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A Study of the Regulation of the Glucose Transporter in the Plasma Membranes of Hepatoma Cells Induced by 3'-Me DAB

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5'-nucelotidase and glucose-6-phosphatase are liver plasma and microsomal membranes markers and their respective activities were determined. In the liver homogenate, the activities of 5'-nucleotidase were 0.58±0.08 and 0.29±0.07 μmols/mg protein/10min in the control and 3'-methyl-4-dimethyl aminoazobenzene (3'-Me DAB) groups respectively. The enzyme activities in the partially purified plasma membranes were 2.15±0.25 and 1.31±0.23 μ mols/mg protein/10min in the control and 3'-Me DAB groups respectively. The glucose-6-phosphatase activities in the homogenates of the control and 3'-Me DAB groups were 0.23±0.10, and 0.45±0.25 μmols/mg protein/10min, and in the microsomal fraction, 1.14±0.32, and 0.63±0.11 μmols/mg protein/ 10min, respectively. The concentrations of glucose carrier in the plasma membranes from the control and 3'-Me DAB group were 25, and 35 pmols/mg membrane protein, respectively, and the Ka values for cytochalsin B in each group were 5.20×10^9. and 5.14×10^9ml/mol, respectively. However, in the microsomal fraction, no significant differences of glucose carrier were found between the control and 3'-Me DAB groups from the DEAE Sephadex A-50 ion exchange chromatography, fractions I and II were obtained. Electrophoretic analysis of fraction I revealed a major protein band with a molecular weight of 45,000 and minor bands with MWs of 50,000, 55,000 and 15,000. Following AcA 34 gel filtration, a major protein band with a MW of 45,000 was obtained.

From these results, it can be concluded that the glucose carrier protein was increased on plasma membrane of hepatoma induced by 3'-Me DAB, and the carrier protein showed similar molecular weight to other glucose carrier found in the RBC, muscle cells and adipocyte.

Key Words: Glucose transport, 5'-nucleotidase, glucose-6-phosphatase, cytochalasin 8

One of the earlist events during carcinogenesis by a chemical carcinogen or an oncogenic virus is the stimulation of glucose transport (Warburg 1956: Lo *et al.* 1963), and this phenomenon may be related to changes in the glucose carrier (Weber 1973: Elbrink and Bihler 1975). Kinetic studies have shown that there is an increase in the Vmax with little change in the Km for the transport system, which indicates that the quantity of functional transporter in the plasma membrane increases during the carcinogenesis (Hatanaka 1974, Lee and Weinstein 1979). However, the data obtained from kinetic analysis alone can not satisfactorily distinguish between an increase in the rate at which the already functioning transport systems operate. It is known that there are two kinds of glucose carriers in animal cells, that is, insulin-dependent and -independant carriers (Wheeler and Hinkle 1985; Simpson and Cushman 1986). Those that respond to insulin are present on the plasma membranes of myocytes or adipocytes and those that do not respond to insulin are located on the plasma membranes of RBC, brain cells and hepatocytes. The glucose carriers on the plasma membranes of insulin responsive cells were translocated to microsomes in the insulin deficient state and then, insulin induced translocation of glucose carriers from the microsomes to the plasma membrane was accomplished. (Cushman and Wardzala 1980; Cushman *et al.* 1984). However, little is known about the relationship between insulin concentration and the translocation of glucose carriers in hepatocytes and RBCs. Especially, the mechanism of increased glucose transport in insulin-indepenent transformed cells is not known. Us-

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ing cytochalasin B, a competitive inhibitor of the glucose carrier, the quantitation of glucose carriers on fibroblasts transformed by the Rous sarcoma virus (Salter and Weber 1979) and on hepatocytes from fasting rats (Pessin *et al.* 1982) was performed. The present study was designed to investigate whether there is an increase in the quantity of glucose transporter or an increase in the rate of already functioning glucose transporter in the hepatoma cell membrane induced by 3'-Me DAB as compared with that of normal rat liver cell, and to purify the glucose carrier expressed on the hepatoma cells.

