 8.5) at 4°C and centrifuged for 20 min at 48,000 × *g*. The supernatant following centrifugation was aspirated, and the membrane pellets were washed with the corresponding TEN buffer, pH 7.6 or 8.5. The membrane pellets were then resuspended with TEN buffer at the appropriate pH. This procedure effectively brings the DTT concentration below detectable levels (*data not shown*). Samples of the resuspended pellets were solubilized with 1.0% Triton X-100 and 10 μM leupeptin for 30 min at 4°C followed by microcentrifugation at 13,000 × *g* for 30 min at 4°C.

Insulin Binding

Insulin binding to placenta membranes, Triton X-100 solubilized membranes or Bio-Gel A-1.5m gel filtration column fractions was performed by the addition of 0.25 nM monoiodinated-[^{125}I]A_{14}-insulin for 16 hr at 4°C in a final volume of 0.2 ml with KRH buffer (50 mM HEPES, pH 7.6, 130 mM NaCl, 5.2 mM KCl, 1.3 mM $CaCl_2$ and 1.3 mM $MgSO_4$) plus 0.1 mg/ml bacitracin and 0.1% bovine serum albumin. Free ^{125}I-insulin was separated from the bound hormone by the addition of 0.5 ml of 0.1% bovine gamma globulin and 0.5 ml of 25% polyethylene glycol at 4°C, followed by microcentrifugation at 13,000 × *g* for 10 min. The supernatant was aspirated off, and the pellets were washed with 1.0 ml of 10% polyethylene glycol. Nonspecific binding was determined in the presence of 1.0 μM unlabeled insulin in the binding assays. Binding data for Scatchard experiments were generated by incubating the various insulin receptor samples with 0.1 nM ^{125}I-insulin plus increasing concentrations of unlabeled insulin (0.1–100 nM). Scatchard data [33] were analyzed with a modification of the computer program LIGAND [25] kindly provided by the Diabetes and Endocrinology Research Center, University of Iowa. The concentration of Triton X-100 in all the soluble insulin binding assays was maintained at 0.025%.

Bio-Gel A-1.5m Gel Filtration Chromatography

Solubilized insulin receptors, after various treatments as described in the figure legends, were resolved on separate Bio-Gel A-1.5m (1.6 × 46 cm) gel filtration columns equilibrated with 50 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 150 mM NaCl and 0.02% NaN_3 at 4°C. The columns were run at a flow rate of 15 ml/hr and 20 ml were voided before collecting 0.4 ml fractions. Fractions from the Bio-Gel A-1.5m gel filtration columns were then assayed for ^{125}I-insulin binding and affinity crosslinking.

Insulin Affinity Crosslinking

Insulin receptor samples (membranes, solubilized or gel filtered) were incubated for 16 hr at 4°C in the presence of 0.25 nM monoiodinated-[^{125}I]B_{26}-insulin in KRH buffer plus 0.1 mg/ml bacitracin and 0.1% bovine serum albumin. Affinity crosslinking was initiated by the addition of 5.0 mM DSS in 10% dimethylsulfoxide, 45% ethanol to yield a final DSS concentration of 0.1 mM [4]. The samples were then incubated for 5 min at 4°C followed by the serial addition of 1.0 M Tris, pH 10.5, 0.5 ml of 0.02% bovine gamma globulin plus 0.1% bovine serum albumin and 0.5 ml of 25% polyethylene glycol. The use of 1.0 M Tris, pH 10.5 is necessary to prevent nonspecific DSS crosslinking of ^{125}I-insulin. The samples were centrifuged, washed as described earlier for insulin binding assays, resuspended in water and subjected to SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

Insulin affinity crosslinked samples were run under nonreducing conditions using 3–10% linear gradient SDS-polyacrylamide gels containing a 3% stacking gel (acrylamide: bis-acrylamide ratio of 37.5 : 1.0) according to the procedure of Laemmli [20]. ^{125}I-insulin affinity crosslinked receptors were mixed with Laemmli sample buffer (50 mM Tris-HCl, pH 6.9, 10% glycerol, 1.0% SDS and 0.05% bromophenol blue) and applied to the 3–10% gels without heating. The gels were stained with Coomassie brilliant blue R,

