Glucose Regulation of Glucose Transporters in Cultured Adult and Fetal Hepatocytes

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GLUT2 is the major glucose transporter of adult hepatocytes. In vivo, membrane GLUT1 is localized to a ring of perivenous cells and increases slightly after fasting or insulin deprivation. GLUT1 also increases in vitro after prolonged culture of isolated adult hepatocytes. We have previously shown that GLUT1 mRNA, protein, and activity are present in the rat fetal hepatocyte, and that both GLUT1 and GLUT2 are important for the pattern of glucose transport in the fetal hepatocyte. We tested the hypothesis that the postnatal increase in circulating glucose is one of the regulators of the changed pattern of GLUT1 and GLUT2 in the hepatocyte after the fetal to neonatal transition. Fetal and adult rat hepatocytes were cultured for 45 hours in supplemented Dulbecco's modified Eagle's medium at glucose concentrations of 1, 8.3, or 30 mmol/L. Culture at 8.3 and 30 mmol/L glucose diminished GLUT1 mRNA as compared with culture in 1 mmol/L glucose in both fetal and adult hepatocytes ($P < .05$), but GLUT1 mRNA levels were lower in adult versus fetal hepatocyte cultures at 8.3 and 30 mmol/L ($P < .05$). Similarly, GLUT1 protein levels were significantly diminished in hepatocytes cultured at higher medium glucose ($P < .05$ for fetal cells at 30 *v* 1 mmol/L; $P < .05$ for adult cells at 8.3 and 30 *v* 1 mmol/L). GLUT2 mRNA abundance was enhanced by medium glucose in adult hepatocytes ($P < .05$ at 8.3 and 30 *v* 1 mmol/L) and was unchanged by medium glucose in fetal hepatocytes. In contrast, GLUT2 protein level was unchanged by medium glucose in adult hepatocytes, and was diminished at 30 mmol/L as compared with 1 mmol/L glucose in fetal hepatocytes ($P < .05$). In confirmation of these findings, uptake of 2-deoxyglucose (2-DOG) by fetal hepatocytes was significantly diminished after culture in 8.3 or 30 mmol/L glucose versus 1 mmol/L glucose ($P < .05$ and $<.01$, respectively). These studies confirm that the fetal hepatocyte glucose transporter pattern could be maintained in part by low fetal portal glucose levels. However, the resistance of the fetal hepatocyte glucose transporter pattern as compared with that of the adult hepatocyte to the effects of hyperglycemia suggests additional undefined control mechanisms.

TRANSPORT OF GLUCOSE across the liver cell membrane does not appear to be rate-limiting for glucose metabolism.[1] Nonetheless, transport capacity of the hepatocyte is developmentally regulated in the rat and contributes to changes in glucose metabolic capacity from fetal to extrauterine life. The major glucose transporter of the adult rat hepatocyte is GLUT2, a high-K_m glucose transporter that facilitates glucose exchange in a cell bathed intermittently in high-glucose portal blood. GLUT1 expression in the adult hepatocyte is limited to a few perivenous cells.[2] GLUT1 protein increases in vivo in rat liver only during fasting or insulin deficiency.[3,4] GLUT1 mRNA expression also increases when isolated adult hepatocytes are placed in culture.[5] In contrast, in a previous study we have found GLUT1 mRNA in large quantity in freshly isolated fetal hepatocytes, and increased GLUT1 mRNA when these hepatocytes are cultured. Moreover, the remarkable capacity for glucose transport that we identified in cultured fetal hepatocytes correlated with the presence of abundant GLUT1 and GLUT2 protein.[1]

During the fetal to neonatal transition, there is a shift from abundant GLUT1 in the hepatocyte to an adult pattern.[6,7] Fetal hepatocytes are normally exposed to a circulating glucose concentration that is two thirds the maternal arterial glucose concentration. Because GLUT1 mRNA is regulated by glucose in some cultured cell lines (LLC-PK1 cells,[8] fibroblasts,[9] and rat brain glial cells[10]), we postulated that the increase in portal glucose that follows delivery might be one of the regulatory factors controlling the loss of GLUT1 and conversion of the fetal hepatocyte to the adult hepatocyte glucose transporter pattern. We therefore examined the effect of glucose concentration over 45 hours in culture on GLUT1 and GLUT2 mRNA and protein and on glucose transport in fetal hepatocytes, and compared glucose transporter mRNA and protein levels with those of adult hepatocytes exposed to similar culture conditions.

