Fetal Programming of Perivenous Glucose Uptake Reveals a Regulatory Mechanism Governing Hepatic Glucose Output During Refeeding


Increased hepatic gluconeogenesis maintains glycemia during fasting and has been considered responsible for elevated hepatic glucose output in type 2 diabetes. Glucose derived periportally via gluconeogenesis is partially taken up perivenously in perfused liver but not in adult rats whose mothers were protein-restricted during gestation (MLP rats)—an environmental model of fetal programming of adult glucose intolerance exhibiting diminished perivenous glucokinase (GK) activity. We now show that perivenous glucose uptake rises with increasing glucose concentration (0–8 mmol/l) in control but not MLP liver, indicating that GK is flux-generating. The data demonstrate that acute control of hepatic glucose output is principally achieved by increasing perivenous glucose uptake, with rising glucose concentration during refeeding, rather than by down-regulation of gluconeogenesis, which occurs in different hepatocytes. Consistent with these observations, gluconeogenesis in vivo commenced in the perivenous cells during refeeding, MLP livers accumulating less glycogen than controls. GK gene transcription was unchanged in MLP liver, the data supporting a recently proposed postranscriptional model of GK regulation involving nuclear-cytoplasmic transport. The results are pertinent to impaired regulation of hepatic glucose output in type 2 diabetes, which could arise from diminished GK-mediated glucose uptake rather than increased gluconeogenesis. Diabetes 52:1326–1332, 2003

The precise mechanism(s) by which the liver normally switches rapidly from glucose production during fasting to glucose uptake and gluconeogenesis after refeeding or a glucose load remain unclear. Net hepatic glycolysis is promptly curtailed during glucose infusion, but gluconeogenesis is not decreased (1). These and other observations (2) have contributed to the view that the liver is mainly synthesized via an “indirect” pathway during the early postprandial phase, in which gluconeogenic glucose-6-phosphate (G6P) is diverted to glycogen (3,4), rather than via the “direct” pathway from glucose to glycogen. Experiments in humans and in rats have demonstrated that if insulin is maintained at constant levels but glycemia is increased by glucose administration, then hepatic glucose output is rapidly suppressed and hepatic glucose uptake rises (1,5–8; postprandial hyperglycemia in type 2 diabetes is partly caused by failure of this mechanism. Hyperglycemia per se has no significant effect on hepatic gluconeogenic flux (1), and refeeding does not suppress rat liver G6P (9) in the time frame in which glucose production is switched off in individuals without diabetes (6,7). These observations raise the problem of how the balance between glycogen synthesis, gluconeogenesis, and glucose output is regulated from a generally assumed common pool of G6P.

However, none of these studies takes account of metabolic heterogeneity along the radius of the hepatic lobule. If glucose output and uptake occurred in different hepatocytes, then a relatively simple explanation as to how hepatic glucose output is controlled could be achieved. We have reexamined the pathways of glycogen synthesis during refeeding in starved rats, mapping glucose metabolism (10) and glycogen formation within the hepatic lobule in intact liver. Using this technique, we have previously shown that in starved rats, gluconeogenesis is confined to periportal cells (70–80% of lobular volume) (10,11), whereas in the perivenous cells, where glucokinase (GK) activity was most concentrated (12,13), uptake of glucose occurs (12,14).

