Foxa3 (Hepatocyte Nuclear Factor 3γ) Is Required for the Regulation of Hepatic GLUT2 Expression and the Maintenance of Glucose Homeostasis during a Prolonged Fast*

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The winged helix transcription factors, hepatocyte nuclear factors 3α, -β, and -γ (HNF-3, encoded by the Foxa1, -α2, and -α3 genes, respectively), are expressed early in embryonic endoderm and play important roles in the regulation of gene expression in liver and pancreas. Foxa1 has been shown to be required for glucagon secretion in the pancreas, whereas Foxa2 is critical for the regulation of insulin secretion in pancreatic β-cells. Here we address the role of Foxa3 in the maintenance of glucose homeostasis. Mice homozygous for a null mutation in Foxa3 appear normal under fed conditions. However, when fasted, Foxa3<sup>−/−</sup> mice have a significantly lower blood glucose compared with control mice. The fasting hypoglycemia in Foxa3<sup>−/−</sup> mice could not be attributed to defects in pancreatic hormone secretion, ketone production, or hepatic glycogen breakdown. Surprisingly, mRNA levels for several gluconeogenic enzymes were up-regulated appropriately in fasted Foxa3<sup>−/−</sup> mice, despite the fact that the corresponding genes had been shown to be activated by FOXA proteins in vitro. However, the mRNA for the plasma membrane glucose transporter GLUT2 was decreased by 64% in the fasted and 93% in the fed state, suggesting that efflux of newly synthesized glucose is limiting in Foxa3<sup>−/−</sup> hepatocytes. Thus, Foxa3 is the dominating transcriptional regulator of GLUT2 expression in hepatocytes in vivo. In addition, we investigated the hepatic transcription factor network in Foxa3<sup>−/−</sup> mice and found that the normal activation of HNF-4α, HNF-1α, and PGC-1 induced by fasting is attenuated in mice lacking Foxa3.

Recent studies suggest that no single transcription factor on its own controls hepatocyte differentiation and metabolism. Instead, a transcription factor network including HNF-3α, HNF-3β, HNF-3γ, HNF-1α, HNF-4α, HNF-6, C/EBP (CCAAT/enhancer-binding protein), and GATA proteins appears to function cooperatively (1–6). The genes encoding the HNF-3 proteins are thought to play an important role in this network based on the observation that embryonic stem cells lacking HNF-3β differentiated toward visceral endoderm in vitro fail to activate the normal transcription program, including the activation of HNF-1α and HNF-4α (5). HNF-3γ is a member of the winged helix/forkhead transcription factor gene family that also includes HNF-3α and HNF-3β (7). According to a recent change in nomenclature, the genetic loci encoding HNF-3α, HNF-3β, and HNF-3γ are now known as Foxa1, Foxa2, and Foxa3, respectively (Fox refers to forhead box) (4).

During formation of the definitive endoderm, Foxa2 is activated first, followed by Foxa1, and finally Foxa3 (8, 9). The targeted null mutation of the Foxa2 gene results in a missing or abnormal node and endoderm, which leads to early embryonic lethality (10, 11). Embryos deficient in Foxa1 develop to term but have abnormal glucagon secretion and die of hypoglycemia around postnatal day 10 (12). In contrast to the Foxa1<sup>−/−</sup> and Foxa2<sup>−/−</sup> mice, Foxa3<sup>−/−</sup> mice develop normally and are fertile (13). The consensus DNA binding sequence for FOXA1, FOXA2, and FOXA3 overlaps, indicating that their molecular physiology may be redundant (7, 14–17). This putative redundancy is supported by the fact that in Foxa3<sup>−/−</sup> mice expression of Foxa1 and Foxa2 is increased (13). The lack of a development phenotype raises the following issue: is Foxa3 required for the maintenance of liver differentiation and function in the adult, and if not, why is the Foxa3 gene maintained during evolution?

In vitro studies have suggested that members of the Foxa family are required for proper regulation of hepatic gluconeogenesis (14, 18). For example, the promoters of three key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2,6BPase), contain FOXA binding sites (15–17, 19). In addition, the glucocorticoid response of PEPCK is dependent on the binding of FOXA proteins to one of the accessory elements in its promoter region (15, 16). Here we report that Foxa3 is required for the maintenance of glucose homeostasis during a prolonged fast and for the metabolic regulation of the hepatic transcription factor network. We also show that Foxa3 is the dominant regulator of GLUT expression in the liver, suggesting that reduced efflux of glucose from hepatocytes contributes to the relative hypoglycemia observed in fasted Foxa3<sup>−/−</sup> mice.