MATERIALS AND METHODS

Hepatocyte Isolation and Culture

Hepatocytes were isolated from Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). All laboratory animal studies were conducted humanely according to National Institutes of Health guidelines for the use and care of laboratory animals, and were approved by the Institutional Committee on Research. Pregnant rats at 20 days' gestation and male rats weighing 160 to 200 g were anesthetized with ketamine/xylazine. Fetal rat hepatocytes were isolated using the method reported by Freemark and Handwerger,[11] and adult male rat hepatocytes were isolated using standard collagenase perfusion techniques.[12] Isolation and primary culture were as previously described.[13] Cells (0.5×10^6 for adult and 1.5×10^6 for fetal per 35-mm dish) were cultured in 1.5 mL Dulbecco's modified Eagle's medium (D5030; Sigma, St Louis, MO) supplemented with 0.2 mmol/L glutamine, aspartate, and serine, 0.1 mmol/L pyruvate, 10 μmol/L cortisol, 1 μmol/L porcine

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insulin, 50 μg/mL gentamycin, 5 μg/mL amphotericin B, 10% fetal bovine serum, and 1, 8.3, or 30 mmol/L glucose as designated.

RNA Extraction and mRNA Quantitation

RNA was extracted from cells after 45 hours of culture, using the method reported by Cathala et al.[14] Total RNA samples (18 μg) were denatured at 75°C for 5 minutes, separated by electrophoresis on 1% formaldehyde agarose gels, transferred to Hybond membranes (Amersham, Arlington Heights, IL), and ultraviolet cross-linked. Rat brain RNA (5 μg) and adult rat liver RNA (18 μg) were used as positive controls for GLUT1 and GLUT2, respectively. Rat GLUT1 cDNA (kindly supplied by Dr. Morris Birnbaum) and GLUT2 cDNA (kindly supplied by Dr Bernard Thorens) were used to prepare ^{32}P end-labeled probes (Quikprime; Pharmacia, Piscataway, NJ) for Northern blots.[15,16] The GLUT1 cDNA probe was a 1.65-kb *Eco*RI/*Sac*I fragment and the GLUT2 cDNA probe was a 0.9-kb 5′ fragment corresponding to the coding region of the gene. The probe was denatured and hybridized with the RNA blots overnight at 42°C. Blots were washed at high stringency at 65°C with a solution of 15 mmol/L sodium chloride and 1.5 mmol/L sodium citrate, pH 7. After washing, membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) using an intensifying screen at −80°C. Specific mRNAs for GLUT1 and GLUT2 were quantified by scanning densitometry. Adequacy of transfer of RNA was assessed by quantitation of total RNA using ethidium bromide staining, as used previously.[17] Normalization to tubulin or actin is not possible in cultured hepatocytes because these are not stable during culture. The Zeineh Videophoresis II program (BioMed Instruments, Fullerton, CA) on an Apple IIe computer (Cupertino, CA) was used to determine total integrated spot intensities. Linearity was assessed with a densitometry curve. Results of densitometry are expressed as relative intensity compared with zero-time fetal samples run on the same blot and designated 100.

