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GLYCOSYLATION OF CELL SURFACE RECEPTORS: TUNICAMYCIN TREATMENT DECREASES INSULIN AND GROWTH HORMONE BINDING TO DIFFERENT LEVELS IN CULTURED LYMPHOCYTES

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Summary: Treatment of IM-9 human cultured lymphocytes with tunicamycin, an antibiotic that specifically inhibits the asparagine-linked N-glycosylation of proteins, resulted in a time and dose-dependent decrease in insulin binding. Carbohydrate incorporation into protein fell, while leucine incorporation was slightly elevated. Specific growth hormone binding also decreased, with an identical tunicamycin dose-response, to 40% of control levels whereas insulin binding decreased to 16%. For both hormones, Scatchard analysis indicated that these decreases were mostly in receptor binding capacity, not affinity. These data are consistent with a differential decrease in the receptor steady-states due to inhibited glycosylation.

INTRODUCTION: There is strong evidence that the insulin receptor from various tissues is a glycoprotein (1-6). Some evidence suggests that the growth hormone (GH) receptor from pregnant rabbit liver membranes is also glycosylated (7). The role of carbohydrate moieties in the function of the receptors, however, is unknown. A glycoprotein that did not bind insulin was found solubilized with the insulin receptor from liver membranes (8). Recently Hedo et al reported an increase in the affinity of the solubilized receptor from human placenta or IM-9 lymphocytes after passage through certain lectin columns (9). They postulate the removal of a glycoproteic affinity regulator of the also glycosylated receptor.

The antibiotic tunicamycin (TM) (10) specifically inhibits N-glycosylation (11), which is the predominant type of all protein glycosylation and occurs via the dolichol phosphate pathway (12,13). In 1979, Rosen et al demonstrated that TM decreases the appearance of functional insulin receptors in differentiating and differentiated 3T3-L1 pre-adipocytes (5). Recently TM was shown to prevent the conversion of ST 13 fibroblasts to adipocytes and the increase in the number of insulin receptors that accompanies such development (14).

In this study, we found that TM decreases the expression not only of insulin but also of GH receptors in human cultured lymphocytes, one of the cell

Abbreviations used: GH, growth hormone; TM, tunicamycin.

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lines most commonly used for studying the regulation of these receptors (15, 16). The conditions employed effectively inhibited carbohydrate and slightly increased leucine incorporation into protein. The data provide new evidence that the GH receptor is a glycoprotein and suggest that preventing N-glycosylation of insulin and GH receptors results in a decreased steady-state level of each in the membrane, but to different extents.

MATERIALS AND METHODS:

Materials: Porcine monocomponent insulin (a gift from NOVO Research Institute; lot 141042) was iodinated with Na ^{125}I(New England Nuclear) as previously described (17) to a specific radioactivity of 200 μCi/μg (0.55 mol ^{125}I/mol insulin) and purified by fractionation on a Sephadex G-75 column. Human GH (a gift from Dr. Alejandro C. Paladini, Departamento de Quimica Biologica, Facultad de Farmacia y Bioquimica, Buenos Aires, Argentina) was iodinated (17) to a specific radioactivity of 60 μCi/μg (0.55 mol ^{125}I/mol GH) and purified on a column of Sephadex G-50. Stock solutions of TM (a gift from Dr. Robert L. Hamill, Lilly Research Laboratories) were made by weight in 0.01N NaOH and stored frozen in aliquots. L-[4,5-^{3}H]-leucine (60 Ci/mmol), D-[1-^{3}H]-mannose (13.2 Ci/mmol) and D-[6-^{3}H]-glucosamine HCl (19 Ci/ mmol) were from New England Nuclear. Lumasolve and Lipoluma were from Lumac Systems. RPMI 1640 was from GIBCO. All other materials were reagent grade.

Cell Culture: Human IM-9 lymphocytes were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, by feeding 3 times a week (17). For experiments, cells at stationary growth were diluted 1:2.5 with fresh medium and incubated (37°C) with or without TM (0-2 μg/ml). Control cells received equivalent amounts of 0.01 N NaOH. TM was added either at the time of dilution, or 24 hr later, in which case the preincubated cell stocks were pooled and divided into portions for testing the various amounts of TM. After incubation as indicated in the text, cells were collected by centrifugation (10 min x 800 g) and prepared for the assays as described.

