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EVIDENCE THAT INSULIN AND CONCANAVALIN-A CAN CO-BIND TO SOLUBILIZED INSULIN RECEPTORS WITHOUT INHIBITING EACH OTHER

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SUMMARY: Concanavalin A (Con A) precipitates detergent-solubilized insulin receptors (free of or bound to [^{125}I]insulin) prepared from fat cell membranes resulting in a loss of [^{125}I]insulin binding capacity (or bound hormone) in the soluble fraction. The losses can be recovered by redissolving the precipitates with methyl-α-D-mannoside; the sugar also inhibits precipitation. [^{125}I]insulin also binds to insoluble Con A-occupied receptors. At all concentrations of Con A tested, the initial amounts of free or hormone-bound receptors were completely accounted for by their distribution between soluble and insoluble states. We conclude that Con A and insulin can co-bind to independent sites on the insulin receptor without inhibiting each other and that previously reported decreases in insulin binding to solubilized insulin receptors were likely due to precipitation by Con A of the receptors.

INTRODUCTION: Con A exhibits a variety of insulin-like bioactivities, and there is evidence that these effects are mediated by insulin receptors (1-3). Con A also inhibits the binding of [^{125}I]insulin (1-4) and insulin-Sepharose (5) to intact cells. Moreover, Con A-treatment of detergent-solubilized insulin receptors results in a decrease in the insulin binding capacity of the remaining solution (1-3). Solubilized insulin receptors also bind to immobilized lectin on affinity columns (1,6). It has therefore been assumed that the observed inhibition of insulin binding to intact cells is due to a direct interaction of the lectin with insulin receptors. However, it has also been proposed, upon finding various insulin-like and antagonistic glycosides, that there might exist on cell membranes a carbohydrate-containing component separate and distinct from, but functionally linked to, insulin receptors that might modify insulin binding (5).

In the present studies, evidence is presented that the ability of Con A to "inhibit" detergent-solubilized insulin receptors prepared from adipocyte

Abbreviation: Con A, Concanavalin A

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surface membranes is due to the precipitation from solution of the receptors as a result of their binding to Con A. The evidence indicates that Con A can co-bind with insulin to the receptors without each ligand inhibiting the other.

MATERIALS AND METHODS: Isolated fat cells (7) were derived from the epididymal fat pads of albino Charles River CD rats weighing 185-210 gm and fed Purina Chow ad libitum. Fat cell ghosts (8) were solubilized by incubating them in Krebs-Ringer Tris (0.1M) buffer, pH 7.5, containing 0.5 percent Triton X-100 for 30 min at 22°, centrifuging the resultant mixture at 105,000 x g for 30 min, and then filtering the supernatant through an HAWP millipore filter yielding soluble insulin receptors. Highly purified membranes (9) were solubilized by incubating them in 0.1M phosphate buffer containing 1.0 percent Triton X-100 for 60 min at 22°, followed by centrifugation at 105,000 x g for 30 min yielding the soluble receptors in the supernatant. [^{125}I]insulin was from New England Nuclear and further purified by DE-23 chromatography (10). Con A (affinity chromatographically purified and designated homogeneous by acrylamide electrophoresis) was from Pharmacia. The Sephadex G-100 assay for measuring soluble bound [^{125}I]insulin is based on the ability of this gel to separate bound from free [^{125}I]insulin after incubation of the labeled hormone with the solubilized receptor preparation (11,12). The area measured under the void volume fractions that eluted from the column represents the amount of bound [^{125}I]insulin added to the column, and is dose responsive to both membrane protein and hormone (12).

RESULTS AND DISCUSSION: Table 1 shows the effects of incubating a Triton-solubilized extract of insulin receptors (from fat cell ghosts) with Con A and of separating the resultant insoluble fraction from the soluble fraction by centrifugation prior to assaying the binding capacities of these fractions. Soluble bound [^{125}I]insulin was determined by the Sephadex G-100 assay. In the absence of Con A, receptors remained soluble. Con A, in the absence of receptors, did not bind [^{125}I]insulin. When Con A was incubated with receptors and the resultant visibly cloudy mixture was incubated with labeled hormone, 75-80 percent less receptor-bound hormone was detectable. Removal of the precipitate from the incubation mixture prior to incubation with [^{125}I]-insulin consistently increased the inhibitory effect to about 95 percent. This suggests that the loss of insulin binding resulted from a Con A-produced removal of receptors from solution.

