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Effect of tunicamycin on hexose transport in mouse embryo fibroblast Swiss 3T3 cells

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The role of glycosylation of the carrier in the transporting activity was investigated in Swiss 3T3 cells. Inhibition of protein glycosylation by tunicamycin resulted in the decrease of hexose uptake in a dose- and time-dependent manner without a cytotoxic effect. From kinetic analysis, a decrease in the number or availability of hexose carriers in the plasma membrane was suggested. This was in good correlation with the decrease in the amount of photoaffinity cytochalasin B binding in the plasma membrane by the treatment with tunicamycin. The rate of phorbol 12,13-dibutyrate-induced translocation of the hexose carrier from microsomal to plasma membrane was reduced in tunicamycin-treated cells, which may be correlated with the decrease in the number of the completely glycosylated carrier translocatable from the microsomal membrane. In both tunicamycin-treated and untreated cells, the stimulation of hexose transport by phorbol 12,13-dibutyrate was abolished by the removal of phorbol 12,13-dibutyrate, and upon its readdition the stimulation recovered to the same degree as before the removal. Thus, the recycling of the functionally mature hexose carrier appeared not to be affected by the treatment with tunicamycin. These results suggested that complete glycosylation of the carrier may be necessary for the translocation of the carrier from microsomal to plasma membrane to accomplish its function on the cell surface.

Introduction

The oligosaccharide moiety of glycoproteins is important in maintaining protein conformation and solubility [1,2], proteolytic processing and stabilization of the polypeptide against uncontrolled proteolysis [3,4], mediation of biological activity [5–7], intracellular sorting and externalization of glycoproteins [3,8,9], and embryonic development and differentiation [1,3].

The hexose transport across the plasma membrane is thought to be mediated via glycoprotein [10], and experiments with tunicamycin have shown that the oligosaccharide moieties of glycoprotein are suggested to play important roles in the expression of its function, insertion into plasma membrane and degradation process [11,12]. Oligosaccharide moiety is said to be required for the transporting activity of the hexose carrier in chick embryo fibroblast [13] and glycosylation of the carrier may also be involved in the control of hexose carrier. Moreover, many receptor proteins of the plasma membrane, including transport proteins are believed to be recycled in the cells [14–16].

In this study, we further investigated the role of glycosylation of hexose carrier in its activity, distribution and recycling in the cell using tunicamycin which inhibits the synthesis of *N*-acetylglucosaminylpyrophosphoryl polyisoprenol [17–19].

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Chemicals. 3-*O*-[^{3}H]Methylglucose (5 Ci/mmol), L-[^{3}H]glucose (10.7 Ci/mmol), [^{3}H]cytochalasin B (14.9 Ci/mmol) and [^{3}H]AMP (50 Ci/mmol) were purchased from New England Nuclear. Phorbol 12,13-dibutyrate was obtained from P-L Biochemicals. Cytochalasins B and E were obtained from Nakarai Chemicals. All other chemicals were obtained from commercial sources in either reagent grade or highest purity available.

Cell culture. Swiss 3T3 cells [20] wre prepared by plating $1 \cdot 10^5$ cells/ml in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum. Cells were grown in plastic tissue plates (35 mm in diameter) in a humidiated CO_2 incubator at 37°C. After 3 days, cultures were used for experiments.

Measurement of hexose uptake. Specific 3-*O*-methylglucose uptake was assayed as described previously [21]. Cells were incubated for a designated period of time, and rinsed twice with 2 ml of phosphate-buffered saline. The uptake was initiated by addition of 1 ml phosphate-buffered saline containing 3-*O*-[^{3}H]-methylglucose (4 μM, 5 μCi/ml) at 20°C. After a designated period of time, hexose uptake was stopped by washing the plates three times with 2 ml ice-cold phosphate-buffered saline. Carrier-mediated uptake was calculated after correction of nonspecific uptake determined with L-[^{3}H]glucose.

