Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5958–5961, October 1980
Cell Biology

Effects of combined glutamine and serum deprivation on glucose control of hexose transport in mammalian fibroblast cultures

(glucose metabolism/2,4-dinitrophenol/transformed cells)

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Contributed by Herman M. Kalckar, June 30, 1980

ABSTRACT **Regulation of hexose transport in NIL hamster fibroblasts has been studied in confluent cultures preconditioned for 24 hr in media deprived of glutamine or of serum or of both. Cultures maintained in media containing dialyzed fetal calf serum and 4 mM glutamine accumulated up to 72 nmol of glutamine per mg of cell protein; in contrast, cells deprived of glutamine contained less than 1 nmol/mg of cell protein. Glutamine elicited a general enhancement of hexose transport compared with transport in glutamine-deprived cultures. This enhancement was particularly pronounced in glucose-fed cultures which in the absence of glutamine showed conspicuously low transport activity. When maintained in glucose media, cultures deprived of serum also showed a marked loss of hexose transport which, in this case, was not compensated for by addition of glutamine. However, regardless of the presence or absence of glutamine, these cultures were able to develop the usual transport enhancement response to glucose starvation. Moreover, 2,4-dinitrophenol was also able to elicit a pronounced enhancement of hexose transport in the glucose-fed cultures; this effect surpassed even the transport derepression observed in the glucose-starved cultures. In polyoma-transformed cultures maintained in serum-free media, hexose transport remained relatively high, even in the presence of glucose. However, addition of glutamine brought about an enhancement in both the presence and absence of serum. The various phenomena are discussed in regard to protein turnover in general and more specifically the turnover of hexose transport carriers.**

Glucose starvation or replacement of glucose by fructose permits cultures of NIL hamster fibroblasts to develop a marked enhancement or "derepression" of the hexose transport system. Readdition of glucose (or D-glucosamine), brings back a "repression" of this system, regardless of the lactic acid production (1, 2). In order to test whether an oxidative metabolic pathway might be involved in exerting further repressive control, the effect of L-glutamine on hexose transport activity has been investigated.

In several types of mammalian cell cultures, L-glutamine contributes significantly to oxidative and even to glycolytic catabolism (3, 4). We have recently found that confluent or near-confluent NIL cultures as well as Swiss 3T3 mouse fibroblast cultures can readily be maintained for 24–48 hr in standard culture media (with 10% dialyzed fetal calf serum) without any supplement of glutamine (unpublished data). In the present article we describe the regulation of the hexose transport system largely as a function of the presence or absence of glutamine. Are the regulatory responses of the hexose transport in the presence or absence of glutamine in the medium different from those observed in glucose-fed or starved cultures?

Because the regulatory enhancement of hexose transport by glucose starvation is arrested not only by cycloheximide but also by the transcriptional inhibitors actinomycin D and camptothecin (5+7), the enhancement has been classified as a transcriptional event (5)—i.e., as a "derepression" (1). Conversely, the metabolic glucose effect, restraining the transport, has been classified as "repressive control" (1).

In the NIL cultures used, glutamine in the absence of hexoses seems only to be able to generate traces of lactic acid (8). If any pyruvate is formed from glutamine (3, 4) it does not bring about any detectable repressive control, nor does added pyruvate (8, 9). However, this does not exclude that glutamine itself or one of its other catabolic products might on its own promote catabolic control of the transport system. This problem requires studies of hexose transport in the absence of glucose as well as glutamine.

As will be shown here, the regulatory responses of the hexose transport system are greatly influenced by the presence or absence of glutamine. However, the responses turned out to be governed by several factors of which the presence or absence of serum was one of the dominating ones. It was also unanticipated that the strongest regulatory perturbations were found in the glucose-fed cultures rather than in glucose-starved cultures. No repressive effects from glutamine were observed; on the contrary, in the presence of serum, glutamine seemed to enhance the hexose transport markedly.

The corresponding responses in polyoma-transformed NIL cultures were also studied. It is well known that, for growth, transformed cells are less dependent on high serum concentrations than are untransformed cells. In contrast, the latter have lower glutamine requirements than do tumor cells (10).