Quantitation of GLUT1 and GLUT2 Protein

Cells (5 to 10 × 10^6) were homogenized, and the homogenates were centrifuged at 2,800 × *g*. Membrane proteins in the supernatant were collected by ultracentrifugation at 120,000 × *g*. Proteins were precipitated and resuspended, and 50 μg protein was electrophoresed on sodium dodecyl sulfate gel before transfer to a polyvinylidene fluoride membrane (PVDF-Plus; Micron Separations, Westboro, MA).[5,18] GLUT1 and GLUT2 protein were then estimated by Western blot analysis using specific rabbit antibodies (1:5,000 dilution) raised against synthetic carboxy-terminal peptides of the glucose transporters (East-Acres Biologicals, Southbridge, MA) and an enhanced chemiluminescence technique (ECL; Tropix, Bedford, MA). After confirming linearity of the densitometric reading, densitometry was used to compare each blot with zero-time fetal samples, designated 100.

Measurement of Glucose Analog Uptake

Uptake of 2-deoxyglucose (2-DOG) was assessed at room temperature in fetal hepatocytes cultured with Dulbecco's modified Eagle's medium and 1, 8.3, or 30 mmol/L glucose for 45 hours. After cells were washed three times in glucose-free medium, uptake was initiated by adding 0.5 mL medium containing ^{14}C-2-DOG at a concentration of 0.25 mmol/L (0.2 μCi/mL). Uptake was terminated at 10 seconds with 0.75 mL iced stop solution (160 mmol/L NaCl, 0.2 mmol/L $HgCl_2$, 0.8 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, and 5 mmol/L KCl). After washing four times with stop solution, cells were solubilized in 0.5 mL 0.2N NaOH, and an aliquot was taken for determination of protein and 2-DOG uptake (liquid scintillation counting). Data were corrected for extracellular trapping of 2-DOG determined on appropriate controls in which stop solution was added before uptake medium.[1] Uptake was linear to 30 seconds.

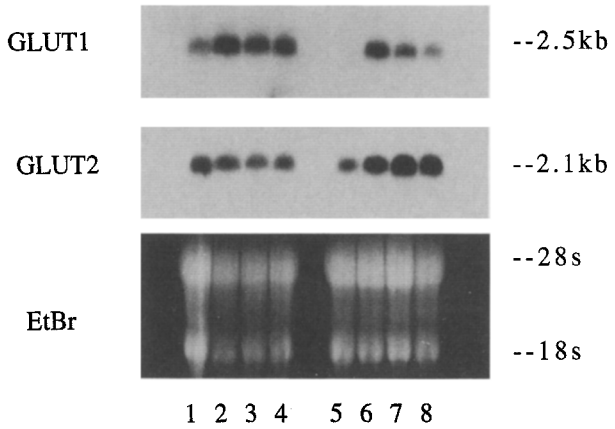

Fig 1. Representative Northern blot of GLUT1 mRNA and GLUT2 mRNA in fetal and adult hepatocytes at isolation (0) and cultured at 1, 8.3, and 30 mmol/L medium glucose for 45 hours. The ethidium bromide (EtBr)-stained ribosomal band is presented for comparison. Lanes 1 to 4, mRNAs from fetal hepatocytes at isolation and cultured in 1, 8 and 30 mmol/L glucose, respectively. Lanes 5 to 8, mRNAs from adult hepatocytes at isolation and cultured at 1, 8, and 30 mmol/L glucose, respectively.

Protein Determination

Protein for Western blot and transport studies was assayed using the Bradford dye method and bovine serum albumin as standard (BioRad, Hercules, CA).

Statistical Analysis

All data are expressed as the mean ± SEM. Logarithmic transformation was used as necessary to normalize values before analysis. ANOVA with post-hoc Tukey test was used for comparisons.

RESULTS

Culture for 45 hours in 8.3 mmol/L glucose diminished GLUT1 mRNA concentration in both fetal and adult hepatocytes as compared with culture in 1 mmol/L glucose (Figs 1 and 2). However, GLUT1 mRNA levels were lower in adult versus fetal hepatocyte cultures at 8.3 and 30 mmol/L ($P < .05$ and $P < .02$, respectively). GLUT2 mRNA abundance was enhanced by medium glucose at 8.3 or 30 mmol/L in adult hepatocytes ($P < .05$ *v* 1 mmol/L) and remained constant in fetal hepatocytes (Figs 1 and 2). Glucose transporter mRNA is normalized to total cellular RNA. There were no systematic differences in total RNA recovery from cultures maintained at the different glucose levels; therefore, changes in mRNA likely reflect changes in the quantity of glucose transporter transcript per cell, rather than an alteration in total RNA production per cell.