Hormone Binding Assays: ^{125}I-insulin binding to control or TM-treated lymphocytes was assayed as previously described (17). Centrifuged cells were washed 3 times in assay buffer (100 mM Tris, 120 mM NaCl, 1.2 mM Mg sulfate, 1 mM EDTA, 10 mM glucose, 15 mM Na acetate, and 10 mg/ml bovine serum albumin, pH 7.6), then resuspended for cell counts and binding experiments. Cell viability was determined in two representative experiments by the trypan blue exclusion method (18) and at all concentrations of TM the dye was excluded by 95% or more of the cells. When both ^{125}I-insulin and ^{125}I-GH were assayed for binding, a single batch of cells was washed in GH assay buffer (same as above except it is 25 mM Tris and pH 7.0), resuspended in a small volume, and a portion was used for GH binding studies (16,17). The remaining cells were diluted to 5 x 10^{6} cells/ml with insulin assay buffer and ^{125}I-insulin binding was determined. All values were corrected for nonspecific binding (17).

Sugar and Leucine Incorporation: Cell cultures were diluted as described, incubated at 37°C without TM for 24 hr, then pooled and divided into 50 ml batches. TM (0-2 μg/ml) was added, and after 18 hr more incubation, 3 sets of duplicate 5 ml aliquots were removed from each batch. Each set received 4 μCi/ml of either [^{3}H]-leucine, [^{3}H]-mannose, or [^{3}H]-glucosamine HCl. The remaining 20 ml of cells for each control or TM concentration were given equivalent amounts of ethanol:water, 9:1 (4 μl/ml). All cells were incubated for 6 hr

more at 37°C and the 20 ml lots of cells were used for cell counts and ^{125}I-insulin binding assays. In the 5 ml-aliquots, ^{3}H-precursor incorporation into protein was determined essentially according to Housley et al(19): cells were centrifuged (10 min X 800g), washed 3 times with phosphate buffered saline, and dissolved by heating (100°C) in 1 ml of 1% sodium dodecyl sulfate, 25 mM EDTA for 10-25 min. Aliquots (200 µl) were removed and stored at -70°C for protein determinations by a modified Lowry method (20). The remaining 800 µl were incubated at 4°C for 1 hr with 800 µl of 20% TCA. Precipitate in each sample was collected by centrifugation and washed sequentially with 5 ml each of methanol, methanol:ether (1:1) and ether. The pellets were air dried and dissolved by heating (45°C) in 100 µl water and 500 µl Lumasolve. Lipoluma (5 ml) was added and radioactivity determined in a liquid scintillation counter.

RESULTS:

Effect of 65 hr growth in TM on ^{125}I-insulin binding to IM-9 lymphocytes: IM-9 cells at stationary growth were diluted 1:2.5 with medium containing 0-2 µg/ml TM and grown at 37°C for 65 hr. ^{125}I-insulin binding per cell decreased at all insulin concentrations in a dose-dependent fashion when compared to controls (Fig. 1A), with increasing TM concentration during growth. The data were normalized to the concentration of cells in the control assay (the only significant decrease in total cell number was 20-30% at 2 µg/ml TM). Scatchard plots (17, 21) (Fig. 1B), which were all curvilinear and essentially parallel, showed that the decrease was in the receptor binding capacity R_0 (horizontal intercept) rather than in affinity. The competition curves of Fig. 1A also suggested no major affinity changes since 50% inhibition of binding was obtained with similar concentrations of unlabeled insulin in all sets. The dose-response relationship (inset of Fig. 1B) approached a limiting level at 2 µg/ml TM. A 30% binding decrease was observed at the lowest TM concentration and a maximum loss of 85% was seen at the highest (Table 1, (A)).

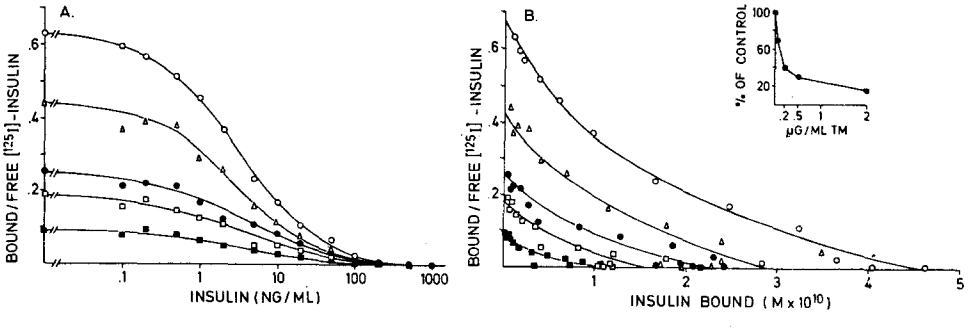

Fig.1. Competitive binding of ^{125}I-insulin to cells treated with TM for 65 hr. Control (○); TM: 0.05 µg/ml (△), 0.2 µg/ml (●), 0.5 µg/ml (□), 2 µg/ml (■). (A) Competition curves; (B) Scatchard plots; inset: dose-response curve for tracer binding (no competing insulin) for each set as % of control.