MATERIALS AND METHODS

Hamster fibroblasts (NIL strain) and polyoma-transformed NIL (PyNIL) were obtained from K. J. Isselbacher (Gastrointestinal Unit, Massachusetts General Hospital). 3T3 mouse fibroblasts were obtained from D. V. Young (Chemistry Department, Boston University). Cells were grown to near-confluence in Dulbecco's modified Eagle's medium with glucose at 4 mg/ml and 10% (vol/vol) calf serum in 35-mm plastic culture dishes (Falcon) in a humidified CO_2 incubator. Sixteen to 24 hr before the uptake tests, cells were rinsed twice with medium free of glucose, glutamine, and serum. They were then maintained for 20 hr in the growth medium lacking pyruvate, glycine, serine, and $Fe(NO_3)_3$. Glucose was omitted or replaced by other sugars; glutamine and dialyzed fetal serum were occasionally omitted and inhibitors were added as indicated for the individual experiments. Before the uptake test, the cells were rinsed three times with Dulbecco's phosphate-buffered saline (pH 7.4) (P_i/NaCl) and then given [*U*-^{14}C]galactose (100 nmol/ml or less in P_i/NaCl) for 10 min at 37°C. Cultures were then rinsed three times with ice cold P_i/NaCl and then extracted for at least

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Abbreviations: P_i/NaCl, phosphate-buffered saline, pH 7.4; DNP, 2,4-dinitrophenol.

15 min with 500 μl of 70% (vol/vol) ethanol. To test for leakiness of the cells and completeness of washing, L-[^{3}H]glucose was included with the [^{14}C]galactose. The ethanol extract was assayed in a Beckman scintillation counter.

Media and serum were obtained from GIBCO and Microbiological Associates (Bethesda, MD). Radioactive sugars were from New England Nuclear, and 2,4-dinitrophenol (DNP) was from Sigma.

For glutamine determination, the cells were rinsed seven times with P_i/NaCl and extracted with ice cold 5% (wt/vol) $HClO_4$. The $HClO_4$ extract was adjusted to pH 3–4 with KOH. The precipitate was removed by centrifugation and the supernatant was analyzed by using a Beckman 119CL amino acid analyzer and a modification of the lithium physiological fluid system described in the Beckman application notes (118/119CL AN-002, April 1977). The column (0.6 × 22 cm) was eluted with 0.2 M lithium citrate (pH 2.83) at 40°C for 50 min and then regenerated. Under these conditions, asparagine, glutamic acid, and glutamine are well resolved. The detection limit for glutamine was about 0.1 nmol. Samples (200 μl) from replicate experiments were mixed with 50 μl of pH 2.2 citrate buffer and injected into the analyzer.

For protein determination, the ethanol-insoluble fraction of the dish was dissolved in 1 ml of 0.1 M NaOH, and protein was determined by a slight modification of the Lowry method, in which the stock alkaline copper reagent was 5 times more concentrated. To a sample made up to 1 ml was added 0.2 ml of the concentrated alkaline copper reagent and, after 15 min, 0.1 ml of Folin reagent was added. This modification is especially suited to sparse cultures or to cases in which more than half of the cells become detached.

RESULTS

The hexose transport was monitored routinely as uptake of galactose in 10 min. This assay was chosen because it has been shown that it closely represents the initial hexose transport activity (1, 11).

Near-confluent NIL cultures maintained in media with 10% dialyzed fetal calf serum with or without glucose and exposed to glutamine deprivation in general showed low hexose transport activity. The greatest decline in hexose transport was in the glucose-fed cultures (Table 1). The presence of glutamine at 4 mM in the maintenance media preserved the hexose transport activity of the cultures. Regardless of the presence or absence of glutamine, glucose starvation was able to induce a pronounced enhancement of the hexose transport system. There was no sign that glutamine exerted any type of catabolic repressive control. This should have been detected in the glucose-starved cultures; instead, glutamine still exerted the opposite effect—i.e., further enhancement of the system. The dialyzed fetal calf serum could not have contained more than traces of glutamine because the medium fortified with 10% dialyzed serum was unable to provide even a nonproliferating glucose-fed culture with detectable levels of glutamine.

Although the glutamine-deprived cultures fed glucose showed low hexose transport, addition of DNP was very effective in overcoming the combined losses in transport, enhancing hexose transport more than 10-fold. Many of the same features were found in studies on Swiss 3T3 cultures (data not shown). Neither in this case did glutamine bring about any detectable repressive control of hexose transport. On the contrary, enhancement ensued, and DNP also was able to counteract the glucose effect in glutamine-deprived 3T3 cultures. In neither type of culture did DNP exert any significant effect on transport of amino acid analogues (data not shown).

Glutamine effects were also examined with regard to the amino acid transport systems (A and L systems) and glutamine was not found to stimulate these transport systems. If anything, an inhibition was discernable in some cases (data not shown).