MATERIALS AND METHODS

Induction of Tumors

Liver tumors were induced in male Sprague Dawley rats weighing approximately 100g by the protocol shown in Table 1 using 3'-Me DAB (Eastman Kodak Co.). The rats were fed 0.06% 3'-Me DAB in their diet for 12 weeks followed by the control diet ad libitum for 4 weeks (Miller *et al.*1948). The rate of tumor production was greater than 99% at the end of diet scheme (Bebawi *et al.* 1970: Lim *et al.* 1982)

Table 1. Diet composition

Constituent (Kg)	Diet group	
	Control	3'-Me DAB
Casein, gm	180	180
Corn oil. gm	50	50
Glucose monohydrate, gm	770	770
Salt mixture, gm	40	40
Riboflavin, gm	0.001	0.001
Vitamin mixture*, ml	5	5
3'-Me DAB, gm	–	0.6

* cod liver oil 20 gm, choline chloride 1.5 gm. pteroylglutamic acid 0.6 gm, biotin 1.5 mg, thiamine–HCl 20mg, pyridoxine-HCl 20mg, menadione 50 mg(in 20gm of cod liver oil), nicotinamide 50 mg, potassium p-amino benzoic acid 50 mg, calcium pantothenate 60 mg, inositol 100 mg and cyanocobalamin 40 ug.

Fractionation of the plasma membrane and microsomes from the hepatocytes

Plasma and microsomal membranes were prepared from the control and 3'-Me DAB group by differential centrifugation according to the method described by Kitagawa *et al.*(1985). Livers obtained from each group were minced and suspended in 5 volumes of 20 mM HEPES (pH 7.4), and then was mixed with an equal volume of 20 mM HEPES containing 40% (W/V) sucrose. 1 mM of EDTA(pH 7.0) was rapidly added to the suspension and adjusted to a final concentration of 1 mM of EDTA. Hypotonic shock was accomplished by dispersing the cell pellet rapidly in 30 volumes of 1 mM HEPES(pH 7.0) at 0°C followed by homogenation with a Teflon homogenizer. The supernatant was aspirated and saved for the preparation of microsomal membranes. The pellet was washed once and resuspended for preparation of the plasma membrane with a solution containing 10 mM HEPES, 1 mM EDTA and 255 mM sucrose. Plasma membranes were obtained by centrifugation at 23,000 × g for 60 min on a discontinuous 1.12 M sucrose gradient. The plasma membranes were washed twice and the membranes were obtained from the inital supernatant by centrifugation at 180,000 × g for 80 min. The pellet was washed once and resuspend to a final concentration of 3 to 5 mg of protein/ml. The purity of the plasma membranes was determined by 5'-nucleotidase activity. Microsomes were further purified by centrifugation at 105,000 × g for 60 min and identified by glucose-6-phosphatase activity. 5'-Nucleotidase and glucose-6-phosphatase activities were determined by the methods of Hepple and Hilmoe (1955) and Nordlie and Arion (1955), respectively. Protein was measured by the method of Lowry *et al.* (1951).

Quantitation of glucose carriers on the plasma membranes and microsomes

0.1 mg of the plasma membranes and microsomes were mixed with 25.3 nM of [3H]-cytochalasin B, and the adjusted concentration of cold cytochalasin B ranged from 0–1.000 mM. Then binding activity was determined according to the method of Kitagawa *et al.* (1985). The number of glucose carriers on the plasma and microsomal membrane was analyzed by Scatchard plot (1949).

Partial purification of glucose carriers from hepatoma cell membranes

The integral proteins from the partially purified plasma membranes of the hepatoma cells described above were extracted with 0.5% Triton X-100 containing 25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.5 at 4°C. The extracted proteins were further purified by DEAE Sephadex A-50 (Pharmacia Chem Co.) ion exchange and AcA 34 (LKB Co.) gel filtration chromatography. Glucose carrier activities were assayed by measuring the amount of 14C(U) glucose in the liposomal preparation containing a constant

amount of protein from the purification procedures (Carruther and Melchior 1984).

Sodium docecyl sulfate–polyacrylamide gel electrophoresis

Samples obtained in each purification step from the 3'-Me DAB induced hepatoma cell were boiled for 2 min in the presence of 1% SDS, then applied to a 7% polyacrylamide gel electrophoresis by the method of Laemmli (1970).