Low birth weight predisposes to increased risk of type 2 diabetes in humans (15). In the maternal low protein (MLP) model of fetal programming of glucose intolerance (16), GK activity is deficient in the perivenous cells (12). From the age of 12 months, glucose tolerance declines in
MLP rats faster than in controls (16). Adult MLP livers exhibited increased hepatic glucose output on perfusion with lactate, primarily caused by decreased perivenous glucose uptake (PVGU) (12). We hypothesized that if PVGU were normally mediated by GK, then it might increase substantially as blood glucose increased, because of the kinetics of this enzyme (half-saturated at $S_{0.5}$ at 8 mmol/l glucose). GK-mediated PVGU would then make a potentially important contribution to net hepatic glucose balance, but this might be diminished in MLP liver. Furthermore, the relevance of PVGU at physiological glucose concentrations needed to be established because in our previous work (12), the concentration of glucose reaching the perivenous cells was <1 mmol/l, derived entirely from portoportal gluconeogenesis. We have examined the distribution of glycogen formation during early refeeding, because the most simple consequence of the indirect pathway would be that glycogen synthesis commenced in the gluconeogenic perportal cells. The current and previous studies suggested that failure of PVGU in MLP liver was caused by perivenous depletion of GK, and we have explored the question of whether GK gene expression in MLP liver is attenuated during refeeding.

RESEARCH DESIGN AND METHODS

Animals. The feeding regimens for the control and MLP rats were as previously described (12); briefly, pregnant dams were fed ad libitum 20% (control) or 8% (MLP) protein diets during pregnancy and lactation. After weaning, control and MLP offspring received identical RM1-chow (SDS, Witham, Essex, U.K.). In each of the studies, male and female control and MLP animals were studied as adults in a blocked design such that comparisons between type of animal, age, and sex could be made. The total number of animals studied was 90 controls (age 8.5 ± 0.4 months) and 100 MLPs (age 9.1 ± 0.4 months). No significant differences were found between age or sex, and data were pooled for analysis between control and MLP for each study. All experiments on animals were conducted under ethically reviewed Government License (Scientific Procedures [Animals] Act 1986, U.K.).

Liver perfusion. Rats were studied at a mean age of 7.4 ± 0.4 (n = 24) and 8.0 ± 0.5 (n = 24) months for control and MLP, respectively (NS). Nonrepressing isolated liver perfusions were established (14, 17) from 48-h fasted (19). GK activity. Perivenous glucose uptake was calculated by difference from hepatic glucose output/uptake was calculated by the Fick principle. Perchloric acid mixing with 4% perchloric acid. All samples were stored at −80°C until C until 0.92 ± 0.12 (mean ± SE; n = 7; P > 0.3), demonstrating stability of glycogen during mapping. GK gene expression. Rats were studied in two age groups for both Northern and in situ hybridization, 4–6 months and 12–17 months of age. No differences were found between age groups, and the data were pooled for comparison between control and MLP. For Norms, mean age was 11.9 ± 2.3 (n = 6) and 10.6 ± 2.1 (n = 6) months (control and MLPs, respectively, NS). Total RNA was prepared from rat liver tissues using QIAgen RNAeasy protease kit according to the manufacturer’s protocol. Five micrograms of total RNA/lane was denatured with glyoxal/dMSO, run on 1% agarose gel, and transferred to a nylon membrane as described in Northern Max-Gly protocol (Ambion). Blots were probed with a GK exon nine probe generated by PCR and labeled with [32P]dCTP (Amersham) by random priming using NEBuffer kit (BioLabs). The probe was purified by gel filtration on Micro Bio-Spin P-30 column (BioRad). GK transcript levels were determined by comparison with the signals obtained with a β-actin–specific probe using a Kodak ID 3.5 with Kodak ID Image Analysis Software.

For in situ hybridization studies, rats were studied at a mean age of 5.7 ± 0.6 (n = 19) and 6.8 ± 0.8 (n = 20) months for control and MLPs, respectively (NS). Livers and hearts were removed, frozen in liquid nitrogen, and stored at −70°C. Ten-micrometer sections were cut and 100 MLPs (age 6.8 ± 0.8 months) and 12–17 months of age. No differences were found between age groups, and the data were pooled for comparison between control and MLP. For Norms, mean age was 11.9 ± 2.3 (n = 6) and 10.6 ± 2.1 (n = 6) months (control and MLPs, respectively, NS). Total RNA was prepared from rat liver tissues using QIAgen RNAeasy protease kit according to the manufacturer’s protocol. Five micrograms of total RNA/lane was denatured with glyoxal/dMSO, run on 1% agarose gel, and transferred to a nylon membrane as described in Northern Max-Gly protocol (Ambion). Blots were probed with a GK exon nine probe generated by PCR and labeled with [32P]dCTP (Amersham) by random priming using NEBuffer kit (BioLabs). The probe was purified by gel filtration on Micro Bio-Spin P-30 column (BioRad). GK transcript levels were determined by comparison with the signals obtained with a β-actin–specific probe using a Kodak ID 3.5 with Kodak ID Image Analysis Software.