Table 2 illustrates the effects of serum deprivation on the activity of hexose transport in NIL cultures. The glucose-fed cultures showed very low activity even in the presence of glutamine. Yet, hexose transport activity could readily be enhanced 10-fold or more by two devices. (*i*) Addition of DNP together with the usual glucose medium effectively counteracted the serum deprivation effect. (*ii*) Replacement of glucose by fructose also counteracted most of the effect in the glucose medium deprived of serum. DNP was able to counteract the combined losses in hexose transport which developed in glucose-fed NIL cultures deprived of serum whereas glutamine was ineffective in this case.

Table 3 illustrates how differently the hexose transport system of the transformed PyNIL responded to serum deprivation. In this case the presence or absence of serum made little difference. However, the absence of glutamine did make a difference, further curtailing the hexose transport activity in the PyNIL culture maintained in glucose medium.

DISCUSSION

The original question posed regarding glutamine metabolism was the possible role of glutamine catabolism in the catabolic repressive control of the hexose transport system. This in turn was governed by our observations that the glycolytic pathway

Table 1. Effect of glutamine deprivation on derepression of [^{14}C]galactose uptake by NIL cells

Additions	[*U*-^{14}C]Galactose uptake, nmol/mg protein*	
	Without glutamine	With 4 mM glutamine
Glucose	0.20 ± 0.05	1.32 ± 0.09
Glucose + DNP†	2.59 ± 0.03	4.33 ± 0.77
Fructose	1.94 ± 0.04	3.05 ± 0.32
No hexose	1.90 ± 0.19	3.94 ± 0.29

NIL cells were grown to near confluence in Dulbecco's modified Eagle's medium with 10% calf serum. The cultures were then rinsed twice and maintained for 20 hr in the same medium with 10% dialyzed fetal calf serum with or without glutamine and with sugars and additions as indicated. They were then rinsed with P_i/NaCl and assayed for galactose uptake: [*U*-^{14}C]galactose, 0.1 mM; 10 min; 37°C. Results are shown as mean ± 5%.

* Glutamine concentrations (per mg of cell protein) in cell extracts were as follows: in the cultures without glutamine, <1 nmol; in the cultures with glutamine, 72.5 nmol.

† DNP at 0.2 mM in 0.1% dimethyl sulfoxide.

Table 2. Effect of glutamine deprivation on derepression of [^{14}C]galactose uptake by NIL cells in serum-free medium

Additions	[*U*-^{14}C]Galactose uptake, nmol/mg protein	
	Without glutamine	With 4 mM glutamine
Glucose	0.26	0.16
Glucose + 0.2 mM DNP	1.90	2.18
Fructose	1.37	0.92

NIL cells were grown to near confluence in Dulbecco's modified Eagle's medium with 10% calf serum. The cultures were then rinsed twice and maintained for 20 hr in the same medium but without serum and with or without glucose and with or without glutamine and additions as indicated. All samples contained 0.1% dimethyl sulfoxide. They were then rinsed twice with P_i/NaCl and assayed for galactose uptake: [*U*-^{14}C]galactose, 0.1 mM; 10 min; 37°C. Use of 3-*O*-[^{14}C]-methyl glucose in 15-sec transport tests revealed the same features (unpublished data).

Table 3. Effect of serum and glutamine deprivation on [^{14}C]galactose uptake in PyNIL cells

	[*U*-^{14}C]Galactose uptake, nmol/mg protein	
	Without glutamine	With 4 mM glutamine
With serum:		
Glucose	0.70	2.10
Without hexose	3.12	4.19
Without serum:		
Glucose	1.55	2.34
Without hexose	3.64	4.70

is not involved in the repressive control of transport because D-glucosamine also exerts a marked repressive control (1); yet, only traces of lactic acid were formed from this amino sugar (1). Moreover, anaerobiosis tends to counteract repressive control from glucose or from glucosamine (unpublished observations). These findings prompted us to focus on aerobic pathways in carbohydrate metabolism as sources of transport control. Because uncouplers, including DNP, enhance hexose transport (1, 12) presumably by effectively abolishing the metabolic glucose control of hexose transport (3) our first thoughts focused on the tricarboxylate oxidative cycle. Formation of pyruvate from glutamine would proceed along this pathway; furthermore, DNP is known to uncouple oxidative phosphorylation coupled to this pathway. The tricarboxylate pathway therefore had to be considered first as a possible source of repressive control of hexose transport.

From the data of the present paper, glutamine can be discounted as a source of aerobic repressive control of transport. Our arguments for considering the various aerobic hexose pathways as a source for generation of metabolites critical in the control of the hexose transport system will be presented elsewhere. In the present discussion, it seems more pertinent to try to analyze the data shown here.