RESULTS

1. Partial purification of plasma membranes and microsomes of rat river

5'-Nucleotidase and glucose-6-phosphatase activities from the partially purified plasma membranes and microsomes of liver cells from the control and 3'-Me DAB groups are summarized in Table 2.

The activities of 5'-nucleotidase, a marker enzyme for plasma membranes, in the homogenates of livers from the control group were 0.58±0.08 μmols/mg protein/10 min and 0.29±0.07 μmols/ mg protein/10 min in the 3'-Me DAB group.

In the post-microsoml fractions, the enzyme activities were 0.64±0.28 and 0.59±0.04 μmols/mg protein/10 min in the control and 3'-Me DAB groups, respectively. In the partially purified plasma membrane fractions, the enzyme activities were 2.15±0.25 and 1.31±0.28 μmols/mg protein/10 min in the control and 3'-Me DAB groups, respectively, and these data support the results of Chatterjee *et al.* (1976). The activities of glucose-6-phosphatase. a marker enzyme for microsomal fraction, were 0.28±0.10, 0.45 ± 0.25 μmols/mg protein/10 min in the homogenates of the livers from the control and 3'-Me DAB groups, respectively. In the partially purified microsomal fractions, the enzyme activities were 1.14±0.32 and 0.63±0.11 μmols/ mg protein/10 min in control and 3'-Me DAB group, respectively. These plasma and microsomal membrane fractions were used for assaying the glucose carriers

2. The quantitative analysis of glucose carrier from plasma membranes and microsomes of rat livers

The numbers of glucose carriers present in the plasma membranes and microsomes from the control and 3'-Me DAB groups were analyzed by Scatchard plot (1949) and the results are summarized in Table 3, Figs. 1 and 2.

The concentrations of glucose carrier in the plasma membranes from the control and 3'-Me DAB group

Tabel 2. Isolation of plasma membranes and microsomes from the control and 3'-Me DAB treated rat livers, 5'-nucleotidase and glucose-6-phosphatase activities in each fractions were assayed as described by the methods of Happel *et al.* (1955) and Nordlie *et al.* (1955).

Fractions	μmols/10min/mg protein			
	5'-Nucleotidase		Glucose-6-phosphatase	
	Control	3'-Me DAB	Control	3'-Me DAB
Homogenate	0.58±0.08	0.29±0.07*	0.23±0.10	0.45±0.25
Post-microsome	0.64±0.28	0.59±0.04	–	–
Plasma membrane	2.15±0.25	1.31±0.23	–	–
Microsome	–	–	1.14±0.32	0.63±0.11

* Mean ± S.E.

Table 3. Percent of cytochalasin B binding to the plasma membranes of the control and 3'-Me DAB groups. Plasma membranes were incubated for 2 hours in a shaking water bath in the presence of 2 μM cytochalasin E: [3H]-cytochalasin B (25.3 nM) and unlabeled cytochalasin B were added to final concentrations ranging from 0 to 1,000 nM.

Cold cytochalasin B (nM)	Control	3'-Me DAB
0	11.0±0.3*	14.5±0.2
1	9.0±0.2	10.5±0.2
10	6.5±0.4	8.5±0.4
100	4.5±0.3	7.2±0.4
1000	3.6±0.2	3.8±0.2

* Mean ± S.E.

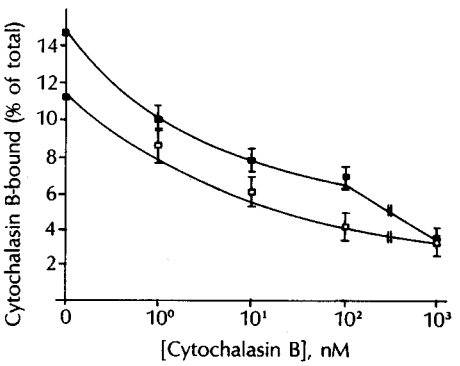

Fig. 1. *Cytochalasin B binding to plasma membranes from the control (□) and 3'-Me DAB (■) groups. Plasma membranes were incubated for 2 hours in a shaking water bath in the presence of 2 μM cytochalasin E; 3H-cytochalasin B (25.3nM) and unlabeled cytochalasin B were added to final concentrations ranging from 0 to 1,000 nM.*

