Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 275-281

THYROID HORMONE ACTION ON GLUCOSE TRANSPORTER ACTIVITY IN ASTROCYTES

Lois M. Roeder, Irene B. Hopkins, Jeffrey R. Kaiser, Lea Hanukoglu and J. Tyson Tildon

Department of Pediatrics, University of Maryland
School of Medicine, Baltimore, Maryland

Received August 8, 1988

In astrocytes from rat brain cultured in thyroid hormone-deficient media cytochalasin B-binding was decreased 80%; addition of L-T3 increased binding to 75% of control levels. Saponin-treatment of controls increased accessibility of binding sites to 60% above untreated cells. Saponin also increased binding in deficient cells; however, the level was less than in treated controls, suggesting L-T3 deficiency decreases total glucose transporters. Addition of L-T3 appeared to convert most (90%) of the binding sites from unavailable to accessible status. Changes in binding to plasma membranes in response to L-T3 level were similar to those in intact cells. No binding to Golgi was detectable, thus no evidence for translocation of carriers was obtained. L-T3 may activate the glucose transporter by increasing its accessibility in brain cells.

Data from our laboratory showed that primary cultures of rat brain astrocytes maintained in the absence of 3, 5, 3'-triiodo-thyronine (L-T3) exhibited low rates of uptake of 2-deoxyglucose (1). The addition of L-T3 to the deficient cells quickly (in minutes) restored uptake rates to those observed in control cells. The rapidity of the effect indicated that synthesis of new carrier protein would not account for this action of L-T3. Rather, the mechanism may involve translocation of existing glucose transporters to more available sites as proposed for the effect of insulin on adipocytes (2-4) and diaphragm (5,6) and for L-T3 action on thymocytes (7) and cardiac cells (8). Alternatively, L-T3 may activate glucose tranporters residing in the brain cells plasma membrane. To further investigate the mechanism of L-T3 action in brain cells, cytochalasin B binding was used to detect glucose transporters in control, L-T3 deficient, and L-T3 replenished rat brain astrocytes. Since saponin has been shown to increase permeability of plasma membranes (9) and has been used effectively to distinguish plasma membrane-associated from intracellularly-located Ca^{++}-pump mechanisms (10).

0006-291X/88 $1.50

we employed this agent to aid in delineating the mechanisms of L-T3 action. In addition to intact cells, plasma and Golgi membranes isolated from the astrocytes were also used for these binding studies.

MATERIALS AND METHODS

Cell Cultures and Treatments

Pregnant rats of the Sprague-Dawley strain were obtained from Zivic Miller Laboratories (Allison Park, PA, U.S.A.). They were maintained in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care in strict accordance with USDA regulations and NIH guidelines.

Brains were removed from 1 to 2 day-old pups and astrocytes prepared as previously described (1). The cells were plated in 35 mm dished (9 dished per brain) and incubated at 37°C in an atmosphere of 95% air-5% CO_2, with 90-95% humidity. The culture medium was replaced after 3 days; after an additional 3 days, medium containing 10% fetal calf serum was used and replaced twice weekly thereafter. Thyroid hormone-deficient cells were prepared by changing the medium to one containing thyroid-hormone-free serum prepared by an anion-exchange method (11). Some of the cells grown in L-T3-deficient serum were incubated with 50 uM L-T3 for 10 minutes either prior to cytochalasin B binding measurement or before isolation of the membrane fractions.

Plasma membranes were isolated from these cells as follows: plated cells were washed 3 times with phosphate-buffered saline (PBS) and then TES buffer (10 mM Tris-HCl, 5 mM EDTA, 0.32 M sucrose, pH 7.4) containing 24 m units of Aprotinin per ml was added (1.0 ml per 35 mm dish). Cells were harvested by scraping the dish with a rubber policeman and homogenized for 2 minutes using a motor-driven teflon pestle in an ice-jacketed tube. Nuclei and cell debris were removed by centrifugation at 250 x g for 5 minutes. Plasma membranes were obtained from the resulting supernatant by centrifugation at 13,000 x g for 10 minutes. Golgi membranes were pelleted from the post 13,000 x g supernatant by spinning at 100,000 x g for 30 minutes. The membranes were gently resuspended in 0.32 M sucrose and aliquots of each used to determine cytochalasin B-binding. In some experiments, the plated cells were permeabilized by incubation in the presence of saponin (0.0025%) for 10 minutes at room temperature prior to assay.