The tendency of glutamine to enhance hexose transport in glucose-repressed cultures seems reminiscent of the ability of ammonia to overcome the glucose effect (13). However, the glutamine effect seems more complex than the ammonia effect. Perhaps more noteworthy is the tendency of the glutamine-deprived cultures to show a marked decline in the hexose carrier system if maintained with glucose at the same time. This state of affairs, however, could readily be averted, if DNP also were present. The situation seems in several ways reminiscent of the striking ability of DNP to overcome the combined losses of hexose transport activity that ensue when cycloheximide is added to glucose-fed cultures (1). In the latter case, the curtailment of protein synthesis combined with a possible high rate of protein degradation, characteristic of glucose-fed dense cultures (2), could lead to such a decline of V_{max} of the hexose transport carriers (14).

DNP is known to interfere with intracellular protein turnover (1, 15). It seems legitimate to sketch an analogous scenario in order to try to explain the dramatic ability of DNP to overcome the combined losses in hexose transport activity in glucose-fed cultures deprived of glutamine as well as serum. These deprivations would probably curtail the rate of protein synthesis. The protein degradation induced by glucose would be arrested by DNP which in turn would stabilize the population of carriers at high levels. The ability of DNP to arrest protein turnover may be complex and requires additional studies. However, the ability of fructose or hexose starvation to restore hexose transport activity in the doubly deprived cultures could perhaps be accounted for more directly by protein turnover, and this in the following way.

Certain intracellular proteases, among them lysosomal cathepsins, show high activity in glucose-fed NIL cultures and low activity in glucose-starved cultures (13). This finding in turn seems to correlate well with the regulative patterns of the hexose transport system. And this correlation may imply the existence of a mechanism (probably proceeding through several steps) by which the hexose transport carriers are becoming internalized and degraded, whenever the intracellular cathepsin level is high (13).

Moreover, it has been reported that transformed cultures maintained in media with glucose show apparently only low levels of intracellular protease activity (16). This seems to fit well with the fact that transformed cells, among them PyNIL, seem to be exempt from the dramatic losses in hexose transport activity in serum-free maintenance media (17). Glutamine deprivation, however, did bring about a lowering of transport activity in these cultures.

Previous studies on the regulatory responses of the hexose transport system from transformed cultures to the presence or absence of glutamine have been conducted on a special hamster PyBHK tumor line (a variant of BHK Py 6 line) (18). This variant is able to grow in the absence of glutamine and was therefore called the "glutamine independent variant," GIV (19). Even in this case, hexose transport was found to be regulated upward by the presence of glutamine.

There is a fairly extensive literature regarding the effects of serum on transport, including the hexose transport system, especially in chicken embryo fibroblasts. Most of the cultures maintained in standard media containing glucose showed only low hexose transport activity if deprived of serum prior to the test, whereas addition of chicken serum (20) or fetal calf serum (17) permitted high transport activities, especially in the hexose transport system (17, 20). An important variation to this pattern is the observation that chicken embryo fibroblasts deprived of serum can muster a marked enhancement of the hexose transport system if they first were starved for glucose (21).

It seems clear from the data presented here that a combination of serum deprivation with a drastic lowering of glutamine levels may constitute a simpler way of studying regulatory events.

Transformed cultures in standard media with glucose show, in general, high hexose transport activity (22), even in the absence of serum (17). However, deprivation of the transformed cells by withholding glutamine from the maintenance medium seems to lead to a noticeable decline of the transport activity, especially in the glucose-fed cultures.

The hypothesis that proteolytic breakdown of hexose transport carriers may play a major role in the regulation of the transport system (13) needs more experimental proof than can be derived from this series of experiments. However the observations available are not in conflict with this hypothesis. On the contrary, some of the phenomena seem to be easier to interpret on the basis of this hypothesis as well as previous discussions about protein turnover (23).

We want to express our gratitude to Dr. C. W. Christopher (Biology Department, Boston University), Dr. S. P. Colowick (Department of Microbiology, Vanderbilt University School of Medicine), Dr. K. J. Isselbacher (Gastrointestinal Unit, Massachusetts General Hospital, Boston), and Dr. D. V. Young (Chemistry Department, Boston University) for their interest and encouragement during this study. This project was supported by grants from the American Cancer Society, New York (BC-120F) and from the National Science Foundation (PCM 7922889) to H.M.K. and (PCM 79049-10) to R.A.L.

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