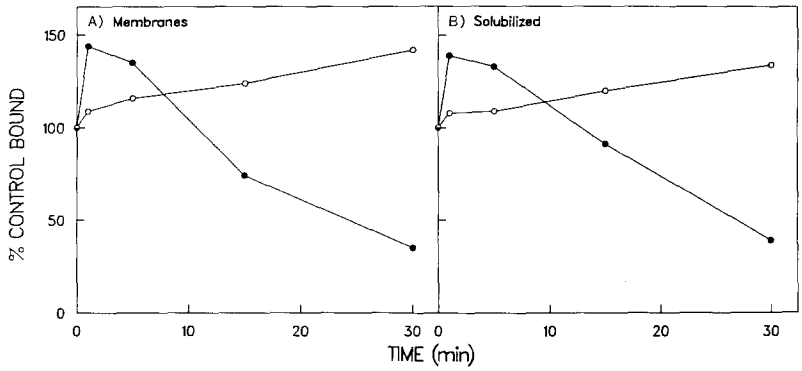

Fig. 1. Effect of DTT treatment on insulin binding to placenta membranes and Triton X-100 solubilized membranes at pH 7.6 or 8.5. Placenta membranes (10 mg/ml) were incubated at 23°C at pH 7.6 or 8.5 in the absence or in the presence of 2.0 mM DTT for 1, 5, 15 and 30 min. The placenta membranes were diluted, centrifuged, washed, resuspended and solubilized as described under Materials and Methods. (*A*) Placenta membranes (30 μg/assay) were treated at pH 7.6 (○) or 8.5 (●) and assayed for tracer insulin binding (0.25 nM) as described under Materials and Methods. (*B*) Solubilized placenta membranes (30 μg/assay) were assayed for tracer insulin binding subsequent to incubation of the placenta membranes at pH 7.6 (○) or pH 8.5 (●) in the absence or presence of 2.0 mM DTT

dried and autoradiographed with Kodak XAR-5 film using Cronex lightning-plus intensifying screens.

Results

We and others have previously reported that a combination of alkaline pH and DTT treatment of the human placenta $\alpha_2\beta_2$ heterotetrameric insulin receptor results in the formation of a functional $\alpha\beta$ heterodimeric complex [3, 37]. However, it was also observed that although 50 mM DTT was fully capable of reducing all the disulfide bonds responsible for the covalent association of the $\alpha_2\beta_2$ heterotetrameric complex, no dissociation of insulin receptor subunits was detectable at neutral pH [38]. To further examine the effects of pH and DTT on insulin receptor structure and function, a time course of DTT treatment on insulin binding to human placenta membranes at pH 7.6 or 8.5 was performed (Fig. 1). Placenta membranes at pH 7.6 or 8.5 were incubated with 2.0 mM DTT for various times, followed by a 10-fold dilution, centrifugation and resuspension into either the pH 7.6 or 8.5 TEN buffer, respectively. Under these conditions, DTT treatment at pH 7.6 was consistently observed to increase tracer insulin binding activity in the placenta membranes to approximately 140% of control by 30 min (Fig. 1*A*). In contrast, DTT treatment of placenta membranes at pH 8.5 was found to initially induce an increase in tracer insulin binding (145% at 1.0 min) followed by a progressive decrease to 35% of the control insulin binding by 30 min. Similarly, Triton X-100 solubilization of the placenta membranes previously treated at pH 7.6 or 8.5 in the presence of 2.0 mM DTT, also demonstrated the increase in tracer insulin binding at pH 7.6 and the biphasic time dependence of insulin binding at pH 8.5 (Fig. 1*B*). ^{125}I-insulin crosslinking demonstrated that the combination of alkaline pH and DTT resulted in the reduction of the insulin receptor into an $\alpha\beta$ heterodimeric state determined by denaturing SDS-polyacrylamide gel electrophoresis conditions (*data not shown*).