Measurements

Glucose binding sites were detected in suspensions of the membrane preparations and in plated cells using the cytochalasin B binding method (2,12). Siliconized glassware and pipet tips were used throughout the procedure. Cytochalasin B (approximately 10^{-4} M was dissolved in 100% ethanol and the molarity determined by absorbance at 210 nm. Various dilutions of this stock solution were mixed with ^3H -cytochalasin B, evaporated to dryness under N_2 and diluted in the appropriate volume of H_2O. Each of these solutions was used to prepare 2 reaction mixtures, one with and one without D-glucose. All other components were prepared in TES buffer (10 mM Tris HCl, 5 mM EDTA and 0.32 M sucrose). The final mixtures contained ^3H -cytochalasin B (concentrations ranged from 1.67 x 10^{-5} mM to 8 x 10^{-4} mM), 2.1 uM cytochalasin E and, either 500 mM D-glucose or TES buffer. All samples were pre-incubated with 2.1 uM cytochalasin E for 10 minutes at 0° to suppress cytochalasin B binding to non-D-glucose inhibitable sites (2).

For plated cells, 1 ml of each reaction mixture (containing the same final concentrations of components as for membrane assays) was added to triplicate samples (35 mm dishes) which were then incubated for 10 minutes at 0°C. The reaction mixture was then removed by suction, and the cells were washed 3 times with PBS. After drying the cells were dissolved in 1.5 ml of 0.1 M NaOH. An aliquot (0.5 ml) was transferred to a scintillation vial, and 1 drop of glacial acetic acid plus 4.5 ml of fluor was added. The radioactivity was determined in a liquid scintillation spectrometer.

When binding was measured using isolated membrane preparations, 500 ul of each suspension was pre-incubated with 40 ul cytochalasin E (final concentration = 2.1 uM) for 10 minutes at 0°C. Two aliquots (250 ul each) were mixed with 200 ul of either D-glucose or TES buffer and then distributed into 4 cellulose propionate tubes (100 ul per tube). The various concentrations of ^{3}H -cytochalasin B (40 ul each) were added and the samples incubated for 30 minutes at 0°C. Samples were centrifuged in a 42.2 Ti rotor in a Beckman model G; plasma membrane samples were spun at 20,000 rpm for 30 minutes and Golgi membrane samples at 42,000 rpm for 2 hours. Two aliquots (50 ul each) of each supernatant were transferred to scintillation vials for determination of free ^{3}H -cytochalasin B. To each aliquot was added 110 ul of a 1:5:5 mixture of 7% acetic acid, H_2O and 0.2 M NaOH plus 50 ul of glacial acetic acid and 4.5 ml of fluor. The remainder of the supernatant was carefully removed by suctioning and discarded. The pellet was dissolved in 50 ul of 0.2 M NaOH with vortexing and incubation in a shaking water bath for 1 hour at 55°C. The entire tube was transferred, with inversion, to a scintillation vial, 60 ul of a 5:1 mixture of H_2O and 7% acetic acid plus 50 ul of glacial acetic acid and 4.5 ml fluor were added. Radioactivity was determined in a liquid scintillation spectrometer.

Protein contents were determined by the method of either Lowry et al. (13) or Smith et al. (14).