That the apparent precipitation of the receptor was due to receptor-bound Con A rather than to co-precipitation of free receptors is suggested by the ability of methyl-α-D-mannoside to block the effect of Con A. When the mix-

Table 1: Effects of Con A on Solubilized Insulin Receptors and on [^{125}I]insulin Binding as Measured by Sephadex G-100 Assay After Centrifugation

Initial Supplements (Incubation I)	Centrifugation I (±)	Additions to Supernatant, Pellet or Uncentrifuged Incubation (Incubation II)	Specifically bound [^{125}I]insulin in Incubation III (cpm)
R	(+)	none (Sup.)	2170
R	(+)	αMMan (Sup.)	2065
R	(-)	αMMan	2005
Con A	(+)	none	15
R + Con A	(-)	none	475
R + Con A	(+)	none (Sup.)	125
R + Con A + αMMan	(+)	none (Sup.)	1900
R + Con A	(+)	αMMan (Sup.)	145
R + Con A	(+)	αMMan (pellet)	1795

Insulin receptors (R) solubilized from fat cell ghosts were incubated (2.48 mg of membrane protein per ml) with and without 250 μg Con A per ml and 0.3 M methyl-α-D-mannoside (αMMan) in Krebs Ringer Tris (15 mM) buffer, pH 7.4, containing 0.5% Triton X-100, in a final volume of 0.5 ml for 30 min at 22° (Incubation I). After centrifugation (+) at 105,000 x g for 20 min, or uncentrifuged (-), the resultant supernatant (Sup.) or pellet (supernatant decanted) was incubated with or without 0.3M αMMan in a final volume of 0.55 ml (Incubation II). The insulin binding capacity of incubation II was determined by adding [^{125}I]insulin (to 2 x 10^{-9}M) and incubating 20 min at 22° (Incubation III) followed by determination of bound [^{125}I]-insulin by Sephadex G-100 assay. Specific binding was determined by subtracting binding (non-specific) in the presence of 5 x 10^{-6}M insulin. Values are recorded to the nearest 5 cpm; experiments were repeated twice yielding similar results.

ture was centrifuged prior to sugar-treatment, the sugar could not reverse the loss in binding capacity. Consistent with the view that Con A binds to, and thereby precipitates, the insulin receptor as a glycoprotein, methyl-α-D-mannoside was able to visibly redissolve the Con A-formed precipitate ("pellet" in Table 1) yielding a solution containing nearly all of the original [^{125}I]insulin binding capacity.

Although the evidence suggested that Con A reduces the insulin-binding capacity of a solution of insulin receptors by binding to free receptors and thereby removing them from solution by precipitation, it was unclear whether each ligand (lectin or hormone) can bind to, and precipitate with, receptors already occupied by the other ligand. Thus, the ability of either ligand to

prevent the binding of the other remained a question. To help answer this, Con A was incubated with receptors before and after incubation of receptors with [^{125}I]insulin, and the soluble and Con A-rendered insoluble products were separated by millipore filtration (Table 2).

Table 2 shows the data on the ability of Con A to bind to, and precipitate, [^{125}I]insulin-occupied insulin receptors. As expected from the conclusions drawn from Table 1, the large increase in radioactivity rendered insoluble by Con A (Table 2) was accompanied by a concomitant decrease in soluble bound [^{125}I]insulin. In view of the requirements for both Con A and receptor for precipitation of radioactivity, the complete recovery of receptors in the combination of soluble and insoluble fractions supports the notion that the insoluble radioactivity represents a tertiary complex of [^{125}I]insulin and Con A co-bound to individual insulin receptors. The ability of the sugar to redissolve the insolubilized receptors further supports this view.

As in Table 1, precipitation of the receptor with Con A prior to incubation with labeled hormone leads to an even greater loss in the hormone-binding capacity of the soluble fraction (Table 2). However, even though incubation of receptors with Con A was sufficient to precipitate most of the receptors prior to exposure to hormone ("Filtration I" experiment in Table 2), a considerable amount of insoluble bound hormone resulted after the hormone treatment if the Con A-bound receptors were not filtered. This suggests that the insulin binding site on the receptor remains accessible despite the insolubility of the Con A-occupied receptors. This latter binding argues against non-specific entrapment of hormone during the formation of, and within, a matrix of Con A-induced crosslinked or aggregated receptors, inasmuch as any crosslinked insoluble matrices should have been formed prior to exposure to hormone. The decreased recovery seen in Table 2 (third experiment listed), resulting from a decrease in both insoluble and soluble receptor-bound [^{125}I]insulin, likely reflects the physical separation, by precipitation, of the receptors from the subsequently-added free hormone.

Table 2: Effects of Con A on Solubilized Insulin Receptors and on [^{125}I]insulin Binding Measured by Sephadex G-100 Assay After Millipore Filtration

Initial Supplements (Incubation I)	Filtration I (±)	Supplements after Filtration I (Incubation II)	Filtration II (±)	Specifically Bound [^{125}I]insulin: in Filtrate II (cpm)	on Filter II (cpm)	Recovery (cpm)
[^{125}I]ins + R	(-)	none	(+)	2320	41	2361
[^{125}I]ins + R	(-)	Con A	(+)	990	1522	2512
Con A + R	(-)	[^{125}I]ins	(+)	520	832	1352
Con A + R	(+)	[^{125}I]ins	(-)	165	-	-
Con A + [^{125}I]ins	(-)	none	(+)	0	38	-

Insulin receptors (R) solubilized from fat cell ghosts (2.5 mg membrane protein per ml) were incubated with 2 x 10^{-9}M [^{125}I]insulin ([^{125}I]ins), 25 µg Con A per ml, or both, for 30 min at 22° (Incubation I). Resultant mixtures were filtered (+) through EAWP millipore filters (Filtration I), or unfiltered (-), as designated. To the filtrates (or non-filtrates) were added Con A or [^{125}I]insulin to the same concentrations as above, or with buffer alone, and incubated for 30 min at 22° (Incubation II). Resultant mixtures were then filtered through fresh filters (Filtration II) and the insoluble radioactivity on the filters was counted. Specifically bound [^{125}I]insulin in the filtrate II (soluble) was determined by the Sephadex G-100 assay. Other details are as in Table 1.

