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Galactose and Glucose Metabolism by Cultured Hepatocytes: Responsiveness to Insulin and the Effect of Age

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Age changes in utilization of glucose and galactose were studied in primary cultures of rat hepatocytes. (1) With increasing age of donor from 2 to 10 weeks, the rate of galactose utilization fell while that of glucose utilization increased from a negative value (net production) to a level approximately double that of galactose. Glucose production could account for about 80% of the galactose taken up by cells from 2- to 3-week-old rats. (2) With increasing time of culture there was a fall in galactose utilization and an increase in glucose utilization comparable to the changes with increasing age *in vivo*. (3) The change in utilization of each sugar was independent of the change in utilization or availability of the other. It is suggested that the switch from galactose to glucose as the preferred substrate *in vivo* is not determined by dietary availability of these hexoses. (4) The increased utilization of glucose by cells of older animals was associated with a decrease in responsiveness to the glycogenic action of insulin. The change in responsiveness was not due to a decrease in insulin binding capacity of the cells.

INTRODUCTION

In the neonate about 50% of the dietary carbohydrate is contributed by galactose. This is metabolized almost entirely by the liver, much of it being converted to glucose and secreted into the circulation (Segal and Blair, 1961). During late fetal life the liver is prepared for a high rate of galactose utilization by an increase in the specific activities of galactokinase, galactose-1-phosphate uridyl transferase, and uridine diphosphate galactose-4-epimerase (Cuatrecasas and Segal, 1965; Bertoli and Segal, 1966; Cahn and Segal, 1969). These enzymes reach their peak activity 6–10 days after birth and then decline to adult levels. With aging there is a decrease in the amount of galactose in the diet and a decrease in the ability to metabolize a galactose load (Segal *et al.*, 1963; Haworth and Ford, 1963; Vink and Kroes, 1959; Sparks *et al.*, 1976).

Since the increases in enzyme activity occur prior to a change in the amount of galactose delivered to the liver, it is not certain that the decrease from peak activity to adult level is related to a decrease in dietary intake with aging. We have examined one aspect of this question by studying the hexose metabolism of hepatocytes in primary culture.

METHODS

Cell preparation and culture. Hepatocytes were isolated from Sprague–Dawley rats previously maintained in a controlled-temperature and -light environment, with free access to food (Purina laboratory chow) and water. Cells were isolated by enzymatic digestion of liver slices essentially as described by Gebhardt *et al.* (1978), but several minor modifications were made in the procedure: (1) Swim's medium S-77 was used as the basic medium throughout the washing and digestion processes. It was supplemented with 2 g of bovine serum albumin and 2.2 g $NaHCO_3$ per liter; (2) the enzyme solution for digestion contained collagenase, hyaluronidase, and DNase but the trypsin was omitted.

Cells were plated at a density of approximately 1.5×10^6 cells/dish, in 35-mm plastic dishes previously coated with rat tendon collagen (Michalopoulos and Pitot, 1975). Four hours later the medium was replaced with Leibowitz medium L-15 containing 20% (v/v) fetal calf serum, 5% (v/v) tryptose phosphate broth, 15 m*M* HEPES buffer, 2 m*M* glutamine, and 1 m*M* oxidized glutathione. Glucose or galactose was added at a concentration of 8 m*M* to give final hexose concentrations of glucose 8 m*M* and galactose 5 m*M*, or galactose 13 m*M* with no glucose.

For measurement of cell constituents, medium was removed by aspiration, the cells were washed rapidly four times with ice-cold phosphate-buffered saline, then dissolved in 1 *N* NaOH. Protein was measured by the method of Lowry *et al.* (1951). For isolation of labeled glycogen, cells were digested in 30% KOH, then glycogen was precipitated with 65% ethanol in the presence of Na_2SO_4 (van Handel, 1965) and 1 mg carrier glycogen; the precipitate was dissolved in 1 ml H_2O then reprecipitated with 65% ethanol without further addition of Na_2SO_4. This procedure gave 93–98% recovery of added labeled glycogen. Glucose was measured with a glucose oxidase procedure (Beckman glucose analyzer) and ga-