Preparation of plasma and microsomal membranes. Plasma and microsomal membranes were prepared from incubated cells by differential centrifugation methods described previously [22]. In brief, cells were disrupted by hypotonic cold shock and then homogenized. Each homogenate was centrifuged at $18000 \times g$ for 20 min. The supernatant was aspirated and served for preparation of microsomal membrane. The pellet was washed once and resuspended for preparation of plasma membrane in 10 mM Hepes, 1 mM EDTA and 255 mM sucrose. The plasma membrane was obtained by centrifugation at $23000 \times g$ for 60 min on a discontinuous 1.12 M sucrose gradient. The plasma membrane was washed twice and resuspended to final concentration of 3–5 mg of protein/ml. The microsomal membrane was obtained from the initial supernatant by centrifugation at $180000 \times g$ for 80 min. The pellet was washed once and resuspended to a final concentration 3–5 mg protein/ml. NADH-cytochrome *c* reductase and 5′-nucleotidase activities were measured by the methods of Dallner et al. [23] and Avruch and Wallach [24], respectively. Protein concentration was determined by the method of Markwell et al. [25].

Photoaffinity cytochalasin B binding assay. The membrane protein suspension (1 mg) was mixed with 2 μM cytochalasin E containing 200 mM D-glucose or sorbitol. [^{3}H]Cytochalasin B was used at final concentration of 200 nM. The sample was placed on ice in the dark for 30 min and then irradiated with 300 W mercury lamp at a distance of 20 cm at 0°C for 10 min. The samples were centrifuged and the pellets were suspended in distilled water. Specific cytochalasin B binding activity was adjusted to those which would have been observed had the membrane fraction been free of cross-contamination. Adjustments were based on the enzyme marker specific activity and protein recovery.

Results

Exposure of Swiss 3T3 cells in the stationary phase to tunicamycin induced a decrease of 3-*O*-methylglucose uptake (Table I). The initial rates of uptake were decreased to 50–60% of the control value after 15 h of treatment with 0.05 μg/ml of

TABLE I

EFFECT OF TUNICAMYCIN ON 3-*O*-METHYLGLUCOSE UPTAKE

Confluent cultures of Swiss 3T3 cells were pre-exposed to tunicamycin (0.05 μg/ml) for 15 h at 37°C. Then, the cultures were used for uptake assay for up to 2 min. The linearity of the uptake was maintained for at least 2 min. The number of viable cells were determined by Trypan blue exclusion. The data are expressed as the mean ± S.E. ($n = 3$).

	Condition	
	− tunicamycin	+ tunicamycin
3-*O*-Methylglucose uptake (pmol/mg protein per min)	2.15 ± 0.48	1.04 ± 0.23
Viable cells (10^6 cells/dish)	1.14 ± 0.08	1.10 ± 0.17

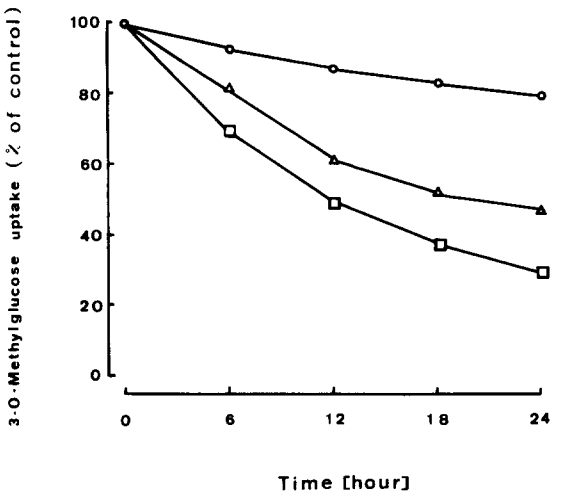

Fig. 1. Effect of time for pretreatment and dose of tunicamycin on hexose uptake. Confluent cultures were incubated in the absence or presence of 0.01 μg/ml (○), 0.05 μg/ml (△) or 0.2 μg/ml (□) of tunicamycin for the indicated time and then 3-*O*-methylglucose uptake was assayed for up to 2 min. The linearity of the uptake was maintained for at least 2 min. Data are expressed as the mean of triplicate experiments.