RESULTS

Fig. 1 is a typical curve for D-glucose inhibitable, non-cytochalasin E, cytochalasin B-binding with increasing concentrations of the ^{3}H-labelled ligand in rat brain astrocytes. Transformation of the data to a Scatchard plot (INSET) yielded a linear

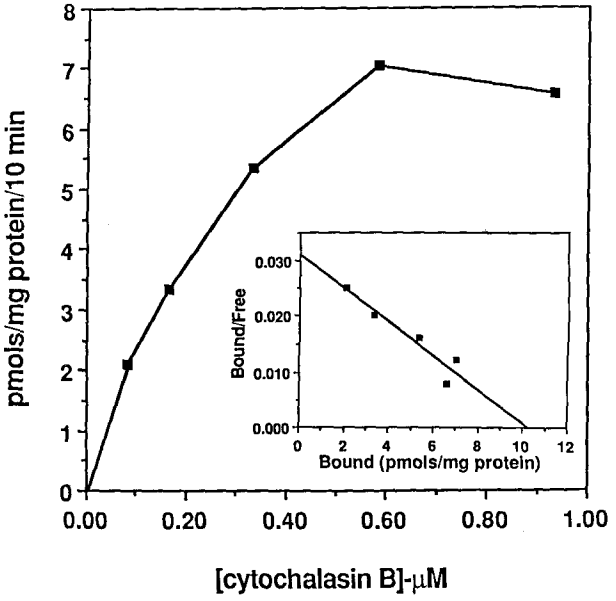

Fig. 1 Cytochalasin B-binding in primary cultures of rat brain astrocytes. Cells plated on 35 mm dishes were pre-incubated with 2.1 uM cytochalasin E for 10 min at 0°C and then incubated in various concentrations of ^{3}H -cytochalasin B (Specific activity approximately 900 DPM per pmol) for 10 min at 0°C. Radioactivity and protein content were determined in separate aliquots of cells from each dish after dissolving in 0.1 M NaOH. Inset shows transformation of the data to a Scatchard plot.

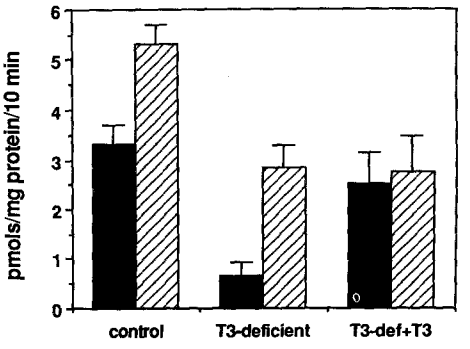

Fig. 2 Effect of L-T3 deficiency and replenishment on cytochalasin B-binding in intact (■) and saponin-treated (▨) rat brain astrocytes. Cells were plated and maintained in medium with complete serum for 7 days. L-T3 deficient cells were prepared by changing the serum in the medium to thyroid hormone-free serum for 6 days. L-T3 (50 uM) was added to the medium of half of the deficient cells for 10 minutes prior to exposure to saponin (0.0025% for 10 min at room temperature). Subsequent determination of cytochalasin B-binding was carried out by incubation in cytochalasin E and ^{3}H -cytochalasin B (0.167 uM) as described in Fig. 1. Vertical bars represent SEM; N = 3 per group.

relationship (r = 0.95) with values for kinetic parameters estimated to be 300 pM for Kd and 9.8 pmol/mg protein for Bmax.

The cytochalasin B-binding assay was carried out in plated control, L-T3 deficient and L-T3 replenished cells using a single concentration of the ligand (0.167 uM); other concentrations of the ligand yielded essentially the same pattern of results . Data were obtained on both untreated and saponin-treated cells. The results are summarized in Fig. 2. In control cultures, exposure to saponin was accompanied by an increase in cytochalasin B-binding of about 60%, suggesting that a large portion of the glucose-binding sites were made more accessible by this treatment. In L-T3-deficient cells, there was a loss (80%) of binding. Saponin treatment increased accessibility of glucose binding sites in L-T3 deficient cells (more than 3-fold). However, the level was less than in saponin-treated controls, suggesting a decrease in total numbers of transporters due to L-T3 deficiency. In the third group of experiments, when L-T3 was added to the cells for 10 minutes prior to the assay, there was an increase in binding and the level achieved was essentially the same after saponin treatment.