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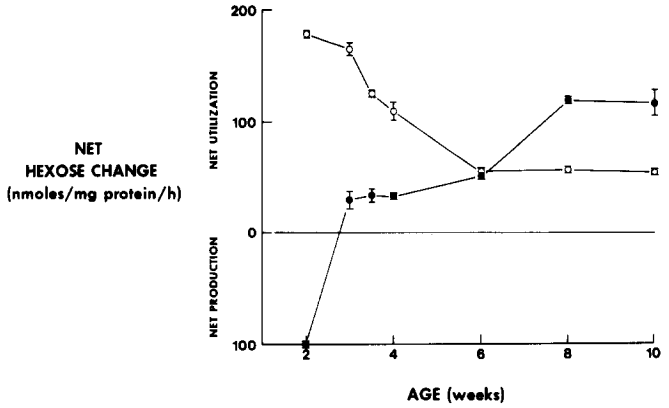

FIG. 1. Changes in utilization of glucose and galactose by primary cultures of hepatocytes obtained from rats of increasing age. Culture medium (Leibowitz L-15) contained galactose 5 m*M* and glucose 8 m*M*. Glucose (●) and galactose (○) were measured in the medium after a 24-hr culture period.

lactose by a galactose dehydrogenase method (Finch *et al.*, 1969).

Insulin binding and degradation. Binding was measured in 24-hr cultures. Cells were washed 2× with fresh medium; then 0.8 ml of medium containing 1 ng/ml of ^{125}I-labeled insulin with or without unlabeled insulin (40 μg/ml) was added and the cells were incubated at room temperature for the times indicated. The medium was then aspirated, the cells were washed 4× rapidly with ice-cold phosphate-buffered saline, then dissolved in 1 *N* NaOH; aliquots of the solution were taken for counting of radioactivity and for protein determination. To measure degradation of insulin cells were incubated with medium containing ^{125}I-labeled insulin as for binding studies; 0.1 ml of the medium was then added to 0.2 ml normal serum and 0.7 ml of ice-cold phosphate buffer pH 7.4, 0.3 ml of cold 40% (w/v) trichloroacetic acid was added, and after centrifugation and complete removal of the supernatant the pellet was assayed for radioactivity.

Glycogenic response to insulin. The incorporation of [^{14}C]glucose into glycogen was measured by the procedure of Plas *et al.* (1973) with a few minor changes in detail. Four hours after cells were plated, the medium was replaced with medium L-15 as described above with the addition of glucose 8 m*M* and dexamethasone (10^{-7} *M*). After culture for 48 hr the medium was changed and 50 μl of L-15 containing insulin and [U-^{14}C]glucose (1.5 μCi, specific activity 240 mCi/nmole) was added to the cells; 4 hr later the medium was removed and the cells were prepared for measurement of protein and for isolation of glycogen.

Collagenase (Type IV), hyaluronidase, DNase, HEPES, glutamic acid, glutathione, EGTA, and bovine serum albumin were from Sigma, media L-15 and S-77 were from Grand Island Biological Corp. or Flow Laboratories, galactose dehydrogenase was from Boehringer-Mannheim Inc., and fetal calf serum was from Microbiological Associates. ^{125}I-labeled insulin was obtained from New England Nuclear Corp. and [U-^{14}C]glucose was purchased from ICN.

RESULTS

A. *Increasing Donor Age*

1. Cell yield and viability. All preparations showed 90–95% viability (Trypan blue exclusion) but the cell yield was much greater from 2- to 4-week-old animals (7–13 × 10^7 cells/g wet weight of liver) than from 6- to 10-week-old animals (13–22 × 10^6 cells/g of liver).

2. Glucose and galactose utilization. Net utilization of each hexose (during a 24-hr culture period) was calcu-

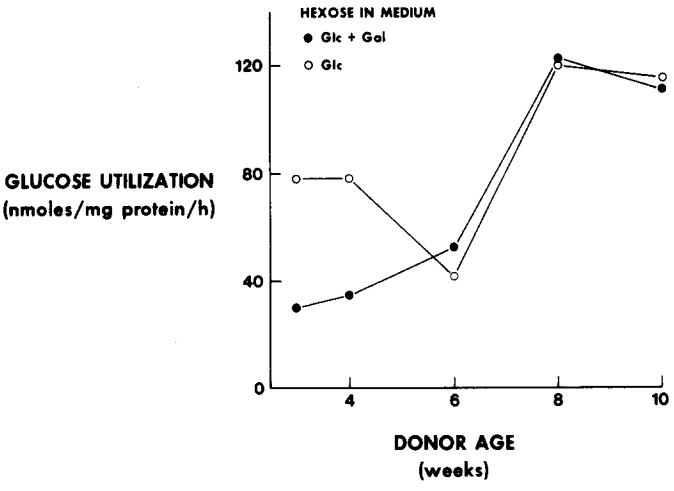

FIG. 2. Effect of donor age on glucose utilization in primary hepatocyte cultures (○). Culture medium was L-15 modified to contain glucose 13 m*M*. Glucose uptake in medium containing glucose 8 m*M* and galactose 5 m*M* is reproduced from Fig. 1 for comparison (●).

