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Research Article

Treatment of human placenta membranes with dithiothrietol (DTT) followed by N-ethylmaleimide results in a 60% reduction in insulin binding. Treatment with N-ethylmaleimide alone has little effect. The decrease in insulin binding that results from DTT treatment is due to a decrease in affinity for insulin, with little change in total receptor number. DTT has similar effects on receptor solubilized from placenta membranes with Triton X-100, indicating that its effects are not attributable to changes in the arrangement of receptors in the membrane. In contrast to placenta membranes, treatment of liver membranes with DTT does not decrease insulin binding. These results suggest that reduction of a critical disulfide bond in insulin receptors from human placenta converts the receptor to a low affinity form.

Disulfide Reduction Converts the Insulin Receptor of Human Placenta to a Low Affinity Form

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ABSTRACT Treatment of human placenta membranes with dithiothietol (DTT) followed by *N*-ethylmaleimide results in a 60% reduction in insulin binding. Treatment with *N*-ethylmaleimide alone has little effect. The decrease in insulin binding that results from DTT treatment is due to a decrease in affinity for insulin, with little change in total receptor number. DTT has similar effects on receptor solubilized from placenta membranes with Triton X-100, indicating that its effects are not attributable to changes in the arrangement of receptors in the membrane. In contrast to placenta membranes, treatment of liver membranes with DTT does not decrease insulin binding. These results suggest that reduction of a critical disulfide bond in insulin receptors from human placenta converts the receptor to a low affinity form.

INTRODUCTION

Insulin receptors are composed of multiple subunits linked by interchain disulfide bonds (1–4). In rat liver, the receptor appears to exist in two redox states (3, 4). The oxidized form can be converted into the reduced form by the disulfide reducing agent dithiothreitol (DTT).[1] Similar forms of the receptor exist in human placenta (unpublished observations). In the present work we have determined the effect of treating the receptor with DTT on the subsequent binding of insulin.

METHODS

Human placenta membranes (5) and rat liver membranes (6) were prepared as described previously, with the modification that 10 μg/ml phenyl methyl sulfonyl fluoride and 5 mM EDTA were included in the homogenization buffer to prevent proteolysis. Placenta membranes (10 mg protein/ml of 50 mM Tris HCl, pH 7.7) were solubilized with 2% Triton X-100, and after centrifugation at 200,000 *g* for 1 h, the supernate was used for studies on solubilized insulin receptors. ^{125}I-Insulin was prepared with chloramine-T (7).

Insulin binding to membranes was measured by filtration with EGWP Millipore filters (Millipore Corp., Bedford, Mass.) (8). Insulin binding to the solubilized receptor was measured by the polyethylene glycol assay (9). In all binding studies, the amount of ^{125}I-insulin bound in the presence of 20 μg/ml of native insulin was determined and used to calculate the nonspecific binding.

To assess the degree of insulin degradation during the binding studies, the membranes were pelleted by centrifugation at 80,000 *g* for 3 min, and the fraction of radioactivity in the supernate that was precipitated by 5% TCA with 1% albumin as a carrier was determined.

RESULTS

The effect of DTT on insulin binding to placenta membranes is shown in Table I. If placenta membranes are incubated with DTT and then with *N*-ethylmaleimide (NEM), which rapidly alkylates accessible sulfhydryl groups, there is a reduction in specific insulin binding. NEM alone causes a considerably smaller, but still significant decrease in insulin binding. Since DTT will rapidly inactivate insulin, it is important that the DTT be fully quenched by NEM before the addition of the ^{125}I-insulin. That the DTT is indeed completely quenched by this procedure is shown by reversing the order of addition of the membranes to the incubation. If DTT is incubated with NEM before the addition of placenta membranes, there is no reduction in insulin binding (Table I). Since membrane preparations contain insulin-degrading enzymes, which are sensitive to sulfhydryl reagents (10), it is also important to determine whether the observed decrease in binding is the result of enhanced insulin degradation. As shown in Table I, at least 92% of the ^{125}I-insulin was trichloroacetic acid precipitable at the end of the incubation with each of the treatments.

Fig. 1 shows a concentration-effect curve for DTT. Under the conditions used, 0.5 mM DTT produces nearly half-maximal inhibition of binding.