We next examined the competition of insulin binding to placenta membranes and solubilized membranes previously treated at pH 7.6 or 8.5 in the presence or absence of 2.0 mM DTT for 5 min. Scatchard analysis of insulin binding to placenta membranes treated at pH 7.6 in the presence of 2.0 mM DTT demonstrated a small increase in the number of high affinity insulin binding sites without any significant change in the number of low affinity or total insulin binding sites compared to the placenta membranes treated in the absence of DTT (Fig. 2*A*). Similarly, insulin binding to placenta membranes treated at pH 8.5 in the presence or absence of DTT also generated curvilinear Scatchard plots with no apparent change in the number of low affinity or total binding sites, but with the same small DTT-dependence increase in the number of high affinity insulin binding sites (Fig. 2*B*). Titron X-100 solubilization of the pH 7.6-treated placenta membranes in the presence of DTT also demonstrated the small increase in the number of high affinity insulin binding sites compared to the control membrane preparations (Fig. 2*C*). Under all these above conditions, the competition of insulin binding generated the typical curvilinear Scatchard plots when a broad range of insulin concentrations (0.1 to 100 nM) was used. Analysis of the insulin binding data using a noninteracting two-site binding model indicated no

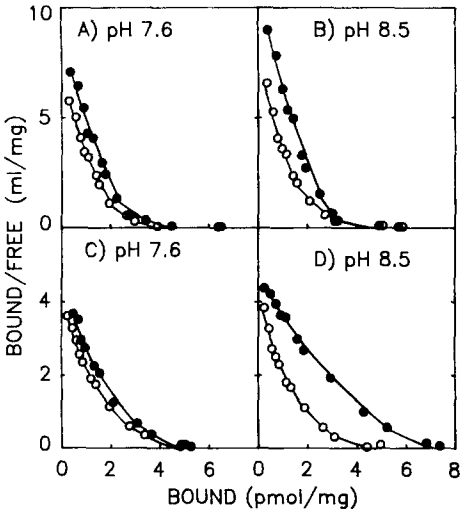

Fig. 2. Scatchard analysis of insulin binding to placenta membranes and solubilized placenta membranes previously incubated in the absence or presence of DTT at pH 7.6 or 8.5. Placenta membranes (10 mg/ml) were incubated at 23°C in the presence or absence of 2.0 mM DTT and subjected to competition of insulin binding and analyzed by the method of Scatchard as described under Materials and Methods. (*A*) Placenta membranes (30 μg/assay) were treated at pH 7.6 in the absence (○) or presence (●) of 2.0 mM DTT for 5 min followed by dilution, centrifugation, and resuspension at pH 7.6. (*B*) Placenta membranes (30 μg/assay) were treated at pH 8.5 in the absence (○) or presence (●) of 2.0 mM DTT as described in *A*. (*C*) Triton X-100 solubilized membranes (30 μg/assay) were prepared from placenta membranes pretreated at pH 7.6 in the absence (○) or presence (●) of 2.0 mM DTT for 5 min as described above. (*D*) Triton X-100 solubilized membranes (30 μg/assay) were prepared from placenta membranes pretreated at pH 8.5 in the absence (○) or presence (●) of 2.0 mM DTT for 5 min

change in the high affinity dissociation constant (K_d = 0.47 nM) and no significant change in the low affinity dissociation constant (K_d = 9.2 *vs.* 6.2 nM) of the solubilized placenta membranes incubated at pH 7.6 in the absence or presence of DTT, respectively. Although a small increase (12%) in the number of high affinity binding sites from the solubilized DTT-treated membranes was consistently observed (R_1 = 1.7 *vs.* 1.9 pmol/mg), no difference between the total number of binding sites was discernable (R_0 = 5.0 *vs.* 5.2 pmol/mg).

In contrast, insulin binding to the Triton X-100 solubilized placenta membranes previously treated at pH 8.5 with 2.0 mM DTT generated a significantly altered Scatchard plot compared to Triton X-100 solubilized placenta membranes treated in the absence of DTT (Fig. 2*D*). The Triton X-100 solubilized placenta membranes that were previously DTT and alkaline pH treated produced a less curvilinear insulin binding isotherm. The insulin receptor high affinity dissociation constant under these conditions was approximately 1.0 nM. A significant increase (1.5-fold) in the total number of insulin binding sites (7.4 pmol/mg) compared to the solubilized placenta membranes incubated at pH 8.5 in the absence of DTT (5.0 pmol/mg) was also observed.