Changes in levels of GLUT1 protein in cells cultured at different concentrations of medium glucose corresponded in direction to changes in abundance of mRNA (Figs 3 and 4). GLUT1 protein levels were diminished in fetal cells cultured in 8.3 and 30 mmol/L glucose, and this difference was significant ($P < .05$) at 30 mmol/L. GLUT1 protein

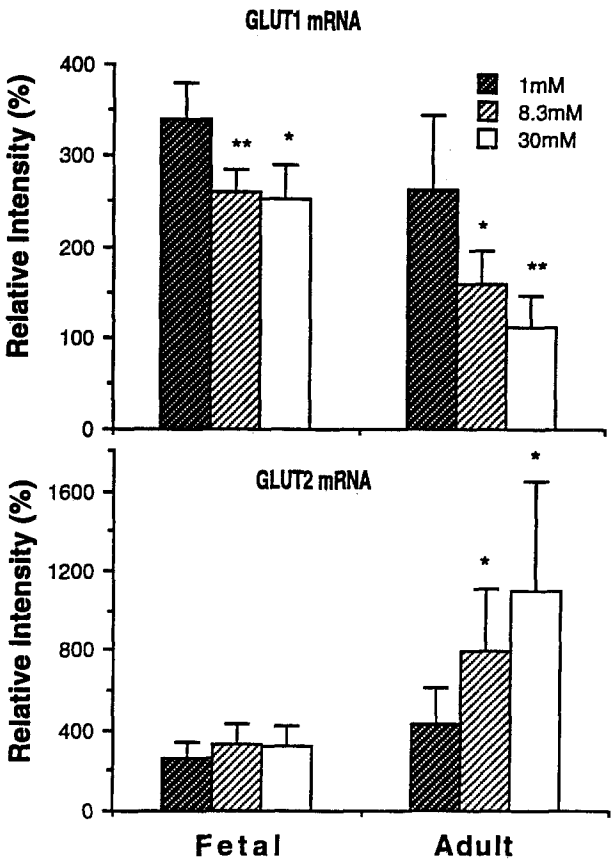

Fig 2. Relative abundance of GLUT1 and GLUT2 mRNA (Northern blot) in fetal (n = 6) and adult (n = 6) hepatocyte preparations (mean ± SEM) plotted as relative intensity compared with abundance in freshly isolated fetal hepatocytes (100 U relative intensity). GLUT1 mRNA expression is decreased in fetal and adult hepatocytes exposed to 8.3 or 30 mmol/L medium glucose. GLUT2 mRNA expression remains constant in fetal hepatocytes and is elevated by medium glucose in adult hepatocytes. *$P < .05$, **$P < .01$.