RESULTS

Effect of glucose concentration on perivenous glucose uptake and net hepatic glucose output. Net hepatic glucose output (Fig. 1A) was progressively suppressed as glucose concentration in the perfusate increased (0–8 mmol/l) in control liver. This direct effect of glucose on regulation of hepatic glucose output in control
Hepatic glucose output is controlled by PVGU. A: Dependence of hepatic glucose output (HGO) on glucose concentration in perfused livers of 48-h-fasted control (●) and programmed (MLP) (○) rats. B: Dependence of PVGU on glucose concentration in control and MLP liver (same convention as A). Mean glucose concentrations reaching the perivenous zones (equal to postdigitonin effluent glucose) were 0.15, 2.15, and 8.1 mmol/l (control) and 0.14, 2.17, and 8.14 mmol/l (MLP) when the perfusate concentrations were 0, 2, and 8 mmol/l, respectively. Unpaired t tests, control versus MLP: *P < 0.05, **P < 0.01. In A and B, data are means ± SE, and the numbers in parentheses refer to the number of animals studied.

Glucose release from the perivenous zone accounts for most of the decline in net hepatic glucose output in control liver. In contrast, in MLP liver, increase in glucose concentration produced no rise in perivenous glucose uptake. Net glucose release from the perivenous cells (<80% of the lobular volume), as a result of gluconeogenesis from lactate, was not significantly affected by glucose concentration in either control or MLP liver, although a trend to decreased gluconeogenesis was apparent at 8 mmol/l glucose in control liver. In controls, gluconeogenesis was 1.47 ± 0.07 (n = 8), 1.49 ± 0.12 (n = 7), and 0.94 ± 0.38 (n = 9) and in MLP liver was 1.41 ± 0.08 (n = 9), 1.68 ± 0.44 (n = 6), and 1.43 ± 0.26 (n = 9) μmol/min · 100 g rat wt⁻¹ when perfusate glucose was 0, 2, and 8 mmol/l, respectively. The main effect of glucose per se on hepatic glucose output was therefore the result of increased glucose uptake by the perivenous cells as glucose concentration rises, rather than downregulation of gluconeogenesis or periportal glucose release. GK activity was significantly decreased in MLP liver (control 2.16 ± 0.16, n = 38; MLP, 1.54 ± 0.13 units/g liver wt wt, n = 39; P < 0.005).

Glycogen synthesis and intralobular distribution in control and MLP liver. After 2 h of refeeding, hepatic glycogen content was significantly decreased in MLP liver (control 13.6 ± 1.2, n = 50; MLP, 9.8 ± 0.7, n = 50, mg/g liver wet wt; P < 0.01). Figure 2 shows the effect of the degree of retrograde digitonin-based destruction on the glycogen content of the remaining viable liver in control and MLP liver, respectively, expressed as a fraction of the predigitonin content. The dotted diagonal is the theoretical locus of data if glycogen distribution were uniform. It can be seen that in control liver when half of the lobule is ablated (FVR = 0.5), glycogen content is decreased by 80%. After 2 h of refeeding, 80% of liver glycogen is therefore contained within the perivenous zone, confirming the perivenous preponderance of glycogen obtained by PAS staining (Fig. 3) (20).