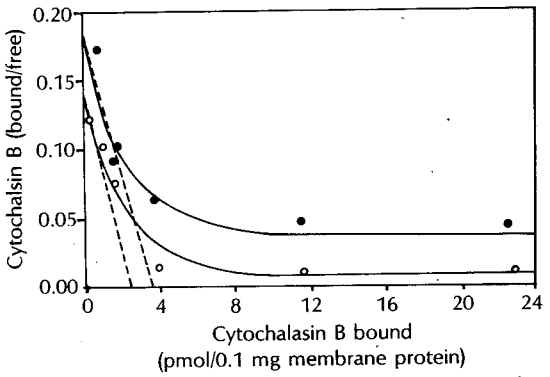

Fig. 2. *Scatchard analysis of cytochalasin B-binding to the plasma membranes. Experimental procedures were the same as described in the text.*

O Control

● 3'-Me DAB

Table 4. Percent of cytochalasin B binding to the microsomes of the control and 3'-Me DAB group. Microsomes were incubated for 2 hours in a shaking water bath in the presence of 2 μM cytochalasin E; [3H]-cytochalasin B (25.8nM) and unlabeled cytochalasin B were added to final concentrations ranging from 0 to 1,000 nM.

Cold cytochalasin B(nM)	Control	S'-Me DAB
0	4.0±0.3*	3.6±0.2
1	1.7±0.2	2.0±0.2
10	1.6±0.2	1.2±0.2
100	1.5±0.1	1.9±0.2
1000	1.6±0.2	1.8±0.3

*Mean ± S.E.

were 25 and 35 pmols/mg protein, respectively, and the Ka values for cytochalasin B in each group were 3.20×10^9 and 5.14×10^9 ml/mol, respectively. These results suggest that increased glucose transport into the 3'-Me DAB induced hepatoma cells is not due to an increase in the affinity of the transporter of glucose but rather is due to an increase in the number of transporters. However, no significant differences in glucose carrier in the microsomal fractions were found between the control and 3'-Me DAB groups (Table 4, Fig. 3).

3. Partial purification of glucose carrier on the plasma membranes of 3'-Me DAB induced hepatoma cell

To purify the glucose carrier in the plasma membranes of hepatoma cells, membrane proteins were

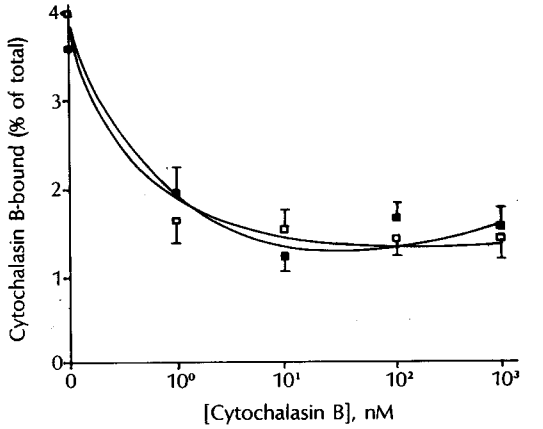

Fig. 3. *Cytochalasin B binding to microsomes from the control (□) and 3'-Me DAB (■) groups. Microsomes were incubated for 2 hours in a shaking water bath in the presence of 2 μM cytochalasin E; 3H-cytochalasin B (25.3 nM) and unlabeled cytochalasin B were added to final concentrations ranging from 0 to 1,000 nM.*

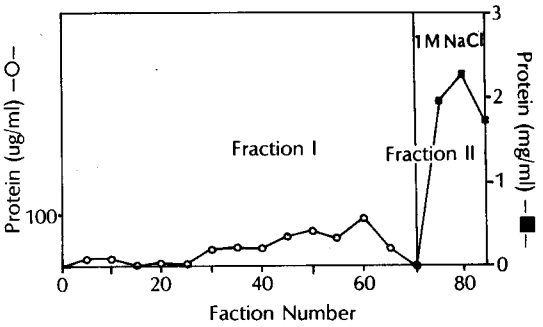

Fig. 4. *DEAE Sephadex A-50 column chromatography of hepatoma cell plasma membrane proteins. The triton extract was prepared with Triton X-100 containing 25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA (pH 7.5) and applied to a DEAE Sephadex column as described in the text. Each fraction was pooled and glucose carrier activities were measured according to Carruther and Melchior (1984).*