To determine if the DTT treatment of placenta membranes at pH 7.6 or 8.5 followed by Triton X-100 solubilization resulted in the formation of an $\alpha\beta$ heterodimeric insulin receptor complex in the absence of SDS, the Triton X-100 solubilized placenta membranes were subjected to Bio-Gel A-1.5m gel filtration chromatography (Fig. 3). Placenta membranes incubated with or without DTT at pH 7.6 prior to Triton X-100 solubilization were resolved by Bio-Gel A-1.5m gel filtration chromatography as a broad peak having a K_{av} of approximately 0.18 (Fig. 3*A*). A small shoulder of insulin binding activity at the void volume of these columns was observed in some of the placenta membrane preparations. Bio-Gel A-1.5m gel filtration chromatography of placenta membranes initially treated with DTT at pH 8.5 followed by detergent solubilization had a decreased mobility with a K_{av} of approximately 0.28 compared to the placenta membranes incubated at pH 8.5 in the absence of DTT (Fig. 3*B*). The formation of the K_{av} = 0.28 species was consistently found to occur with the near complete loss of the material migrating with a K_{av} = 0.18 with no significant change in the insulin binding peak at the void volume of these columns.

To document that Triton X-100 solubilization of placenta membranes initially treated with DTT at pH 8.5 results in the dissociation of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex into an $\alpha\beta$ heterodimeric state, ^{125}I-insulin affinity crosslinking to the Bio-Gel A-1.5m gel filtration fractions was examined (Fig. 4). ^{125}I-insulin affinity crosslinking demonstrated that the material running with a K_{av} = 0.18 from the placenta membranes incubated with or without DTT at pH 7.6 was predominantly the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (*data not shown*). Similarly, the band with a mobility corresponding to the K_{av} = 0.18 from placenta membranes incubated at pH 8.5 in the absence of DTT before detergent solubilization was found to consist primarily of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (Fig. 4*A*). In contrast, ^{125}I-insulin crosslinking of the Bio-Gel A-1.5m gel filtration column fractions obtained from the alkaline pH and DTT-treated placenta membranes demonstrated the presence of the $\alpha\beta$ heterodimeric as well as a small amount of the lower molecular weight proteolytic $\alpha\beta_1$ insulin receptor complex (Fig. 4*B*). Although essentially no ^{125}I-insulin affinity crosslinked $\alpha_2\beta_2$ heterotetrameric complex was found in the Bio-Gel A-1.5m gel filtration column fractions

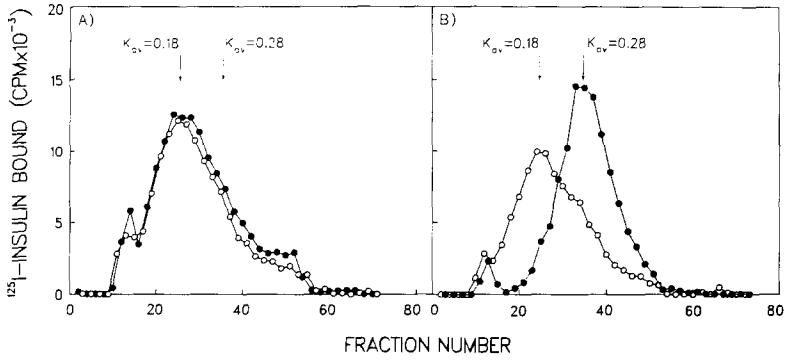

Fig. 3. Bio-Gel A-1.5m gel filtration column profiles of insulin binding to placenta membranes previously treated in the absence or presence of DTT at pH 7.6 or 8.5. Placenta membranes incubated at pH 7.6 (*A*) or 8.5 (*B*) in the absence (○) or presence (●) of 2.0 mM DTT were washed and solubilized with 1.0% Triton X-100 as previously described (Fig. 1). The samples were then applied to separate Bio-Gel A-1.5m columns (1.6 × 46 cm), and 20 ml were voided before collecting 0.4 ml fractions. Aliquots (50 μl) of every other fraction was then assayed for insulin binding activity as described under Materials and Methods