Labeling of glycogen during refeeding with [1-13C]glucose. There was a strong correlation (r = 0.95; P < 0.001) and virtually identical relationships in control and MLP livers between glycogen concentration at 2 h and the [1,13C] abundance in glucose moiety of glycogen (Fig. 4), despite widely varying rates of glycogen synthesis (from 3 to 45 and 1 to 17 mg/g wet wt, respectively, in control and MLP liver). The intercept on the ordinate is significant, although small, accounted for by residual glycogen present after 48 h of starvation. The mean percentage of [1-13C]glucosyl in glycogen in control and MLP liver was 6.69 and 6.35, respectively. No label was detected in the C2, C5, or C6 in liver glycogen using NMR.

Concentration of GK mRNA and distribution within the liver lobule of refeed control and MLP rats. Northern analysis showed no significant difference in GK mRNA in livers from control and MLP animals 2 h after refeeding from a 48-h fast (Fig. 5A). However, because measurements in whole liver could mask zonal changes, the time course and distribution of GK mRNA appearance during refeeding were examined by in situ hybridization. Figure 5B shows GK mRNA in control and MLP liver after 1, 2, and 4 h of refeeding. In accordance with previous findings (21), GK mRNA was almost undetectable in liver of fasted rats (control or MLP), and lobular distribution of GK mRNA in refed control liver was virtually identical to that reported by Moorman et al. (21). By 1 h, GK mRNA is present in all zones in both control and MLP liver. In controls, a signif-
DISCUSSION

Key findings of the present study are 1) glucose concentration per se can regulate the net output of glucose from the perfused livers of normal animals within the physiological range of glucose concentration; 2) suppression of glucose output with rising perfusate glucose is principally achieved by increased PVGU, controlled by GK; 3) glycogen synthesis during refeeding commences in the perivenous cells, which are deficient in gluconeogenic function but which readily take up glucose; and 4) decreased GK activity in MLP liver is not the result of impaired GK gene transcription during refeeding.

The positive dependence in control rats of PVGU on glucose concentrations in the range 0.15–8.1 nmol/l strongly suggests that GK (S0.5 ~8 nmol/l) is the key enzyme in this process. This conclusion is reinforced by the failure of PVGU in MLP liver throughout the perfusate glucose concentrations used, because the perivenous cells in MLP liver are GK-deficient (12). It is noteworthy that mice with one disrupted GK allele exhibit markedly decreased GK activity and failure of hyperglycemic suppression of net hepatic glucose production (22). Rossetti et al. (1) demonstrated that in normal 6-h fasted rats, acute hyperglycemia (but low insulin) caused marked inhibition of net hepatic glucose production through increased uptake of plasma glucose and inhibition of glycogenolysis but without significant effect on gluconeogenesis. PVGU mediated by GK could be responsible for such observations of glucose-dependent glucose uptake in vivo, reinforcing the importance of hepatic GK to glucose regulation (23). The suppression of net hepatic glucose output by rising perfusate glucose is analogous to the phenomenon of “glucose effectiveness,” whereby increments in glycemia per se increase glucose disposal and decrease net hepatic glucose output in nondiabetic subjects (6,8). Hyperglycemia in type 2 diabetes is partly caused by failure of glucose effectiveness (8). The present observations suggest a mechanism for glucose effectiveness and that the postprandial pathophysiology of type 2 diabetes may be based principally on failure of PVGU rather than on increased gluconeogenesis, although a minor component of the latter cannot be excluded.

One fate of glucose taken up by perivenous cells in fasted rats was glycolysis (14), and we speculated that during refeeding an additional fate might be glycogen. Because PVGU in perfused MLP liver was markedly diminished, we anticipated that glycogen synthesis would also be decreased, and indeed the glycogen content was 28% less in MLP liver after 2 h of refeeding. The quantitative mapping of glycogen distribution using direct chemical analysis confirms the predominantly perivenous distribution of PAS staining at 2 h. Corroboration was necessary because of the differing morphology of glycogen deposits (diffuse) and periportally (granular) (20). The glycogen mapping and PAS staining show that the perivenous zone, which lacks gluconeogenic capability (10), accumulates glycogen approximately four times faster than that of the remainder of the lobule in the first 2 h of refeeding.