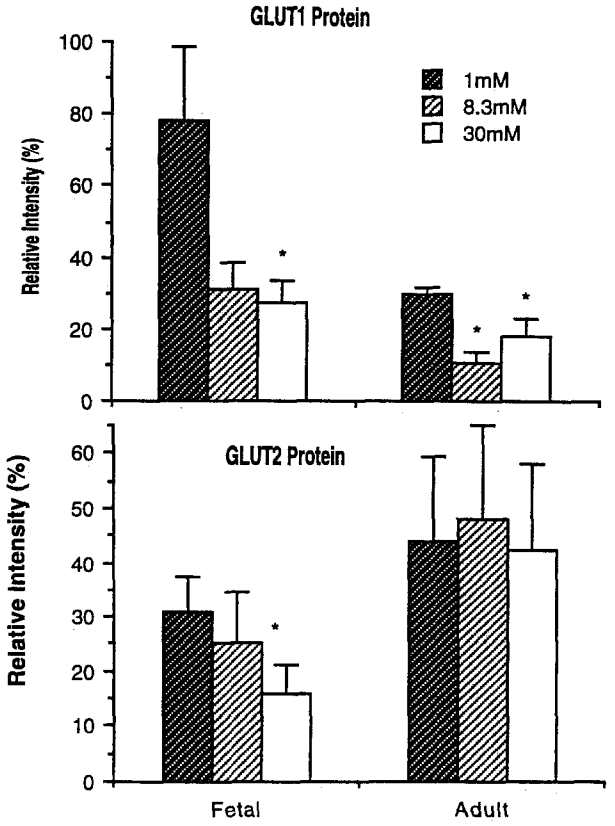

Fig 4. Relative abundance of GLUT1 and GLUT2 protein (Western blot) in fetal (n = 4) and adult (n = 4) hepatocyte preparations (mean ± SEM) at 45 hours' culture plotted as relative intensity compared with abundance in freshly isolated fetal hepatocytes (100 U relative intensity). GLUT1 protein is decreased in cells exposed to 8.3 or 30 mmol/L medium glucose. GLUT2 protein remains constant in adult hepatocytes and decreases in fetal hepatocytes cultured at 30 mmol/L medium glucose. *$P < .05$.

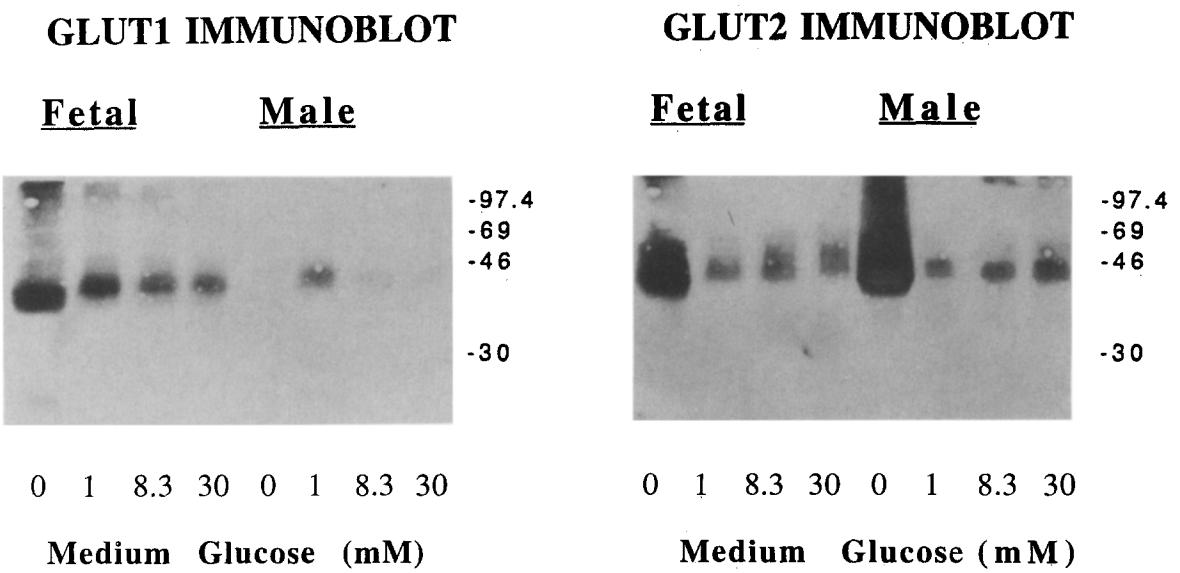

Fig 3. Representative Western blot of GLUT1 and GLUT2 protein in fetal and adult hepatocytes at isolation (0) and cultured for 45 hours at 1, 8.3, or 30 mmol/L medium glucose. Each blot represents 50 μg membrane protein.