In order to determine whether glucose binding sites were associated with Golgi membranes and whether the absence of thyroid hormone in the culture media affected their distribution between plasma and Golgi membranes, cytochalasin-B binding was determined in membranes prepared from control, L-T3 deficient and L-T3 replenished

TABLE 1
Cytochalasin B-Binding to Plasma Membranes and Golgi Preparations From Rat Brain Astrocytes

Group	N	Plasma Membrane	Golgi
Control	4	2.78 ± 1.10	0
T_3-Deficient	3	0.07 ± 0.07	0
T_3-Deficient + T_3	4	2.10 ± 1.34	0

Cells were prepared as described in the Methods section and for Fig. 2. Cells were harvested and homogenized; the plasma and Golgi membranes were obtained as the 13,000 x g and 100,000 x g pellets from the post 250 x g supernatant and used in a cytochalasin B-binding assay as described under Methods. Results are expressed as mean pmol/mg protein ± SEM.

cells. Table 1 shows that binding to the plasma membrane was markedly reduced in T-3 deficiency and was essentially restored when L-T3 was replaced. However, no net binding in the Golgi membrane preparations was detectable within the limits of this assay.

DISCUSSION

The data obtained using the cytochalasin B-binding assay in cultured rat astrocytes showed saturable binding of the ligand and yielded a single-slope plot when transformed for Scatchard analysis (Fig.1). The application of this technique to cultured cells should be useful for studies on the development and regulation of the glucose transport system in specific cell types that can be grown in vitro and exposed to various potential effectors.

Astrocytes cultured in L-T3 deficient media showed less binding of ^{3}H-cytochalasin B than control cells maintained in complete media. This agrees with the effect of L-T3 deficiency on the uptake of ^{3}H -2-deoxyglucose in these cells previously reported from our laboratory (1). Furthermore, the 10 minute exposure of the deficient cells to L-T3 increased binding to 75% control levels. The previous study (1) showed that the uptake of 2-deoxyglucose was equal to or greater than that of controls when the L-T3 deficient cells were replenished with L-T3 for only 60 seconds. Thus, cytochalasin B-binding was not as completely restored as uptake rates. Similarly, in fat cells of diabetic rats, the insulin-stimulated increase in uptake of 3-0 methylglucose exceeded the increase in number of cytochalasin B binding sites (15). These and other studies may indicate that there are 2 forms of the transporter, of which only 1 is translocated (15, 16). These

workers and others (17) have proposed that the stimulation of glucose transport by insulin in insulin-responsive tissues is a 2-step process involving both recruitment of inactive glucose transporters to the plasma membrane and activation of the carrier itself. Our data on L-T3 action in brain cells are consistent with activation of the glucose transporter by the hormone, and further suggest that translocation of carriers from a sub-cellular site such as Golgi is not involved in this process.

The results comparing the cytochalasin B-binding in intact and saponin-treated cells suggest that a portion of the glucose binding sites on the plasma membrane of astrocytes may be unavailable to, or in some way "protected" from, the substrate. When L-T3 was added to thyroid-homone-deficient cells, there was an increase (greater accessibility) of glucose binding sites. The difference in binding between intact and saponin-treated cells (an estimate of "protected" sites) indicated that L-T3 replenishment had made most of these sites accessible to cytochalasin B. The mechanism involved may be similar to that proposed by Karahasanoglu et al (18) for the increased availability of dopamine beta hydroxylase in the presence of acetylcholine in brain. These workers showed that treatment with a detergent increased the activity of the enzyme to the same level seen after addition of the neurotransmitter.