In Table 3 are the results of titrating the effect of Con A in generating insoluble receptor-bound [^{125}I]insulin when the lectin was incubated with receptors in the presence of [^{125}I]insulin. Receptors solubilized from highly purified membranes (9) were used, and the soluble bound [^{125}I]insulin was determined by the polyethylene glycol method (13,14). In agreement with the conclusions drawn from Tables 1 and 2, the losses of insulin-binding capacity in the soluble fraction, brought about by Con A, could be accounted for by corresponding increases in the insoluble (non-filtratable) radioactivity. That the recoveries of receptors at all concentrations of Con A tested corresponded to the amounts present prior to Con A-treatment argues against the possibility, previously noted, that the association of [^{125}I]insulin with the insoluble fraction might reflect non-selective entrapment within a matrix of receptors crosslinked or aggregated with Con A. Moreover, the complete recovery at a concentration of lectin exceeding that required to maximally reduce the binding capacity of the soluble fraction indicates that Con A had little, if any, inhibitory effect on binding of hormone to the receptors.

Table 3: Titration of the Effect of Con A on [^{125}I]insulin Binding to Solubilized Insulin Receptors as Measured by Polyethylene Glycol Assay After Filtration

Supplements (per ml)	Specific Binding in Filtrate aliquot (cpm ± S.E.M.)	Specific Binding on Filter paper (cpm ± S.E.M.)	Total Specific Binding Recovered (cpm)	% Recovery of Bound [^{125}I]insulin
none	-115 ± 362	78 ± 85	-	-
R	5755 ± 561	-5 ± 535	23015	-
R + Con A (50 µg)	3380 ± 474	11136 ± 1096	24656	107
R + Con A (100 µg)	652 ± 189	16865 ± 2104	19473	85
R + Con A (500 µg)	198 ± 233	23054 ± 1873	23852	104
R + Con A (1 mg)	389 ± 340	23627 ± 2284	25183	109
Con A (500 µg)	149 ± 162	98 ± 117	-	-
R + Con A (100 µg) + αMMan	6443 ± 61	-118 ± 125	25772	112

Insulin receptors (R) (135 µg of protein per ml) solubilized from purified membranes (9) were incubated with 1.3 x 10^{-9}M [^{125}I]insulin with and without the listed amounts of Con A in the presence and absence of 0.1M methyl-α-D-mannoside in a final volume of 1.0 ml of 0.1M phosphate buffer, pH 7.5 for 30 min at 22°. The incubation was continued for 17 hrs at 4°. Due to the presence of Triton X-100 in the receptor extract, Triton was at a final concentration of 0.08% in the incubation. Resultant suspensions were filtered through EHWP (0.5 µm pore size) millipore filters and the filters washed 4X with 5 ml buffer per wash and the radioactivity counted. Bound [^{125}I]insulin in 0.25 ml aliquots of the filtrates was determined by the polyethylene glycol (P.E.G.) assay (13,14). Each value in the mean of duplicate incubations and each duplicate value of filtrates was derived from P.E.G. assays run in triplicate. Standard errors are calculated from the duplicate values. Radioactivity on filters is the mean of duplicate experiments, one filtration per experiment. "% recovery" represents combined cpm (filtrate plus cpm "on filter") relative to the 23015 cpm in the control experiment. This experiment was repeated three times yielding similar results.

The finding that methyl-α-D-mannoside completely inhibited the production by Con A of insoluble radioactivity and concomitantly prevented the reduction of soluble binding capacity (Table 3) strengthens the previous argument (Table 1) that the insoluble radioactivity is not due to co-precipitation of either free [^{125}I]insulin or Con A-free receptor-bound [^{125}I]insulin.

The stoichiometric effect of Con A in producing an insoluble form of [^{125}I]insulin at the apparent expense of soluble receptor-bound [^{125}I]insulin

reinforces the view that the insoluble radioactivity trapped on the filter paper represents [^{125}I]insulin and Con A co-bound to the insulin receptor at independent sites in the form of a tertiary complex without each ligand inhibiting the binding of the other. These results further indicate that previously observed decreases of insulin binding to solubilized insulin receptors brought about by Con A (1,2) were likely due to precipitation of the receptors rather than to inhibition of insulin binding. It is therefore suggested that the previously observed inhibition by Con A of insulin binding to the intact cell (1-4) may be due to the interaction of the lectin with a carbohydrate-containing moiety on the cell membrane distinct from, but capable of modifying, the insulin receptor as previously postulated (5).

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