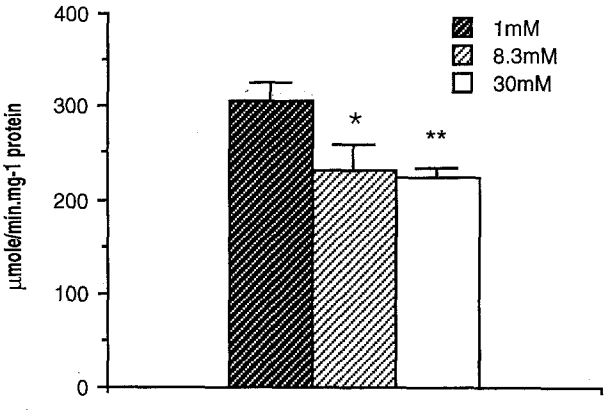

Fig 5. 2-DOG transport activity at 10 seconds measured at 0.25 mmol/L 2-DOG in fetal hepatocytes (n = 4) cultured at 1, 8.3, or 30 mmol/L medium glucose for 45 hours. Transport activity is decreased in cells cultured at higher medium glucose. *$P < .05$, **$P < .01$.

levels in the adult hepatocyte were significantly lower at 8.3 and 30 mmol/L medium glucose versus 1 mmol/L glucose ($P < .05$). In contrast, GLUT2 protein levels did not correlate with mRNA abundance (Figs 3 and 4). In fetal hepatocytes, GLUT2 protein level was significantly decreased in cells cultured at 30 mmol/L glucose versus 1 mmol/L glucose ($P < .05$), although GLUT2 mRNA remained constant. In adult hepatocytes, despite increased mRNA abundance in cells cultured in higher glucose concentrations, GLUT2 protein levels remained constant. GLUT1 protein levels were higher in fetal hepatocytes as compared with adult hepatocytes cultured in 1 and 8.3 mmol/L glucose but not 30 mmol/L glucose. GLUT2 protein levels in fetal and adult hepatocytes were not significantly different at any of the culture medium glucose concentrations. Glucose transporter protein was normalized to total membrane protein. Like total cellular RNA, there was no systematic difference in recovery of total membrane protein from cultures grown at the different medium glucose concentrations. This largely excludes the possibility that changes in glucose transporter protein are an artifact of the normalization method.

To correlate glucose transporter protein abundance with glucose transporter activity, we measured 2-DOG uptake in fetal hepatocytes cultured in 1, 8.3, and 30 mmol/L glucose for 45 hours (Fig 5). Uptake of 0.25 mmol/L 2-DOG at 10 seconds was decreased in cells cultured at 8.3 and 30 mmol/L versus 1 mmol/L glucose ($P < .05$ and $< .01$, respectively).

DISCUSSION

The metabolic adaptation to postnatal life is the result of remarkable changes in various hepatic enzymes and other proteins.[19,20] The glucose transporters of fetal liver are among those proteins that change dramatically within a day of birth in the rat. GLUT1 is found in large quantity in rat fetal liver and rat fetal hepatocytes. Hepatic levels of this relatively ubiquitous glucose transporter decrease precipitously after birth. GLUT1 mRNA can be detected in neonatal liver in greater abundance than in adult liver up to day 3 after birth. GLUT1 protein is not detected after 24 hours of postnatal life.[6,7] GLUT2 mRNA, on the other hand, increases during fetal and neonatal life. GLUT2 protein concentration increases markedly in the first few days after birth and increases again after weaning from high-fat maternal milk.[6] This pattern is likely found in other animal species as well. Chundu and Devaskar[21] reported a shift from a high-affinity to a lower-affinity glucose transporter in rabbit liver during the fetal to neonatal transition.