The initial steps in the interaction of L-T3 with these brain cells are unknown. The binding of L-T3 to its receptor on thymocyte plasma membranes seems to be functionally important to the action of the hormone in increasing 2-deoxyglucose uptake in these cells since mild trypsin-treatment which did not affect basal uptake rates, produced dose-dependent decreases in both L-T3 binding and L-T3 stimulated 2-deoxyglucose uptake (7). Thyroid hormones bind to plasma membranes of cells in several tissues (19), but at present, no data are available to indicate a similar receptor system for L-T3 on the plasma membrane of brain cells.

Information about the glucose transporter and its regulation has been facilitated by the determination of its sequence and structure and the model proposed for its orientation in the plasma membrane by Mueckler et al. (20). The high degree of homology for the glucose transporter protein between species and among tissues (21, 22) suggests that uniqueness of function is regulated by specific effectors that influence recruitment of the carrier molecules and/or their activation. These effectors may be

hormones or intermediates generated in the cascade of events that follow interaction of the hormone with its receptor. On the basis of data from the present study, we propose that, in brain tissue, L-T3 may signal cellular events that promote an increased accessibility of glucose binding sites, perhaps by an activation or shift in the orientation of the glucose transporter in the plasma membrane.

ACKNOWLEDGMENTS: This work was supported in part by NIH Grant HD 16596. We gratefully acknowledge Ms. Bernadette Pasko and Ms. Laura Nasrallah for preparing the typescript and Ms. Dena Yvers for suggestions on the cytochalasin B-binding assay, and we thank Dr. Stephen R. Max for his helpful comments.

REFERENCES

1. Roeder, L.M., Williams, I.B., and Tildon, J.T. (1985) J. Neurochem. 45, 1653-1657.
2. Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758-4762.
3. Suzuki, K. and Kono, T (1980) Proc. Nat. Acad. Sci. 77, 2542-2545.
4. Gorga, J.C. and Lienhard, G.E. (1984) Fed. Proc. 43, 2237-2241.
5. Wardzala, L.J. and Jeanrenaud, B. (1981) J. Biol. Chem. 256, 7090-7093.
6. Wang, C. (1985) Proc. Nat. Acad. Sci. 82, 3621-3625.
7. Segal, J. and Ingbar, S.H. (1986) Endocrinology 118, 1863-1868.
8. Gordon, A., Schwartz, H., and Gross, J. (1986) Endocrinology 118, 52-57.
9. Seeman, P. (1974) Fed. Proc. 33, 2116-2124.
10. Gill, D.L. and Chueh, S-H. (1985) J. Biol. Chem. 260, 9289-9297.
11. Samuels, H.H., Stanley, F., and Casanova, J. (1979) Endocrinology 105, 80-85.
12. Wardzala, L.J., Cushman, S.W. and Salans, L.B. (1978) J. Biol. Chem. 253, 8002-8005.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem.193, 265-275.
14. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mollia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Anal.Biochem. 150, 76-85.
15. Kahn, B.B. and Cushman, S.W. (1986). Ann. N.Y. Acad. Sci. 488, 356-369.
16. Horuk, E., Matthaei, S., Olefsky, J.M., Baly, D.L., Cushman, S.W. and Simpson, I.A. (1986) J. Biol. Chem. 261, 1823-1828.
17. Houslay, M.D. (1985) IN Molecular Mechanisms of Transmembrane Signalling (P. Cohen and M.D. Houslay, Eds.) p. 279-334. Elsevier, New York.
18. Karahasanoglu, A.M., Edwards, E.H., Tildon, J.T. and Ozand, P.T. (1976) J Neurosci. Res. 2:401-412.
19. Krenning, E.P. and Docter, R. (1986) In: Thyroid Hormone Metabolism (G. Henneman, Ed.) p. 107-131, Marcel Dekker, New York.
20. Mueckler, M., Caruso, C., Baldwin, S.A., Ponico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E., and Lodish, H.F. (1985) Science 229, 941-945.
21. Birnbaum, M.J., Haspel, H.C., and Rosen, O.M. (1986) Science 5784-5788.
22. Flier, J.S., Mueckler, M., McCall, A.L., and Lodish, H.F. (1987) J. Clin. Invest. 79, 657-661.