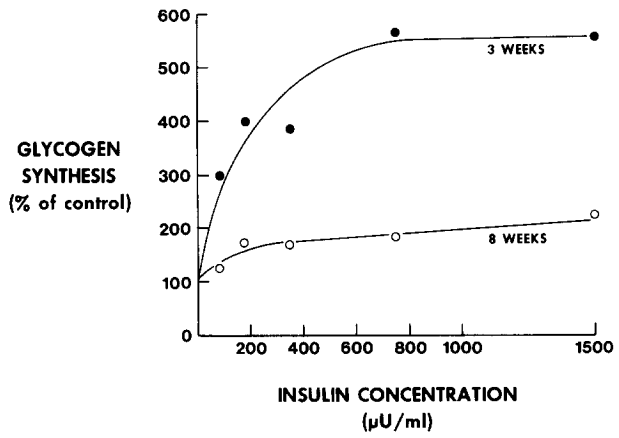

FIG. 3. Effect of insulin on glycogen synthesis (incorporation of [^{14}C]glucose into glycogen) in cultured hepatocytes from rats aged 3 weeks (●) and 8 weeks (○). Values are means of four experiments.

lated from the net change in concentration in the medium. When medium containing glucose and galactose was used (Fig. 1) galactose utilization was initially high and decreased as the age of the cell donor increased to 6 weeks. Glucose utilization followed an inverse course, increasing from a net production at 2 weeks of age to net utilization which exceeded galactose utilization at 8 weeks of age.

When medium containing 13 m*M* galactose and no glucose was used with hepatocytes from 2-week rats, the net rate of galactose utilization was increased to 260 nmoles/mg protein/hr but there was also an increase in net glucose production to 185 nmoles/mg/hr: if glucose was derived only from galactose, it accounted for about 80% of the galactose taken up.

In medium containing 13 m*M* glucose and no galactose (Fig. 2) the rate of utilization was initially higher than in the glucose–galactose medium, but at 6 weeks and later the rates of utilization were almost identical. Consequently, total hexose utilization (glucose and galactose) was greater at all times in the glucose–galactose medium.

3. Insulin stimulation of glycogen synthesis. Insulin increased the incorporation of glucose into glycogen in a dose-dependent manner, with a maximum effect at about 750 μU/ml and a half-maximal effect at approximately 150 μU/ml. The response of cultures from 3-week rats was four to five times greater than that of cells from 8-week animals (Fig. 3).

4. Insulin binding and degradation. The time course of binding is shown in Fig. 4A; specific binding reached equilibrium after about 60 min and was constant for an additional 60 min. Binding curves to cells of 3-week and 8-week rat hepatocytes were identical. On the other hand, the rate of degradation by 8-week hepatocytes was distinctly greater than that of the 3-week cells, largely because of a lag in the onset of degradation by the latter cells (Fig. 4B).

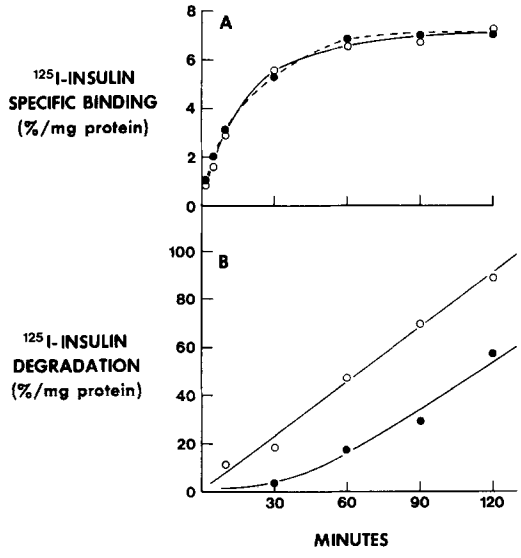

FIG. 4. Time course of insulin binding (A) and degradation (B) in 1-day cultures of hepatocytes obtained from rats 3 weeks (●) and 8 weeks (○) of age. Experiments were carried out at 21°C in the presence of 1 ng/ml of ^{125}I-labeled insulin. Each point is the mean of triplicate plates in two experiments. Values have been corrected for nonspecific binding.