MATERIALS AND METHODS
Derivation and Genotyping of Foxa3<sup>−/−</sup> Mice—The derivation of the Foxa3<sup>−/−</sup> mice has been described previously (13). To allow for the detection of subtle metabolic changes, the Foxa3 null allele was backcrossed 11 times to inbred C57Bl/6 mice to derive an incipient congenic strain. Mice were genotyped by tail-DNA polymerase chain reaction
with three primers: Foxa3-5’ (TCCCAAGCTTTGCCACTGTGTCGCA), Foxa3-3’ (GGTCGACCTAGTTGTGCGCA), and lacZ (GCCCAT-TGCCGTCACGTC). Polymerase chain reactions were carried out for 30 cycles (94 °C, 30 s; 60 °C, 40 s; 72 °C, 60 s). The wild type allele produced a band of 511 base pairs, and the targeted allele produced a band of 236 base pairs. Foxa3+/+ and Foxa3+/− mice were phenotypically indistinguishable and were used interchangeably as controls. All mice were kept in a specific pathogen-free mouse facility, maintained on a 12-h light/dark cycle, and fed a standard rodent Chow (Purina Mills, Inc., St. Louis, MO). Mice of both sexes were randomly selected and used in our experiments.

Blood Glucose, Ketone, and Plasma Hormone Measurements—Five μl of tail venous blood were taken for the measurement of blood glucose levels by a Glucometer device (Bayer, Inc.). Control and homozygous mutant mice (either fed or fasted for 24, 48, and 72 h) were anesthetized with CO₂ before sacrifice. For the determination of hormone and ketone levels, whole venous blood obtained from the inferior vena cava was mixed with an anticoagulant consisting of Trasylol, EDTA, and leupeptin and centrifuged for 5 min at 14,000 x g to obtain plasma. Plasma insulin and glucagon levels were measured by radioimmunoassay in the RIA-Core of the Diabetes Center, University of Pennsylvania. Plasma β-hydroxybutyrate levels were determined by colorimetric assay following the manufacturer’s instructions (Sigma).

Liver Glycogen Content Measurement—Liver glycogen content was determined as described (20). Liver pieces (0.1–0.2 g) were digested in 1 ml of 30% KOH at 95 °C for 30 min, after which time 1.5 ml of 95% ethanol was added. After centrifugation for 20 min at 3000 x g, the pellets containing glycogen were washed with H₂O and 95% ethanol. Final glycerol pellets were dissolved in 0.5 ml of H₂O. An amyloglucosidase solution (1 mg/ml, 0.9 ml, Roche Molecular Biochemicals) was mixed with 0.1 ml of glycerol solution and incubated at 40 °C for 2 h with shaking. In the following colorimetric reaction, 25 μl of the amyloglucosidase-digested solution was first incubated with a mixture of ATP/NADP/glucose-6-phosphate dehydrogenase/Tris-MgCl₂, pH 7.6, loglucosidase-digested solution was first incubated with a mixture of ATP/NADP/glucose-6-phosphate dehydrogenase/Tris-MgCl₂, pH 7.6 (Roche Molecular Biochemicals) for 5 min at room temperature. The absorbance reading (340 nm) at this time was used as the background. Two μl of hexokinase (Roche Molecular Biochemicals, Germany) were then added to the above mixture for 10 min at room temperature, and the absorbance was read again. The difference of the absorbance measurements was plotted along with glucose standards. The glycogen content was finally expressed as mg of glucose/g of wet liver.

RNA Analyses—Total RNA from adult livers was isolated after homogenization in guanidinium thiocyanate (21). For the RNase protection assay, 30 μg of RNA was hybridized with [α-³²P]UTP-labeled riboprobes overnight at 54 °C according to the manufacturer’s protocol (RPA III Kit™, Ambion, Austin, TX). Protected RNA fragments were then added to the above mixture for 10 min at room temperature, and the absorbance was read again. The difference of the absorbance measurements was plotted along with glucose standards. The glycogen content was finally expressed as mg of glucose/g of wet liver.

DNA Analyses—All quantitative data were expressed as mean ± standard errors. Student’s t test or analysis of variance was used to compare values between the control and mutant and treatment groups. A p value of less than 0.05 was considered statistically significant. Microsoft Excel was used to plot data.