Whether hepatic glycogen is synthesized primarily via the direct (G6P via glucose) or indirect pathway (G6P via gluconeogenesis) is pertinent to understanding the bio-

FIG. 3. Liver sections stain for glycogen predominantly in the perivenous zone after 2 h of refeeding fasted rats. A: PAS stain for glycogen in control animals fasted for 48 h and then refed for 2 h. B: Same as A, except in MLP liver. C: PAS-stained liver of control refed for 24 h after a 48-h fast. D: PAS-diastase glycogen digestion in situ of adjacent section to C results in absence of staining of lobular regions, used as a control for C (similar adjacent control sections for A and B gave qualitatively similar results). P, periportal zone; C, perivenous (centrilobular) zone.

FIG. 4. Relationship of glycogen concentration and accumulation of labeled glucose in the diet. Linear relationship between glycogen content and [1-13C]glucosyl labeling in glycogen in control and MLP liver 2 h after start of refeeding with chow containing 10% by weight 99% [1-13C]glucose. , control liver; ○, MLP liver.
chemical mechanism(s) underlying hepatic glucose output (24). In reviewing the evidence in favor of the indirect pathway, McGarry et al. (2) noted that the consequences of hepatocyte heterogeneity were unexplored. Much of the evidence supporting the indirect pathway is based on 1) dilution of specific activity of 3H label in glycogen glycosyls when glycogen is formed from [U-14C, 3-3H]glucose and 2) the randomization of label from [1-14C]glucose among C2, 5, and 6 in the glycosyl moieties of glycogen (2–4). The latter occurs only when the pathway traverses the lactate/pyruvate stage before gluconeogenic conversion into G6P (randomization arising during oxaloacetate ↔ fumarate equilibration). In addition, the administration of 3-mercaptopicolinate (3MP), an inhibitor of phosphoenolpyruvate carboxykinase, a gluconeogenic enzyme, decreases both glycogen synthesis and randomization of label (2, 4). We find that over a wide range of hepatic glycogen formation, the specific labeling of glycogen glycosyls after feeding [1-13C]glucose remains constant in both control and MLP rats. If two pathways had contributed substantially to glycogen synthesis, then this result could have been obtained only if the ratio of the two pathways had not varied with the rate of glycogen synthesis or type of animal, an unlikely situation. The NMR technique detects labeling in C2, 5, and 6 of glucose, which reaches 10% of that in C1; as we did not detect C2, 5, and 6 labeling, a minimum direct pathway contribution of 69% may be calculated, or 64% if randomization is only 80% complete.

These data (glycogen deposition in nongluconeogenic cells and uniformity of labeling) are consistent with glycogen formation during early refeeding principally via the direct pathway. However, they also indicate an alternative interpretation of the data supporting the indirect pathway (2, 4) as follows. Gluconeogenically derived periportal G6P could contribute to glycogen formation perivenously by hydrolysis to glucose, exit from the periportal cells, transport down the sinusoid, and reuptake via GK to form perivenous G6P (Fig. 6). An important difference between the trans-sinusoidal pathway (TSP) and indirect pathways is that in the former, glucose itself is a necessary intermediate. The TSP could account for the labeling observed in C2, 5, and 6 of glycogen glycosyl moieties after administration of C1-labeled glucose (2, 4). The effect of 3MP on randomization of label may be accounted for by deletion of the TSP, and inhibition of glycogen synthesis could be due to incomplete correction of the profound hypoglycemia induced by 3MP (4) (correction was based on arterial rather than portal vein glucose).