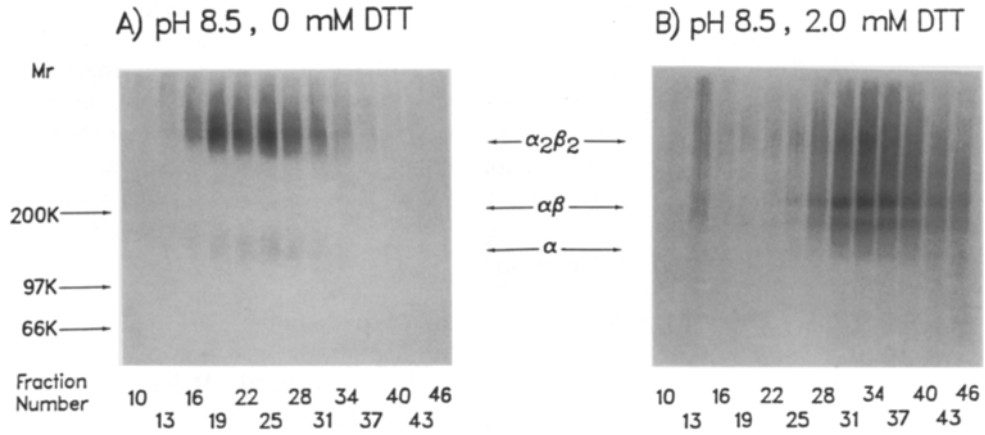

Fig. 4. ^{125}I-insulin affinity crosslinking of Bio-Gel A-1.5m gel filtration column fractions. Aliquots (50 μl) from the fractions of the Bio-Gel A-1.5m gel filtration columns (Fig. 3*B*) were incubated for 16 hr at 4°C in the presence of 0.25 nM monoiodinated-[^{125}I]B_{26}-insulin. The samples were then affinity crosslinked with 0.1 mM DSS and subjected to nonreducing SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. (*A*) ^{125}I-insulin affinity crosslinking profiles from placenta membranes previously incubated in the absence of DTT at pH 8.5 followed by Triton X-100 solubilization and Bio-Gel A-1.5m gel filtration chromatography at pH 7.6. (*B*) ^{125}I-insulin affinity crosslinking profiles from placenta membranes previously incubated in the presence of 2.0 mM DTT at pH 8.5 followed by Triton X-100 solubilization and Bio-Gel A-1.5m gel filtration chromatography at pH 7.6

corresponding to the mobility of $\alpha_2\beta_2$ heterotetrameric complex (K_{av} = 0.18), a significant amount was observed in the column fractions corresponding to the mobility of the $\alpha\beta$ heterodimeric complex (K_{av} = 0.28).

To further examine the structural constraints that help to maintain the $\alpha_2\beta_2$ heterotetrameric association state in the placenta membrane, we next determined the ability of ordered treatments with alkaline pH and DTT to dissociate the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex into an $\alpha\beta$ heterodimeric species (Fig. 5). The placenta membranes were incubated at pH 8.5 for 30 min, diluted, washed by centrifugation and resuspended at pH 7.6. The membranes were then treated for 5 min in the presence or absence of 2.0 mM DTT, solubilized with Triton X-100 and subjected to Bio-Gel A-1.5m gel filtration chromatography (Fig. 5*A*). Under these conditions, no dissociation of the $\alpha_2\beta_2$ heterotetrameric insulin receptors into $\alpha\beta$ heterodimers was detectable. Conversely, placenta membranes were treated at pH 7.6 for 25 min followed by the standard 5.0 min incubation in the presence or absence of 2.0 mM DTT, followed by dilution and