Although an altered hormonal or substrate milieu is often implicated etiologically in the metabolic maturation associated with birth, the mechanism of many of these changes is still unknown. In the rat, the transition from fetal life to life ex utero is accompanied by a change from placentally derived hepatic energy sources to high-fat maternal milk. Despite the relatively low carbohydrate content of rat milk, there is a postnatal increase in circulating glucose. We have shown that peripheral glucose values increase by 4 hours after birth to as high as 8 mmol/L.[22] Portal glucose values in the fed neonatal rat are undefined. High-carbohydrate feedings lead to precocious induction of developmentally regulated proteins such as glucokinase and malic enzyme.[6,23] Because GLUT1 mRNA and protein are diminished in other cell systems cultured in the presence of high glucose, we postulated that hepatocyte exposure to higher glucose levels after birth was one of the triggers of the change in glucose transporter pattern at the fetal to neonatal transition.

To separate out the many changes of the fetal to neonatal transition, we have used a model of cultured fetal hepatocytes, studied after 2 days in culture, that we have previously demonstrated to display at 48 hours, glucose transporter characteristics similar to those of fetal life. To intensify measured differences in glucose transporter response in culture, we have chosen to study hepatocytes cultured in 1 mmol/L glucose as compared with 8 and 30 mmol/L glucose; 1 mmol/L glucose is a low concentration for both fetal and adult hepatocytes, 8 mmol/L is a high concentration for the fetal hepatocyte and normal for the adult hepatocyte, and 30 mmol/L is a high concentration for both fetal and adult hepatocytes. The use of a cultured hepatocyte system standardizes the hormonal or substrate milieu, but introduces issues related to culture-induced alterations in hepatocyte function. Our previous studies have demonstrated some changes in expression of GLUT1 and GLUT2 mRNA and protein in fetal and adult hepatocytes over a 45-hour period in culture at 1 mmol/L glucose. However, GLUT1 and GLUT2 expression, like expression of other hepatocyte-specific genes, is measurable through at least 72 hours in culture. Therefore, studies performed using the cultured fetal hepatocyte complement in vivo studies and can offer insight into factors leading to developmental changes in gene expression.

We show in this study, in contrast to our previously published data, that GLUT2 protein levels are similar in fetal and adult hepatocytes after 45 hours in culture. The previous study showed elevated GLUT2 protein levels in

fetal hepatocytes as compared with adult hepatocytes after 45 hours in culture, but not after shorter periods of culture. The previous finding correlated with an increased maximal rate for 3-*O*-methylglucose transport at a K_m typical for GLUT2 (Eadie-Hofstee plot) in fetal cells. In this report, we could not replicate increased GLUT2 protein levels in fetal hepatocytes after 45 hours' culture. A repeat Eadie-Hofstee plot of 3-*O*-methylglucose uptake using cells cultured for this study (not shown) similarly did not show an increased maximal transport rate for GLUT2, confirming the different behavior of hepatocytes in this study as compared with the previous report. We cannot explain this finding. It is possible that the change is due to a minor temporal alteration in reagent or cell-plating density. All other data are congruent with those previously published.

We now report that glucose is capable of regulating GLUT1 mRNA and protein abundance. Moreover, we show that culture of fetal hepatocytes in high medium glucose reduces transporter activity for low concentrations of 2-DOG. Glucose transporter activity measured in the linear range for 2-DOG uptake was diminished in cells cultured at 8.3 and 30 mmol/L glucose. This is not likely a reflection of changes in phosphorylation, because phosphorylation is not complete at 10 seconds in the fetal hepatocyte.[1]

However, glucose-induced diminution of GLUT1 mRNA and protein abundance was greater in adult versus fetal hepatocytes. This suggests that other metabolic, endocrine, or paracrine mechanisms contribute to the shift in hepatocyte GLUT1 levels in the postnatal period.