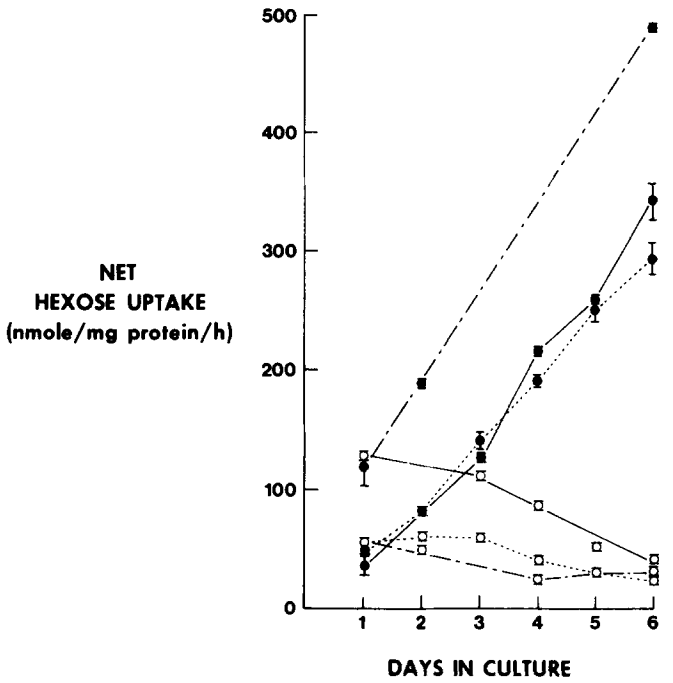

FIG. 5. Utilization of glucose (●) and galactose (○) by hepatocytes maintained in primary culture for 6 days. Medium containing glucose 8 m*M* and galactose 5 m*M* was changed daily. Cells were obtained from rats aged 3 weeks (——), 6 weeks (—·—), and 10 weeks (---).

B. Increasing Duration of Culture

1. Hexose utilization. Cells were obtained from donors of increasing age and maintained in culture in glucose–galactose medium for up to 6 days, with daily change of medium. On each day of the experiment five plates were taken for measurement of glucose and galactose utilization. With increasing time in culture the uptake of galactose fell and that of glucose rose sharply (Fig. 5); as in the preceding experiments, the utilization of galactose fell with donor age while that of glucose rose. In glucose-free L-15 medium, net glucose production accounted for most of the galactose taken up on the first day of culture; it then rapidly decreased to undetectable amounts, while galactose utilization decreased progressively with time (Fig. 6).

2. Insulin binding and degradation. Binding by cells of 3-week rats was the same after 24 and 48 hr (data not shown) of culture but was substantially reduced after 5

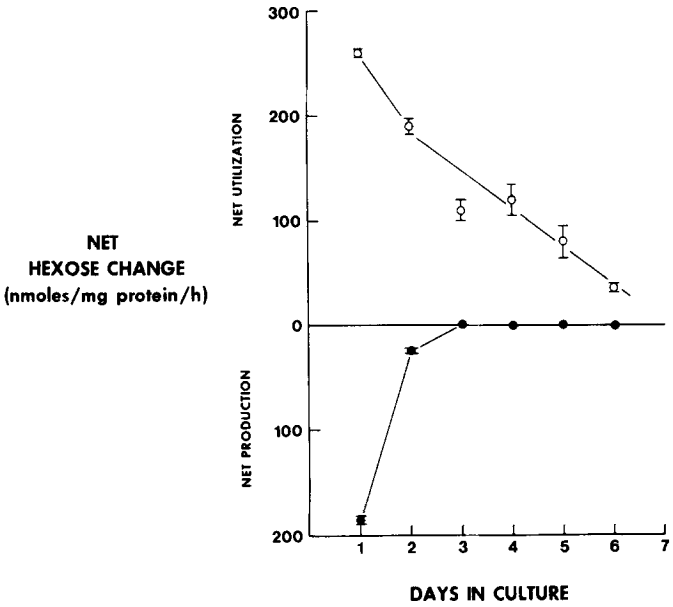

FIG. 6. Galactose utilization (O) and glucose production (●) by hepatocytes in primary culture for 6 days. Medium containing 13 m*M* galactose and no glucose was changed daily. Cell donor was 3 weeks old.

days in culture (Fig. 7A); the degradation rate was considerably increased after 5 days (Fig. 7B).