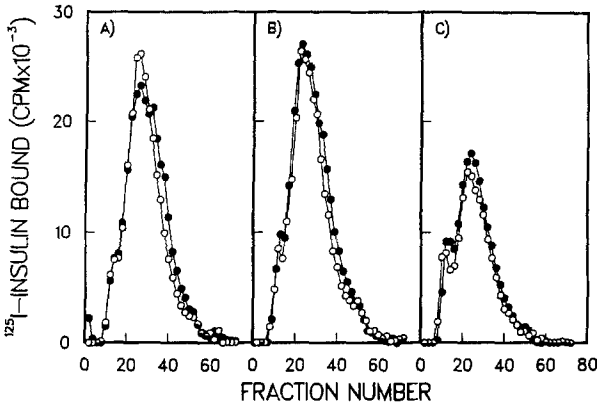

Fig. 5. Effects of sequential DTT and alkaline pH treatments on the dissociation of the placenta membrane insulin receptors. (*A*) Placenta membranes (10 mg/ml) were incubated at pH 8.5 for 30 min at 23°C, then diluted, centrifuged and resuspended at pH 7.6. The membranes were then incubated for 5 min in the absence (○) or presence (●) of 2.0 mM DTT followed by dilution, centrifugation, resuspension and Triton X-100 solubilization prior to Bio-Gel A-1.5m gel filtration chromatography at pH 7.6. (*B*) Placenta membranes (10 mg/ml) were incubated in the absence (○) or presence (●) of 2.0 mM DTT at pH 7.6 for 5 min followed by dilution, centrifugation, resuspension and Triton X-100 solubilization at pH 8.5 before Bio-Gel A-1.5m gel filtration chromatography at pH 7.6. (*C*) Placenta membranes (10 mg/ml) were incubated in the absence (○) or presence (●) of 2.0 mM DTT at pH 7.6 for 5 min. The membranes were then incubated for an additional 15 min with 10 mM NEM followed by dilution, centrifugation, resuspension and Triton X-100 solubilization at pH 8.5 prior to Bio-Gel A-1.5m gel filtration chromatography at pH 7.6

washing as previously described. The membranes were then resuspended at pH 8.5, solubilized with Triton X-100 and subjected to Bio-Gel A-1.5m gel filtration chromatography (Fig. 5*B*). The mobility of the insulin receptor solubilized from these membranes demonstrated only the presence of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex (K_{av} = 0.18). It was possible, however, that the insulin receptor $\alpha_2\beta_2$ complex incubated at pH 7.6 with 2.0 mM DTT, although reduced, may have reoxidized during the dilution and washing procedure before adjustment to pH 8.5. This possibility was examined by first incubating the placenta membranes with or without 2.0 mM DTT for 5 min at pH 7.6 followed by an additional 15-min treatment with 10 mM NEM. The placenta membranes were then diluted, centrifuged, resuspended at pH 8.5 and Triton X-100 solubilized prior to Bio-Gel A-1.5m gel filtration chromatography (Fig. 5*C*). Similar to the previous protocols, no dissociation of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex into an $\alpha\beta$ heterodimeric state was observed. These results demonstrate that reduction of the critical disulfide bond(s) responsible for the covalent association between the $\alpha\beta$ heterodimers under nondenaturing conditions requires the simultaneous treatment of alkaline pH and DTT.

The Bio-Gel A-1.5m gel filtration column fractions (K_{av} = 0.18 and 0.28) were also subjected to Scatchard analysis of insulin binding (Fig. 6). These data likewise demonstrated the typical curvilinear binding plots from all the samples which migrated with a K_{av} = 0.18 ($\alpha_2\beta_2$ complex). Similar to the Triton X-100 solubilized placenta membranes subsequent to alkaline pH and DTT treatment (Fig. 2*D*), the Bio-Gel A-1.5m column fractions with a mobility of K_{av} = 0.28 ($\alpha\beta$ complex) uniquely displayed a single class of homogeneous insulin binding sites over the entire insulin concentration range examined with an approximate K_d = 1.0 nM. The number of insulin binding sites at saturation (Fig. 6*B*) was observed to increase approximately two-fold between the $\alpha_2\beta_2$ heterotetrameric (K_{av} = 0.18) and $\alpha\beta$ heterodimeric (K_{av} = 0.28) complexes, respectively (R_0 = 5.4 *vs.* 11.9 pmol/mg). Competition of ^{125}I-insulin binding by unlabeled insulin and unlabeled IGF-1 [22] to the Bio-Gel A-1.5m gel filtration column isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric receptor complexes demonstrated the specificity of the Scatchard analysis for the insulin receptor species (*data not shown*).