Table 1. Effects of TM on IM-9 lymphocytes – Insulin and GH Binding

TM µg/ml	Growth in TM 65 h (A) [125I]-INSULIN B/F*	(A) %LOSS	Growth 24 h, TM for 24 h (B) [125I]-INSULIN B/F*	(B) %LOSS	Growth 24 h, TM for 48 h (C) [125I]-INSULIN B/F*	(C) %LOSS	(D) [125I]-GH B/F**	(D) %LOSS
0	.224	0	.097	0	.160	0	.091	0
.05	.156	30.4	.069	28.9	.105	34.4	.065	28.6
.2	.090	59.8	.054	44.3	.061	61.9	.050	45.1
.5	.068	69.6	.042	56.7	.034	78.8	.038	58.2
2.0	.033	85.3	.024	75.2	.026	83.8	.036	60.4

*expressed per 10^6 cells ** expressed per 10^7 cells

Effects of 24 hr of TM treatment of rapidly growing lymphocytes on ^{125}I-insulin binding and leucine and sugar incorporation into protein: When human cultured lymphocytes at saturation are diluted sufficiently with fresh medium a lag phase is normally seen before the onset of logarithmic growth (22). We therefore diluted IM-9 lymphocytes 1:2.5 and incubated them at 37°C for 24 hr so that the cells were rapidly growing. We then added 0-2 µg/ml TM and incubated the cells for a further 24 hr at 37°C. No effect on cell number was seen at any TM concentration. ^{125}I-insulin binding decreased in a dose-dependent manner (Table 1, (B)) similar to that observed in cells treated with TM for 65 hr from the onset of dilution (Table 1, (A)), both approaching a limiting level at 2 µg/ml. However, the loss was smaller than in the 65 hr experiment at all TM concentrations except 0.05 µg/ml, where it was the same.

Protein synthesis was assessed by the incorporation of [^{3}H]-leucine during the last 6 hr of TM treatment into the cellular acid-insoluble fraction. It was slightly higher than in control cells for all concentrations of TM (Table 2). The greatest increase was 17% at 0.5 µg/ml TM. Glycoprotein synthesis was measured during the same 6 hr period by the incorporation of [^{3}H]-mannose and [^{3}H]-glucosamine into acid-insoluble material. Incorporation of both sugars decreased with increasing TM concentration down to 28% and 32% of controls at 2 µg/ml TM for [^{3}H]-mannose and [^{3}H]-glucosamine, respectively (Table 2). A similar dose-response was found for ^{125}I-insulin binding (Table 1, (B), expressed as % loss), but the lowest concentration of TM inhibited sugar incorporation much less than it decreased insulin binding. This may indicate a high sensitivity or susceptibility of the insulin receptor to inhibited glycosylation or an underestimation of the inhibition itself.

Table 2. Effects of TM on Radioactive Precursor Incorporation into 10% TCA Precipitable Material

TM	[^{3}H]– LEUCINE		[^{3}H]– MANNOSE		[^{3}H]– GLUCOSAMINE	
μg /ml	CPM*	%CTRL.	CPM*	%CTRL.	CPM*	%CTRL.
0	7.89	100	1.2	100	7.66	100
.05	8.69	110.1	1.17	97.5	7.60	99.2
.2	8.66	109.8	0.92	76.5	5.23	68.3
.5	9.22	116.9	0.50	42.0	4.64	60.6
2.0	8.15	103.3	0.34	28.4	2.48	32.4

* X10 $^{-5}$ / mg protein

Effect of 48 hr of TM treatment of rapidly growing lymphocytes on ^{125}I-insulin and ^{125}I-GH binding: IM-9 cells were diluted and preincubated in the absence of TM at 37°C for 24 hr as in the preceding section. TM (0-2 μg/ml) was then added and to insure more complete turnover of pre-existing receptors, incubation was continued at 37°C for another 48 hr. Separate ^{125}I-insulin and ^{125}I-human GH assays were conducted (Fig. 2A and Table 1, (C)&(D)). The loss in ^{125}I-insulin binding at each TM concentration was greater than in cells similarly treated for only 24 hr (Table 1, (B)). ^{125}I-GH binding also decreased and, like with insulin binding, approached a minimum value (Fig. 2A). The dose-dependencies were identical for both hormones (Fig. 2B), yet while the loss of insulin binding approached 84%, that of GH binding reached only 60%.