Similarly, we investigated whether increased medium glucose could induce increased abundance of GLUT2, as is noted in the immediate postnatal period and at weaning. Our finding that adult rat hepatocyte GLUT2 mRNA levels increased following exposure to high glucose replicates the findings of others. GLUT2 mRNA and protein concentrations in adult liver are decreased in vivo by hyperinsulinemia and are increased by refeeding after fasting, hyperglycemia, or diabetes.[3,24-27] Changes in GLUT2 protein concentration require prolonged exposure, suggesting that GLUT2 protein has a long half-life.[28] In vitro in the isolated cultured adult rat hepatocyte, insulin variably diminishes or does not alter GLUT2 mRNA, whereas glucose, as well as other metabolizable sugars, increases GLUT2 mRNA abundance.[28,29] GLUT2 protein levels have not been shown to change in response to insulin under short-term culture conditions.[28] The lack of GLUT2 protein response to culture of adult and fetal hepatocytes in high medium glucose has not been previously described. Failure of change of GLUT2 protein levels in adult hepatocytes may be attributed to a relatively long half-life of GLUT2 protein. Alternatively, high medium glucose may both increase GLUT2 mRNA abundance and increase GLUT2 protein turnover, so that there is no discernible net effect on protein quantity in these studies.

The lack of significant change in GLUT2 mRNA abundance in fetal hepatocytes exposed to high medium glucose and the small decrease in GLUT2 protein after such treatment suggest that higher blood glucose is not the explanation for increasing GLUT2 with age in the postnatal rat. This is perhaps not surprising, because the change in GLUT2 levels in the postnatal period are gradual and do not demonstrate the remarkable and rapid shift noted for GLUT1. Moreover, the increase in GLUT2 begins in the late prenatal period in the rat. The decrease in GLUT2 protein after glucose exposure may suggest posttranslational control by glucose, as we have discussed. The discordant increase in GLUT2 protein and decrease in GLUT2 mRNA in 45-hour cultured cells as compared with freshly isolated cells may also reflect posttranscriptional regulation, as previously noted by this group[1] and others.

The mechanism of changes in glucose transporter transcript abundance in cultured hepatocytes in response to medium glucose remains to be defined. Two metabolic pathways have been suggested as control mechanisms for glucose-modulated levels of glucose transporter mRNA. In cultured rat pancreatic islets, inhibition of glycolysis by mannoheptulose prevents the stimulatory effect of glucose on GLUT2 mRNA and protein levels. Nonmetabolizable sugars such as 2-DOG have no stimulatory effect on GLUT2 mRNA. Tunicamycin, an inhibitor of *N*-glycosidation, has no effect on levels of GLUT2 mRNA following glucose stimulation. These studies suggest that in the β cell, another of the few tissues expressing GLUT2, this glucose transporter may be regulated through the glycolytic pathway.[30] In contrast, in L6 myocytes, inhibition of the hexosamine pathway to protein glycosylation with various agents leads to upregulation of GLUT1 mRNA and protein levels.[31] Moreover, in isolated cultured adipocytes, glucose-induced desensitization of glucose transport is mediated through the same pathway. It is unclear whether desensitization involves GLUT1, GLUT4, or both adipocyte transporters.[32] However, in other cell types, membrane GLUT1 protein is diminished by tunicamycin treatment. Whether this effect is at the level of GLUT1 gene expression or protein translocation has not been delineated.[9,33-35] We speculate that both the glycolytic and hexosamine pathways would be regulated differently in fetal versus adult hepatocytes. In fetal hepatocytes, the glycolytic pathway would be modified by the presence of hexokinase (enhanced capacity for phosphorylation at low glucose), as compared with glucokinase in adult hepatocytes. The fetal hexosamine pathway would be altered by hexokinase and the very active glutamine transport system of the fetal hepatocyte.[36] Both substrates for the rate-limiting enzyme of this pathway (glutamine:fructose-6-phosphate amidotransferase) would likely be much increased in fetal liver cells.[32]

The pathway of such glucose regulation remains to be determined, as does its mechanism. Some hepatic genes are known to be under transcriptional control by carbohydrate.[37] However, it is unclear whether the alterations in glucose transporter mRNA are transcriptional or related to changes in mRNA stability. Future studies should elucidate these mechanisms.

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