Discussion

Earlier reports have demonstrated that pretreatment of various membrane preparations with low DTT concentrations at neutral pH have either no effect (human placenta) or increased tracer insulin binding from two- (rat liver) to six- (rat adipocyte) fold [11, 34]. We have consistently observed that human placenta membranes treated with DTT at neutral pH results in an approximate 140% increase in tracer insulin binding when protease inhibitors, particularly bacitracin, were included in the binding assays (Fig. 1). The ability of DTT to stimulate insulin binding is presumably due to a direct effect on the insulin receptors since the membranes are initially incubated with DTT and subsequently washed to remove the reducing agent before the addition of ^{125}I-insulin. Furthermore, we have also observed a similar DTT increase in tracer insulin binding activity in purified human placenta and rat adipocyte insulin receptors [36]. In contrast, DTT treatment of placenta membranes at pH 8.5, followed by removal of the DTT and pH adjustment to 7.6, resulted in a biphasic response with respect to insulin binding. DTT treatment at pH 8.5 from 1 to 5 min resulted in an approximate 1.4-fold increase in tracer insulin binding; however, by 30 min the binding was de-

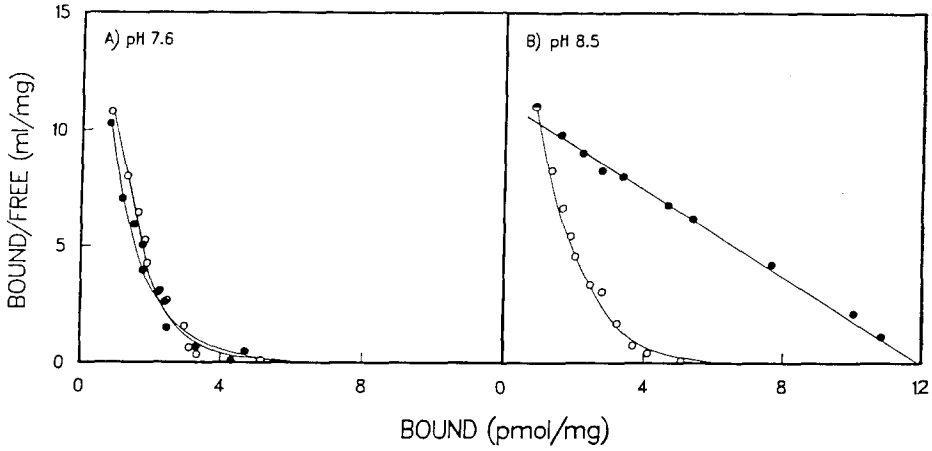

Fig. 6. Scatchard analysis of insulin binding to the isolated $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric insulin receptor complexes. The peak fractions from the Bio-Gel A-1.5m gel filtration columns (Fig. 3) were pooled and subjected to competition of insulin binding (0.1 to 100 nM) as described under Materials and Methods. (*A*) Bio-Gel A-1.5m gel filtration column peak fractions (K_{av} = 0.18) were pooled from placenta membranes previously incubated in the absence (○) or presence (●) of 2.0 mM DTT at pH 7.6, followed by Triton X-100 solubilization and gel filtration chromatography at pH 7.6. (*B*) Bio-Gel A-1.5m gel filtration column peak fractions (K_{av} = 0.18 and 0.28) were pooled from placenta membranes previously incubated in the absence (○) or presence (●) of 2.0 mM DTT at pH 8.5, followed by Triton X-100 solubilization and gel filtration chromatography at pH 7.6

creased to 35% of the control value. Identical results were also obtained when the placenta membranes were solubilized with Triton X-100 after the initial pH and DTT treatments. These data clearly demonstrate that the incubation time of placenta membranes with DTT at pH 8.5 is critical in order to maintain maximal recovery of insulin binding activity with the formation of the $\alpha\beta$ heterodimeric insulin receptor complex.

In contrast to the ability of DTT to generate $\alpha\beta$ heterodimers at pH 8.5, under neutral pH conditions DTT was either unable to reduce the critical disulfide linkage(s) necessary to maintain the covalent $\alpha_2\beta_2$ heterotetrameric structure or, alternatively, the insulin receptor $\alpha\beta$ heterodimers may have rapidly reoxidized after the removal of DTT. This second possibility appears not to be the case since treatment of placenta membranes at pH 7.6 with DTT, followed by addition of excess NEM and solubilization at alkaline pH did not result in the formation of an $\alpha\beta$ heterodimeric state (Fig. 5). It therefore seems likely that the critical disulfide bond(s) necessary for the covalent association of the $\alpha_2\beta_2$ complex is accessible to DTT only under alkaline pH conditions.