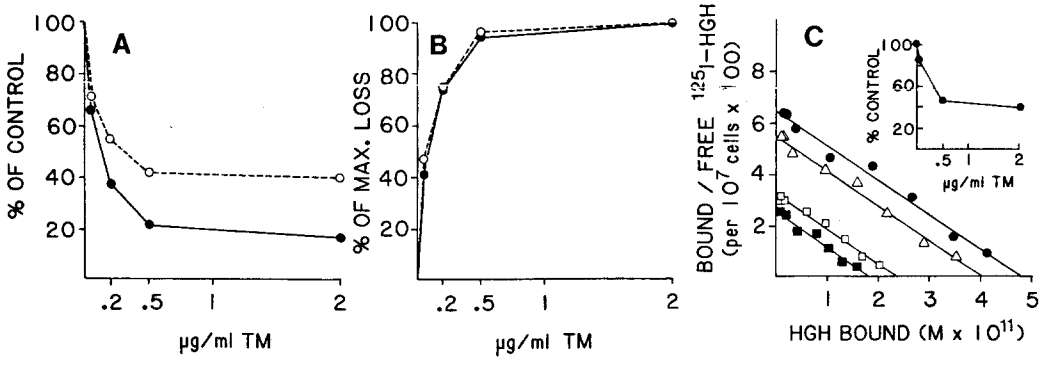

Fig. 2. ^{125}I-insulin and ^{125}I-GH binding to rapidly growing cells treated with TM for 48 hr. ^{125}I-insulin binding (●); ^{125}I-GH binding (○). (A) dose-response curve, bound/free tracer per 10^6 cells as % of control; (B) dose-response for each hormone's binding expressed as % of maximal binding loss; (C) Scatchard plots for ^{125}I-GH binding, in a separate but similar experiment, to cells treated for 48 hr with TM: Control (●), 0.05 μg/ml (△), 0.5 μg/ml (□) and 2 μg/ml (■). The inset shows the dose-response for the effect, as in 1B.

Scatchard plots of GH binding (Fig. 2C) showed a pure decrease in binding capacity with increasing TM concentration.

DISCUSSION: We have shown a dose- and time-dependent decrease in specific insulin binding in IM-9 lymphocytes treated with TM, due to a decreased number of functional receptors with no detectable change in affinity. The GH binding capacity also decreased with a TM dose-dependency identical to that for insulin in the same cells. Neither binding decreased to zero; each approached a limiting value (increasing TM from 0.5 μg to 2 μg/ml had little additional effect after 48 hr or 65 hr treatment). The conditions used with rapidly growing cells produced no appreciable effect on cell growth, a slight elevation in leucine incorporation into protein and a dose-dependent but incomplete decrease in sugar incorporation into protein.

One interpretation of our data is that without N-glycosylation, the insulin receptor is non-functional--either unable to bind insulin or unable to enter the plasma membrane. The residual binding we detected, even after exposure of the cells to high concentrations of TM for long periods of time, would then represent residual N-glycosylation that somehow was still occurring--ie., low levels of normal receptor. This could be supported by the failure of both insulin binding and sugar incorporation to reach zero. However, this interpretation may not be correct. Indeed, N-glycosylation may well have been inhibited more than indicated since mannose and glucosamine can partially be metabolized to forms incorporated into other than N-linked carbohydrate groups (e.g. O-linked glycosyl groups) (23-27). This may explain the residual sugar incorporation found, masking a complete inhibition of N-glycosylation. In support of this, the dose-response curves for insulin and GH binding suggest that essentially maximal effects have been obtained, implying that N-glycosylation inhibition was complete. This, and the difference in the decreases in the binding of each hormone, suggest another interpretation, especially since the levels observed cannot be accounted for solely on the basis of the receptor halflives (6.7 hr for the insulin receptor (28) and 10-12 hr for the GH receptor (29)), even if one allows for a 10 hr lag for the effect (5).

If the persistent levels of hormone binding were not due to residual normal receptors, it may be that non-N-glycosylated insulin and GH receptors bind hormone normally, but have new and differing steady-state levels in the membrane. This may result from a decreased rate of insertion into the membrane, and/or a quicker turnover before or after such insertion.

The elevated leucine incorporation may represent a fraction of protein that accumulated because of slower secretion. Continued but often impaired protein

secretion or membrane insertion after altered glycosylation has been well documented (19,30-33). In addition, an increase in protein degradation may also slightly stimulate protein synthesis. Such increased degradation after inhibition of N-glycosylation (34) was reported for the acetylcholine receptor (35).

Another although less likely explanation of our data is that both hormone receptors have a particular fraction always present which is not affected by N-glycosylation, with binding characteristics identical to a larger fraction which is affected, existing in different proportions in the two receptor populations. It does not appear that TM affected an "affinity regulator" since we detected no appreciable affinity changes in the decreased functional number of either GH or insulin receptors. However, we may not have altered directly the hormone receptors themselves but some other component vital to their expression. Also, our studies do not examine the possible involvement of O-linked saccharides. Further experiments, including the detection of these receptors by means other than hormone binding, will hopefully extend our understanding of the role of covalent carbohydrates in their function and metabolism.

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