RESULTS

Fasted Foxa3+/− Mice Have Significantly Lowered Blood Glucose—Previous studies show that Foxa3+/− (HNF-3y−/−) mice appear normal compared with wild type mice in regard to growth characteristics and morphology of liver, stomach, and intestine (13). To be able to detect possible subtle metabolic abnormalities, we back-crossed the Foxa3 null allele to wild type inbred C57BL/6 mice for 11 generations, at which time the resulting incipient congenic strain is more than 99.9% genetically uniform. To investigate the role of Foxa3 in the regulation of glucose homeostasis, Foxa3+/− and control mice were fasted for 24–72 h and assayed for blood glucose, plasma ketone, insulin, glucagon, and liver glycogen content. We observed no difference in blood glucose levels between fed control and

![Fig. 1. Lowered fasting blood glucose in Foxa3+/− mice. Blood glucose levels were measured in Foxa3+/− (white bars) and Foxa3 control mice (black bars) under fed, 24 h-fasted, 48 h-fasted, and 72 h-fasted conditions. The asterisk above each mutant group indicates a significant difference (p < 0.02) as compared with its control group. N, sample number; P, statistical value by Student’s t test.](image)
Dramatically Reduced in Foxa3 Mice

FIG. 2. Pancreatic hormones levels are appropriate in Foxa3−/− mice. Plasma insulin (A) and glucagon (B) levels from Foxa3−/− and control mice under fed and 72 h-fasted conditions were measured by radioimmunoassay. Multiple samples were assayed and plotted. No statistical differences were detected between Foxa3−/− and control groups for either insulin or glucagon plasma level under fed or 72 h-fasted condition. N, sample number.

Expression of the Facilitative Glucose Transporter GLUT2 Is Dramatically Reduced in Foxa3−/− Mice—Next we considered the possibility that glucose export from the hepatocytes might be affected and contribute to the fasting hypoglycemia in Foxa3−/− mice. Glucose transport across the hepatocyte plasma membrane occurs at least in part via facilitated diffusion through the glucose transporter GLUT2 (24), and the promoter of the GLUT2 gene has been shown to contain a FOXA binding site (25). Prolonged fasting led to an ~80% decrease in GLUT2 mRNA in control plasma (Fig. 5). When we compared control and Foxa3−/− livers, we observed a 93% decrease in the fed state and a 64% reduction after a prolonged fast (Fig. 5). Thus, Foxa3 is the dominant regulator of GLUT2 gene expression in hepatocytes. These data suggest that the dramatic reduction in hepatic GLUT2 expression contributes to the hypoglycemia observed in fasted Foxa3−/− mice by limiting the efflux of newly synthesized glucose from hepatocytes.

Hypoglycemia in Fasted Foxa3−/− Mice—The winged helix transcription factor, Foxa3, is expressed from embryonic day 8.5 in the invading hind gut and ventral endoderm and later in most endoderm-derived tissues (9). Mice carrying a null mutation of the gene encoding Foxa3 develop normally, suggesting that Foxa3 is not required for embryonic development and morphogenesis (13). This lack of a developmental pheno-

DISCUSSION

FIG. 3. Liver glycogen content in Foxa3−/− mice. Liver glycogen content from Foxa3−/− and control mice under both fed and 72 h-fasted conditions were measured as described under “Materials and Methods.” After 72-h fasting, control liver glycogen content was only 10% of the fed level; there was no significant difference between Foxa3−/− and control groups under either fed or fasted condition.
type could be because of functional redundancy with the two other Foxa genes, Foxa1 and Foxa2. Here we show that following a prolonged fast, Foxa3−/− mice have significantly lower blood glucose levels as compared with fasted control mice. We have shown previously that both Foxa1 and Foxa2 are important regulators of pancreatic hormone secretion (12, 22) and speculated that hormone secretion is also affected in fasted Foxa3−/− mice. However, glucagon and insulin levels were appropriate in both fed and fasted Foxa3−/− mice. Hence, the lower blood glucose in fasted Foxa3−/− mice cannot be explained by deficient pancreatic hormone secretion. The slightly higher glucagon level in fasted Foxa3−/− mice indicates that glucagon secretion is intact in these mice, simply reflecting the lower blood glucose. Likewise, mobilization of hepatic glycogen occurred normally in Foxa3−/− mice, and therefore defects of liver glycogenolysis can also be ruled out as a potential cause for the observed hypoglycemia in these mice.