As shown in Fig. 2, a Scatchard plot of insulin binding to placenta membranes that have not been treated with

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[1] *Abbreviations used in this paper*: DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

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TABLE I
Effect of DTT and NEM on Insulin Binding to Placenta Membranes

Treatment	Bound specifically	TCA precipitable
	cpm	%
None	496±22	91±1
DTT then NEM	213±21	91±1
NEM	429±25	92±1
DTT previously alkylated by NEM	505±15	—

Placenta membranes were incubated at room temperature in Krebs-Ringer bicarbonate buffer, pH 7.4, and 0.1% albumin with no additions; 10 mM DTT for 10 min followed by 25 mM NEM for 10 min; no additions for 10 min followed by 25 mM NEM for 10 min; or 10 mM DTT that had been alkylated with 25 mM NEM before the addition of membranes. ^{125}I-Insulin (9,500 cpm) was then added to each tube and the incubation was continued for 90 min at 15°C. The amount of ^{125}I-insulin that was specifically bound and that which was trichloroacetic acid (TCA) precipitable was then determined.

DTT is concave. After treatment with DTT, the Scatchard plot approaches linearity, and the initial high affinity portion of the curve is lost with little, if any, change in the total receptor number.

To determine whether inhibition of insulin binding by DTT is a direct effect on the insulin receptor, or whether it results from changes in the plasma membrane or the arrangement of insulin receptors in the plasma membrane, the effect of DTT on insulin receptors solubilized from placenta membranes was determined (Table II). Both DTT and NEM decreased insulin binding to solubilized receptors in a manner similar to that observed with membrane-bound receptors.

In contrast to the results with placenta membranes, insulin binding to rat liver membranes in not inhibited by DTT (Table III), but in this tissue, DTT causes a slight enhancement of binding. NEM results in a small inhibition of insulin binding, comparable to that seen with placenta membranes.

DISCUSSION

These studies demonstrate that treatment with DTT followed by NEM of insulin receptors from placenta membranes diminishes the affinity for insulin binding, presumably by reducing (and alkylating) disulfide bonds. Despite the similarities in the structure and binding properties of insulin receptors from rat liver and human placenta, DTT does not inhibit insulin binding to rat liver membranes.

It has been reported previously (11) that DTT inhibits insulin binding in solubilized human placenta. However, this was attributed to increased insulin degradation, which was also observed after treatment with DTT. In the present study, insulin degradation was minimal and was not significantly affected by DTT.

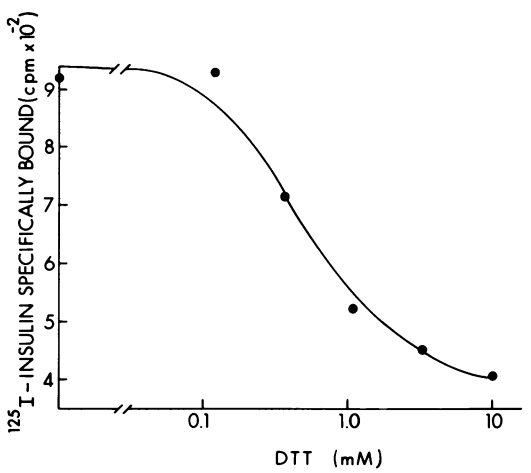

FIGURE 1 The effect of different concentrations of DTT on insulin binding to placenta membranes. Placenta membranes were incubated with the indicated concentrations of DTT at 22°C for 10 min. NEM was added to each tube to bring the concentration to 25 mM. The specific binding of ^{125}I-insulin (12,000 cpm) was then measured after a 90-min period of incubation at 15°C.