Both the proposed TSP and direct pathways require GK. Mean GK activity (1 μmol·min⁻¹·g⁻¹ at S₀.₅ = 8 mmol/l) can produce ~10 mg of glycogen per gram of liver (wet weight) per hour. This calculation requires only that glucose concentration at the perivenous cells is 8 mmol/l during refeeding, justifiable by published data (25). Thus, GK activity is sufficient to account for mean rates of rat liver glycogen synthesis in the present study and those published by other workers (19, 25). In fully refed animals, glycogen is present throughout the lobule (PAS staining remaining weak in the immediate perivenous zone), raising the question of the source of G6P for perivenous glycogen synthesis. We suggest (Fig. 6) that perivenous glycogen is synthesized from G6P derived from cytosolic (active) GK, which is present throughout the lobule in fully refed animals (13) and not from gluconeogenically derived G6P. Several lines of evidence support this contention. Recent work has established that G6P produced by GK but not hexokinase is readily converted to glycogen (26, 27), and furthermore, only the GK-derived G6P pool activates glycogen synthase (26). These data suggest the presence of two noncommunicating intracellular pools of G6P in hepatocytes and help to explain why GK exhibits high metabolic control over glycogen synthesis (28, 29). Glycogen synthesis does not occur during fasting, despite high rates of G6P production via gluconeogenesis, raising the crucial question of why gluconeogenic G6P fails to activate glycogen synthase. We propose that gluconeogenic G6P enters the hexokinase-derived pool (Fig. 6).

The Northern and in situ hybridization studies suggest a posttranscriptional basis to decreased GK in MLP liver, either failure of translation or decreased GK-protein half-life. Refeeding rapidly stimulates GK gene transcription, but very little new enzyme is formed until 4 h of refeeding (30). (The half-lives of GK activity and GK mRNA are 30 h and 45 min respectively [30, 31].) During fasting, GK is tightly bound to the inhibitory GK regulatory protein (GKRP) (32) in the nucleus (33) (except perivenously [13]). Farrelly et al. (34) and Grimsby et al. (35) demonstrated that knockout of the GKRP in mice resulted in decreased hepatic GK activity, suggesting that an impor...
tant function of nuclear sequestration of GK during starvation might be to delay degradation of GK protein, which is relatively easily inactivated by oxidation in vitro (19). This would facilitate rapid nuclear to cytoplasmic transport of “stored” GK when portal glucose concentration rises postprandially, providing increased GK activity before the appearance of newly synthesized GK. In the current studies, although GK-protein concentration was not measured directly, because the GK assay is independent of GKR-P and as GK kinetics in MLP liver are unchanged (Patel et al., unpublished data), the activity data are likely to reflect total protein concentration. Additional studies of GK translocation in MLP liver, including the GKR-P-mediated posttranscriptional regulatory mechanism, should be worthwhile.

In conclusion, we suggest that hepatic glucose output during refeeding is controlled by perivenous glucose uptake, which is responsive to portal blood concentration because of the abundance of high $S_{0.5}$ GK in those cells. In the early fasting → fed transition, the current view of the indirect pathway of glycogen synthesis needs to be modified taking into account hepatic intralobular heterogeneity of function. Previous evidence pointing to an indirect pathway via gluconeogenesis may be the result of intrasinusoidal transfer of glucose and communication with the perivenous cells via a TSP. A dominant direct pathway would have several clear advantages: 1) it is more efficient energetically, 2) it uncouples potentially conflicting demands of glucose regulation and the major hepatic role in acid-base homeostasis effected by lactate disposal (36), and 3) it prevents inappropriate switching of gluconeogenic flux to glycogen. It is not presently possible to analyze directly the specific contribution of PPGU to regulation of hepatic glucose output in humans, although studies of glucose effectiveness provide indirect evidence of such a mechanism. However, it should be pointed out that current measures of hepatic glucose output should be regarded as the sum of gluconeogenesis plus glycogenolysis minus PPGU, rather than consisting of the first two components alone (37).

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**REFERENCES**

10. Burns SP, Cohen RD, Iles RA, Germain JP, Going TCH, Evans SJW,
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