Expression of the Rate-limiting Enzymes in Hepatic Gluconeogenesis Is Induced Normally in Fasted Foxa3−/− Mice—Next we turned to hepatic gluconeogenesis as a possible reason for the decreased resistance to fasting in Foxa3−/− mice. The activation of the gluconeogenic enzymes during fasting occurs in part at the transcriptional level, in particular for PEPCK, G6Pase, and TAT. The FOXA (HNF-3) proteins have been shown to activate the promoters or enhancers of PEPCK, G6Pase, and TAT in vitro, and are involved in the glucocorticoid response of PEPCK (14–17, 19, 23). In light of these findings, we expected decreased expression of one or more gluconeogenic enzymes in Foxa3−/− mice. Surprisingly, the mRNA levels for PEPCK, G6Pase, and TAT were not reduced in fasted Foxa3−/− livers as would have been predicted for the absence of a strong transcriptional activator. Instead, the mRNAs levels for these genes were even higher in Foxa3−/− mice when compared with fasted controls, possibly reflecting the lower blood glucose levels in these mice. Thus, abnormal regulation of the previously established Foxa targets involved in hepatic gluconeogenesis cannot account for the observed hypoglycemia in fasted Foxa3−/− mice.

Foxa3 Is the Dominant Transcriptional Regulator of GLUT2 in the Liver—Another possibility for the hypoglycemia observed in fasted Foxa3−/− mice is reduced efflux of newly synthesized glucose from hepatocytes into the blood stream. The last step in hepatic gluconeogenesis is the hydrolysis of glucose-6-phosphate to glucose by glucose-6-phosphatase in the lumen of the endoplasmic reticulum (26). Newly produced glucose is presumably transported back into the cytoplasm and then exits the cell via the facilitative glucose transporter GLUT2 in the plasma membrane (24). In vitro analysis of the regulatory elements of the GLUT2 gene has identified functional binding sites for both HNF-1α and FOXA in its proximal promoter (25).
HNF-1α has been shown to be critical for GLUT2 expression in pancreatic β-cells in vivo; however, no effect was seen on hepatic GLUT2 expression in HNF-1α-null mice (27). In marked contrast, we have shown that Foxa3 is the dominant regulator of GLUT2 expression in the liver (see Fig. 5), whereas expression in β-cells was unaffected (data not shown). This reciprocal tissue-specific effect of transcription factor mutations on the expression of GLUT2 is interesting and suggests the additional input of other tissue-specific transcription factors and/or co-activators and co-repressors in the transcriptional regulation of GLUT2. The dramatic reduction in hepatic GLUT2 expression likely contributes to the more severe hypoglycemia observed in fasted Foxa3 mutant mice by limiting glucose efflux from hepatocytes into the bloodstream.

**Foxa3 Is Required for the Regulation of the Hepatic Transcription Factor Network**—Hepatocyte-specific gene expression is controlled primarily at a transcriptional level and relies on the activities of multiple transcription factors including HNF-1, CCAAT/enhancer-binding protein (C/EBP), GATA factors, FOXA, HNF-4, and HNF-6 (1–6, 28). It is noteworthy that some cofactors also act with transcription factors to regulate the energy homeostasis. PGC-1, for example, is a transcription coactivator of nuclear receptors including HNF-4 and plays an important role in adaptive thermogenesis (29). Our understanding of the hepatic transcription factor network has been derived from in vitro work such as DNA binding assays and the in vivo analysis of genetically modified mice. These studies established that HNF-4α activates the HNF-1α gene in rat hepatoma cells (30). This regulation of HNF-1α by HNF-4α has also been supported by the analysis of mice deficient in HNF-4α (31).

The FOXA proteins also participate in the hepatic transcription factor network. Embryonic stem cells lacking Foxa1 that are differentiated in vitro toward visceral endoderm have elevated HNF-4α and HNF-1α expression, together with increased mRNAs of several HNF-4 and HNF-1 target genes (5).
In contrast, in Foxa2 null embryonic stem cells differentiated in vitro, HNF-4α and HNF-1α are markedly down-regulated (5). Therefore, we investigated the possibility that Foxa3 participates in the regulation of the hepatic transcription factor network in vivo. When we investigated the expression levels of the genes in the network we discovered a previously unrecognized programmed shift caused by a prolonged fast, with a strong induction of HNF-4α and its target HNF-1α. This induction is blunted in Foxa3−/− livers, indicating that Foxa3 is involved in the regulation of this transcription factor network in vivo. This discovery considerably broadens the spectrum of hepatic targets that need to be investigated in Foxa3−/− mice in the future, as not only genes regulated by Foxa3 directly but also those controlled by HNF-4α and HNF-1α could contribute to the phenotypic consequences observed in Foxa3−/− mice.

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