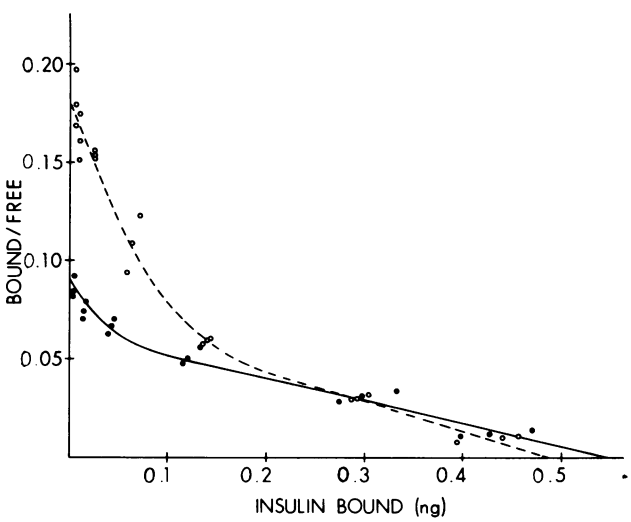

FIGURE 2 Scatchard plots of insulin binding to placenta membranes treated with DTT. Placenta membranes were incubated with or without 5 mM DTT for 10 min at 22°C, and then with 12.5 mM NEM for an additional 10 min at 22°C. The specific binding of various concentrations of ^{125}I-insulin to 40 μg of DTT-treated (●) membranes or to membranes not treated with DTT (○) after a 90-min incubation in 0.2 ml of Krebs-Ringer bicarbonate buffer, and 0.1% albumin at 15°C was then determined. The ordinate is the ratio of counts per minute bound to counts per minute free. The data are the result of a single representative experiment.

TABLE II
Effect of DTT and NEM on Insulin Binding to Solubilized Placenta Membranes

Treatment	Bound specifically
	cpm
None	970±25
DTT followed by NEM	390±16
NEM	770±38

Solubilized placenta membranes were incubated with no additions, 10 mM DTT followed by 25 mM NEM, or 25 mM NEM as described in the legend to Table I. ^{125}I-Insulin (12,500 cpm) was then added to each tube and the incubation continued for 90 min at 15°C. The amount of ^{125}I-insulin bound was then determined.

TABLE III
Effect of DTT and NEM on Binding of ^{125}I-Insulin to Liver Membranes

Treatment	Bound specifically
	cpm
None	950±94
DTT followed by NEM	1182±63
NEM	770±30

Liver membranes were incubated with no additions, DTT followed by NEM, or NEM as described in the legend to Table I. ^{125}I-Insulin (9,600 cpm) was added to each tube and after a 90-min period of incubation at 15°C the amount of ^{125}I-insulin specifically bound was determined.

These different results are probably best explained by the fact that in the present study, insulin binding was performed at 15° rather than 37°C, and DTT was quenched by NEM before the addition of insulin.

Previous reports have described that sulhydryl reagents such as NEM and iodoacetamide have no effect on insulin binding to fat cells (12), liver membranes (13), or placenta membranes (11). We observe a small decrease in insulin binding, but only at considerably higher concentrations of NEM than were used in the previous studies. When we use concentrations of NEM <10 mM, we also observe no significant effect on insulin binding (data not shown).

In a recent paper, the effect of DTT on insulin binding to fat cell membranes was studied (14). As in the present study, DTT tended to linearize the Scatchard plots of insulin binding, and to decrease the affinity of the high affinity sites. However, the affinity of the low affinity sites was increased after treatment with low concentrations (1 mM) of DTT, resulting in a net increase in insulin binding. At high concentrations of DTT, insulin binding decreased below control levels. No such biphasic effects were seen in the present study using placenta or liver membranes. However, we do see similar effects with fat cell membranes (data not shown).

Recent studies have yielded considerable information about the structure of the insulin receptor. It appears to be a tetramer composed of two copies of a subunit having an apparent molecular weight on sodium dodecyl sulfate (SDS)-polyacrylamide gels of about 135,000, and two copies of a lower molecular mass subunit, which may be either 90,000 daltons or 45,000 daltons (1, 3, 4, 15–17). In liver membranes, the receptor exists in different redox states (3, 4). In the oxidized state, the four subunits are linked by interchain disulfide bonds, so that the tetramer remains intact after it is denatured by boiling in SDS (1–4). In the reduced state, the native molecule is also a tetramer; however, when it is denatured, it dissociates into dimers composed of one 135,000-dalton subunit and one of the lower mass subunit (1–4).

In view of the results presented in this manuscript, it is tempting to speculate that these different redox forms of the receptor have different affinities for insulin. This could account for the heterogeneous forms of the receptor that have been described previously on the basis of insulin binding (18). Interconversion of redox forms could also account for the rapid changes in the affinity of insulin receptors that occur under various physiological conditions (i.e., fasting, administration of glucose, after binding insulin). However, it is difficult to explain why the effects of DTT are different in rat liver and human placenta, when these two receptors appear to be structurally similar (1).

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