extracted with 0.5% Triton X-100 and applied to a DEAE Sephadex A-50 ion exchange column (Fig. 4). From this procedure, fractions I and II were obtained. The amounts of 14C (U) D-glucos transported through the liposomal preparations from fractions I and II were 4,740 DPM/mg protein/ 5 min and 2,785 DPM/ mg protein/ 5 min, respectively. SDS-PAGE(7%) analysis of fraction I which contained higher transport activity showed a major protein band with a molecular weight of 45,000 and minor bands of proteins with

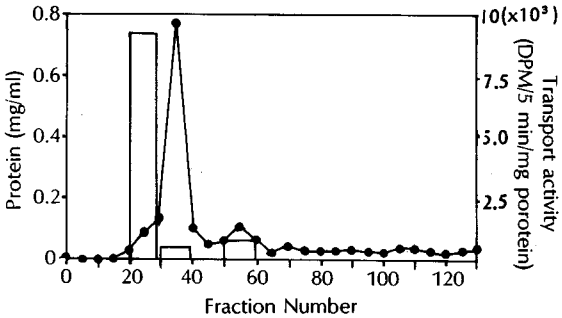

Fig. 5. *AcA 34 gel filtration chromatography of glucose carriers from 3'-Me DAB induced hepatoma cell plasma membranes. Gels were equilibrated with 0.5% Trition X-100 containing 25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA (pH 7.5). Fraction I was obtained from DEAE sephadex A-50 column chromatography, was concentrated to 2 ml in saturated polyethylene glycol, and then was applied to the column (1.5 × 30 cm). The flow rate was 10 ml/hr and 1 ml of each fraction was collected. Carrier activities were measured according to Carruther and Melchior (1984).*

• Shows protein (mg/ml) and bar shows transport activity as DPM/5 min/mg protein.

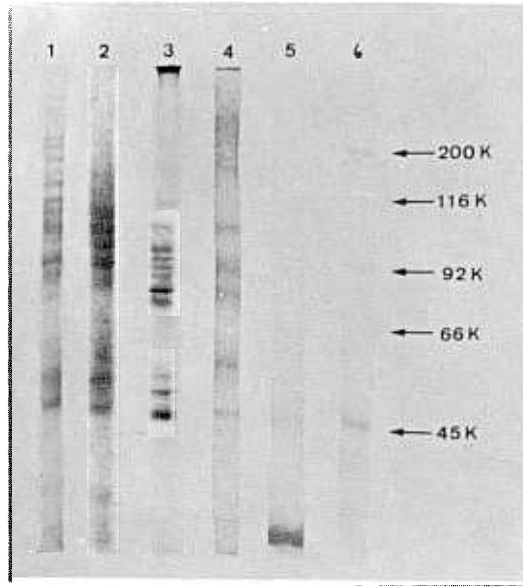

Fig. 6. *SDS-polyacrylamide gel electrophoretic analysis of proteins obtained from 3'-Me DAB induced hepatoma in each purification step.*

1. Homogenate
2. Plasma membrane
3. Triton extract
4. DEAE-Sephadex eluate
5. AcA 34 gel filtration eluate
6. MW Standards, myosin (MW 200,000), β-galactosidase (MW 116,000), phosphorylase B (MW 92,500), bovine serum albumin (MW 66,000), ovalbumin (45,000).

MWs of 50,000, 55,000 and 15,000 (Figs. 4 and 6). For further purification, fraction I obtained from the DEAE Sephadex A-50 ion exchange chromatography was applied to an AcA 34 gel filtration column, and from this, fractions I, II and III were obtained (Fig. 5). Of these, fractions I showed the highest transport activity (8,888 DPM/mg protein/ 5 min), and SDS-PAGE(7%) analysis of this fraction showed a major band with a MW of 45,000 (Fig. 6). However, transport activities from fraction II and III were 475 and 570 DPM/mg protein/ 5 min, respectively.