DISCUSSION

Primary cultures offer a number of advantages over other preparations for the study of hepatocyte function: they permit recovery from the plasma membrane damage resulting from the preparation of isolated hepatocytes and they allow longer periods of study than are possible with the isolated perfused liver.

In primary culture the cells do not proliferate and they maintain liver-specific functions for periods up to 1 week or more (Lin and Snodgrass, 1975; Tanaka *et al.*, 1978; Bissell *et al.*, 1973; Bissell and Guzelian, 1975). In the present work the cells, after 6 days in culture, had begun to lose their polygonal shape, but retained their ability to synthesize serum albumin (data not shown).

The changes in hexose utilization with age of donor confirm in cultured hepatocytes the results previously obtained with the isolated perfused liver: a three-fold higher rate of galactose utilization and a higher rate of conversion of galactose to glucose in the cells of weanling rats than in those of adult rats (Sparks *et al.*, 1976; Berman *et al.*, 1978). Since the high uptake of galactose suppresses glucose production from other sources (Berman *et al.*, 1979), it may be assumed that most of the glucose released into the medium was derived from galactose and thus accounted for about 80% of the galactose taken up; this value is very close to the one determined by isotope exchange from galactose to glucose (Berman *et al.*, 1979) in perfused liver.

It is also evident that with increasing age of donor, as galactose utilization decreased glucose utilization increased. Comparable changes occurred with increasing age of cells in culture, even in the presence of a continuing high concentration of galactose in the medium and in the absence of glucose as an alternative substrate. It would therefore appear that the age-dependent decrease in ability to metabolize a galactose load *in vivo* is not due to prior change in dietary hexose content but to an intrinsically determined change in enzyme pattern, perhaps mediated by changes in the hormonal milieu.

The cultured hepatocytes were very sensitive to insulin, showing a definite response to concentrations within the physiological range of plasma insulin and a maximal response within the range found in the portal vein plasma following a glucose load (Blackard and Nelson, 1970). It has been demonstrated that the presence of a glucocorticoid is essential to permit the development of glycogen synthetase activity (Plas *et al.*, 1973) and glucogenesis from galactose (Simkins *et al.*, 1978) in primary cultures of fetal hepatocytes; similarly, in the present experiments it was necessary to have glucocorticoid, even at a concentration roughly equivalent to that of plasma cortisol, to maintain this enzyme activity, since in the absence of the corticoid we observed a low rate of incorporation of glucose into glycogen and no increase with insulin even at concentrations up to 15 mU/ml (data not shown).

Despite the age-related increase in glucose utilization the responsiveness of the glycogenic pathway to insulin

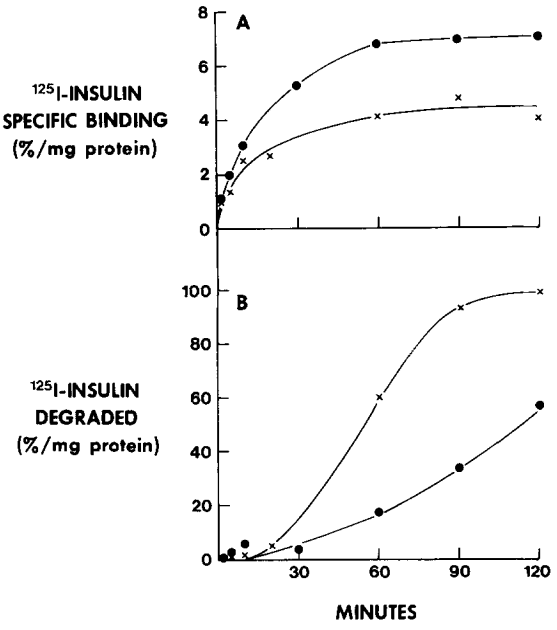

FIG. 7. Time course of insulin binding (A) and degradation (B) by hepatocytes isolated from 3-week rats and maintained in culture for 1 (●) and 5 (×) days. Values have been corrected for nonspecific binding.

decreased; this was not associated with a decrease in insulin binding capacity of the cells but coincided with an increase in the initial rate of insulin degradation. Changes in the activity of insulin-responsive pathways, independent of changes in the number or affinity of insulin receptors, have previously been demonstrated in hepatocytes (Davidson and Kaplan, 1977) and thymocytes (Goldfine, 1975). In the present work the decrease in the response of the glycogen pathway may have been a reflection of a shift in hepatic function away from simple carbohydrate storage to lipogenesis and other synthetic processes or of more rapid insulin degradation. A similar change in insulin degradation occurred with longer life in culture.

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