Consistent with this interpretation, Scatchard analysis of insulin binding to placenta membranes (Fig. 2*A*) or Triton X-100 solubilized placenta membranes (Fig. 2*C*) incubated at pH 7.6 in the presence or absence of DTT displayed the typical curvilinear binding plots with identical number of insulin binding sites at saturation. Similarly, placenta membranes treated with or without DTT at pH 8.5 also generated curvilinear binding plots with the equivalent number of total insulin binding sites (Fig. 2*B*). However, Scatchard analysis performed on the DTT-treated placenta membranes at pH 8.5 which were then subsequently detergent solubilized produced a significantly less curvilinear insulin binding plot with an approximate 1.5-fold increase in the total number of insulin binding sites at saturation (Fig. 2*D*).

To confirm that these different binding curves were reflective of the different association states of the insulin receptor, we separated the solubilized membranes by Bio-Gel A-1.5m gel filtration chromatography (Fig. 3) followed by ^{125}I-insulin binding and ^{125}I-insulin affinity crosslinking. These experiments confirmed the formation of the $\alpha\beta$ heterodimeric insulin receptor complex only when the placenta membranes were DTT treated at pH 8.5 and immediately detergent solubilized (Fig. 4). The appearance of a small amount of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex in the ^{125}I-insulin affinity crosslinking of the peak $\alpha\beta$ heterodimeric Bio-Gel A-1.5m gel filtration column fractions (Fig. 4*B*) is not due to cross contamination with the DTT-treated but nondissociated $\alpha_2\beta_2$ heterotetrameric complex. Previous studies have demonstrated that this occurs as a result of an insulin-dependent covalent reassociation between $\alpha\beta$ heterodimers which can be blocked by addition of sulfhydryl agents [24].

Scatchard analysis of the Bio-Gel A-1.5m gel filtration column fractions documented that the $\alpha\beta$ heterodimeric insulin receptor complex displays homogeneous insulin binding with twice the number of

binding sites compared to the $\alpha_2\beta_2$ heterotetrameric complex (Fig. 6), similar to that observed for the purified insulin receptor complexes [37]. The two-fold increase of the number of insulin binding sites in the $\alpha\beta$ heterodimeric complex compared to the $\alpha_2\beta_2$ heterotetrameric complex is consistent with half-site binding reactivity in the native insulin receptor $\alpha_2\beta_2$ heterotetrameric state [37]. The 1.5-fold increase in insulin binding sites from the detergent solubilized placenta membranes (Fig. 2) probably reflects incomplete dissociation and/or partial reoxidation of the $\alpha\beta$ heterodimers as well as the presence of aggregated insulin receptors, which are effectively resolved by Bio-Gel A-1.5m gel filtration chromatography (Fig. 3). The ability of Triton X-100 solubilization of alkaline pH and DTT-treated placenta membranes to produce a linear Scatchard plot has also been recently observed by others [3] and is consistent with the role of $\alpha\beta$ heterodimeric subunit interactions being responsible for the high affinity curvilinear insulin binding in the $\alpha_2\beta_2$ heterotetrameric complex [3, 37].

In summary, we have observed that a combination of alkaline pH and DTT treatment of placenta membranes can reduce the essential disulfide bond(s) required for the covalent association between $\alpha\beta$ heterodimers. The reduced $\alpha_2\beta_2$ heterotetrameric complex does not dissociate in the membrane, has identical insulin binding properties and can readily reoxidize into a covalent $\alpha_2\beta_2$ heterotetrameric disulfide-linked state. However, detergent solubilization of the reduced $\alpha_2\beta_2$ heterotetramers allows them to dissociate into functional $\alpha\beta$ heterodimers. Moreover, the $\alpha\beta$ heterodimeric insulin receptor displays homogeneous insulin binding in which each α subunit is fully capable of binding insulin, whereas insulin binding to the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex is heterogeneous with half-site insulin binding reactivity.

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