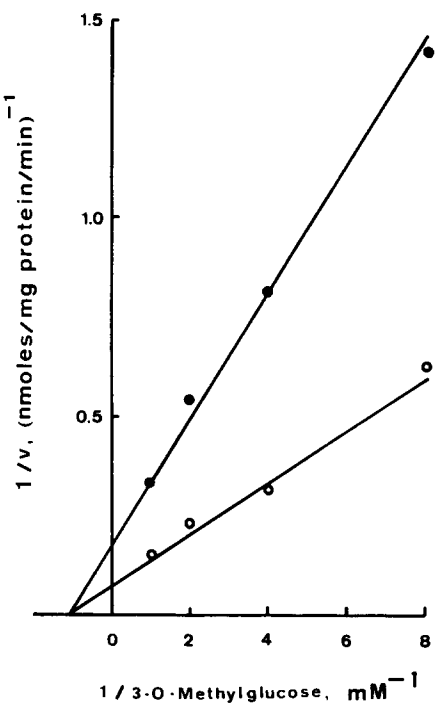

Fig. 2. Effect of tunicamycin on the kinetics of 3-*O*-methylglucose transport. A Lineweaver-Burk plot of the initial rates of 3-*O*-methylglucose uptake was employed. Confluent cells were incubated in the presence (●) or absence (○) of tunicamycin (0.05 μg/ml) for 15 h. 3-*O*-Methylglucose uptake for 30 s at concentrations between 0.125 and 1 mM was determined. The linearity of the uptake was maintained for at least 30 s at a higher concentration of 3-*O*-methylglucose (1 mM). Data are expressed as the mean of triplicate experiments.

tunicamycin. The number of viable cells were hardly affected by the treatment with tunicamycin for 15 h (Table I), and the decrease of L-leucine incorporation into acid-insoluble material was only 10% (data not shown).

When 3T3 cells were pretreated with tunicamycin of three different doses for various durations, the hexose uptake was decreased linearly with time and dose (Fig. 1). Addition of tunicamycin to the assay mixture for measurement of hexose transport activity without pretreatment of cells with tunicamycin did not affect the hexose uptake (data not shown).

Fig. 2 shows the kinetic analysis of hexose transport in tunicamycin-treated cells. The V_{max} value for hexose uptake by tunicamycin-treated cells was significantly lower than that by the control cells (11.5 ± 2.5 vs. 24.5 ± 3.6 pmol/mg protein per min; $n = 3$, $P < 0.01$), whereas the K_m value was scarcely affected (1.10 ± 0.23 vs. 1.28 ± 0.19 mM).

To estimate the alteration of hexose carrier distribution, D-glucose-inhibitable cytochalasin B-binding was assayed in plasma and microsomal membranes. The amount of cytochalasin B binding to the plasma membrane was decreased by treatment with tunicamycin to about half of that of the control, whereas that of the microsomal membrane was not significantly decreased (Table II).

The effect of tunicamycin on the translocation of the hexose transport system was further investigated using phorbol 12,13-dibutyrate. Phorbol 12,13-dibutyrate has been proved to induce the translocation of the hexose carrier into the plasma membrane from its cellular pool, resulting in the increase of hexose transport activity [22]. As shown in Table II, the rate of phorbol 12,13-dibutyrate-induced translocation of the cytochalasin B-bound hexose carrier was decreased in tunicamycin-treated cells, although the amount of cytochalasin B binding in tunicamycin-treated cells in the ab-

TABLE II

EFFECT OF TUNICAMYCIN ON D-GLUCOSE-INHIBITABLE CYTOCHALASIN B BINDING IN PLASMA AND MICROSOMAL MEMBRANES

Confluent cultures were incubated in the presence or absence of tunicamycin (0.05 μg/ml) for 15 h at 37°C. Then, phorbol 12,13-dibutyrate (100 nM) was added and incubation continued for 3 h. Plasma and microsomal membranes were prepared as described in Materials and Methods. The data (pmol/mg protein) are expressed as the mean ± S.E. ($n = 3$).