From these results, it can be concluded that the amount of glucose carrier was increased on the plasma membranes of hepatoma cells induced by 3'-Me DAB, and that the carrier protein had molecular weight similar to that of other reported glucose carriers purfied from the RBCs. myocytes and adipocytes.

DISCUSSION

During the transformation of cells, many changes in the plasma membrane have been reported (Wu *et al.* 1969; Reutter and Bauer 1978; Vischer and Reutter 1978). Notably, glucose transport in virally transformed cells is increased (Kawal and Hanafusa 1971; Weber 1973), and inhibition studies using cytochalasin B have related this to the number of glucose carriers on the cell membrane (Baldwin *et al.* 1979; Salter *et al.* 1982). However, studies on the expression of glucose carrier in cells transformed by chemical carcinogens have not been done although glucose transport in chemically induced cancer cells increased (Lee and Weinstein 1979; Kim *et al.* 1985). In this study, a Scatchard analysis of partially purified plasma and microsomal membranes from the livers of the control and 3'-Me DAB groups was performed.

Two kinds of glucose transport mechanisms are known to be present in most mammalian cells. Active transport is known to occur in the intestinal epithelia and kidney brush border (Ducis and Koespell 1983) and facilitated diffusion occurs in the liver, RBCs, and adipocytes (Wheeler and Hinkle 1985). The facilitated diffusion mechanism is further subdivided

into insulin dependent or independent types. Those cells that depend upon insulin concentrations are adipocytes and myocytes (Wardzala *et al.* 1978; Suzuki and Kono 1980; Kono *et al.* 1982), whereas those that are independent of insulin concentration and known as liver cells and RBCs. (Wheeler and Hinkle 1985). The insulin dependent glucose carrier translocates between the plasma and microsomal membranes. When extracellular insulin concentrations increase, the glucose carriers in the microsomes translocate to the plasma membranes and lowered isulin concentrations induce the glucose carrier in the plasma membranes to migrate to microsomes in fibroblasts transformed by the Rous sarcoma virus, glucose transport was reported to increase (Kletzien and Perdue 1976; Invi *et al.* 1980; Weber *et al.* 1984), and this is due to the increase in the number of glucose carriers on the plasma membranes which were translocated from the microsomes (Kitagawa *et al.* 1985). In this study a 40% increase in cytochalasin B-binding protein with no difference in the Ka value was observed in the 3'-Me DAB group as compared to the control group.

From these results, it can be concluded that glucose uptake into hepatoma cells is not due to an increased affinity of glucose carriers for glucose but due to an increased number of glucose carriers. These data support the results obtained from fat cells (Kono *et al.* 1982) and fibroblasts (Kitagawa *et al.* 1985). In the microsomal fraction, no significant difference in the cytochalasin B-binding protein was found in the Scatchard analysis. This result is not consistent with that of Kono *et al.* (1982) or Kitagawa *et al.* (1985). However, glucose carrier recycling in adipocytes by insulin occurs between plasma membranes and low density microsome but not high density microsomes (Simpson and Cushman 1986).

It's not possible to conclude whether there is a translocation of glucose carrier in hepatoma cells or not. No difference in the glucose carrier concentration on microsomes of normal and hepatoma cell induced by 3'-Me DAB could be detected, because a low density microsomal preparation was not used in the present experiment. Glucose carriers have been purified from various cell types. Human RBC glucose carrier has a MW of 50,000–55,000 (Kasahara and Hinkle 1977: Baldwin *et al.* 1981) and is known to be a glycoprotein (Gorga *et al.* 1979). Also, it has been purified from adipocytes (Lienhard *et al.* 1982), muscle (Klip *et al.* 1983) and embryo fibroblasts (Salter *et al.* 1982), and all preparations have similar molecular weights ranging from 40,000–60,000. Identical antigenic determinants are known to be present on the glucose carrier of RBC, muscle, and adipocytes (Sogin and Hinkle 1980). In the present study, the MW of the glucose carrier was estimated to be 45,000 and it was similar in character to that reported in other cell types. However, detailed investigation is needed for characterization of the physical and chemical properties of the glucose carrier expressed on hepatoma cells induced by chemical carcinogens.

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