Phorbol 12,13-dibutyrate	Specific cytochalasin B binding			
	Plasma membrane		Microsomal membrane	
	− tunicamycin	+ tunicamycin	− tunicamycin	+ tunicamycin
−	9.08 ± 1.79	4.61 ± 0.67	24.5 ± 4.03	22.1 ± 4.21
+	18.02 ± 3.03	8.23 ± 1.26	12.1 ± 2.96	19.1 ± 3.80

sence of phorbol 12,13-dibutyrate was not so different from that in control cells.

Table III shows the effect of tunicamycin on the recycling of the hexose carrier using phorbol 12,13-dibutyrate by washing and readdition. Hexose uptake was stimulated 4-fold by phorbol 12,13-dibutyrate treatment in control cells, whereas in tunicamycin-treated cells, it was hardly increased. This is well correlated with the results shown in Table II. By removal of phorbol 12,13-dibutyrate, the increased hexose uptake induced by phorbol 12,13-dibutyrate was reduced to the basal level, and then the hexose uptake was stimulated by readdition of phorbol 12,13-dibutyrate to the same degree as before its removal.

Discussion

It has been reported that hexose carriers are glycoproteins [10,13] of which the oligosaccharide moieties have important roles in their biological functions [13]. The present study shows that inhibition of protein glycosylation by tunicamycin results in the decrease of hexose uptake in Swiss 3T3 cells. Since tunicamycin inhibits the synthesis of *N*-acetylglucosaminylpyrophosphoryl polyisoprenol, which is required for the synthesis of the core sequence of N-glycosically linked oligosaccharides, it caused the synthesis of glycoprotein deficient in asparagine-linked oligosaccharides [17–19]. From the results of kinetic analysis of the

TABLE III

EFFECT OF TUNICAMYCIN ON HEXOSE CARRIER RECYCLING

Confluent cells were incubated in the presence or absence of tunicamycin (0.05 μg/ml) for 15 h at 37°C. And then, cells were incubated in Dulbecco's modified Eagle medium containing cycloheximide (0.5 μg/ml) and 100 nM phorbol 12,13-dibutyrate. 3 h later, cells were washed and reincubated in Dulbecco's modified Eagle medium containing cycloheximide (0.5 μg/ml). After 3 h, 100 nM phorbol 12,13-dibutyrate was readded and incubation continued for 3 h. Each culture was used for 3-*O*-methylglucose uptake assay for up to 2 min. The linearity of the uptake was maintained for at least 2 min. Data (pmol/mg protein per min) are expressed as the mean ± S.E. ($n = 3$).

	Hexose uptake			
	After first stimulation by phorbol 12,13-dibutyrate		After second stimulation by phorbol 12,13-dibutyrate	
	− tunicamycin	+ tunicamycin	− tunicamycin	+ tunicamycin
Untreated cells	2.15 ± 0.13	1.04 ± 0.07	1.96 ± 0.09	0.79 ± 0.06
Phorbol 12,13-dibutyrate-treated cells	8.01 ± 1.08	3.21 ± 0.24	7.68 ± 1.25	3.10 ± 0.20

hexose uptake and photoaffinity cytochalasin B binding, tunicamycin was strongly suggested to cause insufficient glycosylation of the hexose carrier in 3T3 cells resulting in the decrease of functional carrier molecules in the plasma membrane. It is also conceivable that the effect of tunicamycin on hexose uptake is a secondary consequence of growth inhibition accompanying the decrease of cellular glucose metabolism. However, this possibility seems unlikely, since cellular protein synthesis and the number of viable cells were not affected by tunicamycin treatment. There exists an internal pool of the hexose carrier [15,16] and the carrier recycles in the cell [22,26,27]. The rate of phorbol 12,13-dibutyrate-induced translocation of hexose carrier from microsomal to plasma membrane was reduced in tunicamycin-treated cells. These results suggest that the oligosaccharide moiety of the hexose carrier may play an important regulatory role in the distribution of the hexose carrier in the plasma membrane and microsomal membrane and its recycling in Swiss 3T3 cells.

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