Impaired Regulation of Hepatic Glucose Production in Mice Lacking the Forkhead Transcription Factor Foxo1 in Liver

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SUMMARY

The hallmark of type 2 diabetes is excessive hepatic glucose production. Several transcription factors and coactivators regulate this process in cultured cells. But gene ablation experiments have yielded few clues as to the physiologic mediators of this process in vivo. We show that inactivation of the gene encoding forkhead protein Foxo1 in mouse liver results in 40% reduction of glucose levels at birth and 30% reduction in adult mice after a 48 hr fast. Gene expression and glucose clamp studies demonstrate that Foxo1 ablation impairs fasting- and cAMP-induced glycogenolysis and gluconeogenesis. Pgc1α is unable to induce gluconeogenesis in Foxo1-deficient hepatocytes, while the cAMP response is significantly blunted. Conversely, Foxo1 deletion in liver curtails excessive glucose production caused by generalized ablation of insulin receptors and prevents neonatal diabetes and hepatosteatosis in insulin receptor knockout mice. The data provide a unifying mechanism for regulation of hepatic glucose production by cAMP and insulin.

INTRODUCTION

The mechanism by which hormones regulate hepatic glucose metabolism is a key question in biology with critical ramifications for pathogenesis and treatment of metabolic diseases (Matsumoto and Accili, 2006). Hepatic glucose production (HGP) can be viewed as the product of the opposing actions of glucagon, acting through cAMP-dependent pathways, and insulin, acting through the PI 3-kinase pathway. Acute hormonal effects are independent of protein synthesis (Exton and Park, 1968; Grempler et al., 2005; Sasaki et al., 1984) and transcriptional in nature (O’Brien and Granner, 1996). Ambiguity persists on the identity and interactions of hormone-regulated factors controlling transcription of rate-limiting glucogenic enzymes, such as phosphoenolpyruvate carboxykinase (Pck1) and glucose-6 phosphatase (G6pc) (O’Brien and Granner, 1996).

The Creb-Torc2 pathway is acutely activated by cAMP to promote HGP in a Pgc1α-dependent manner, but does not respond to insulin (Koo et al., 2005). The coactivator Cbp is recruited to Creb when the latter is phosphorylated (Chrivia et al., 1993). It has been shown that insulin phosphorylates Cbp to inhibit gluconeogenesis by disrupting the Creb/Cbp complex (Zhou et al., 2004). However, the recent demonstration that Creb is promiscuously phosphorylated by both cAMP and insulin (Koo et al., 2005) makes this explanation problematic. Among transcriptional coactivators, Pgc1α is induced by fasting and is required for the Creb-Torc2 induction of gluconeogenesis (Koo et al., 2004). But it is disputed whether Pgc1α is itself the target of insulin regulation (Herzig et al., 2001) or is regulated via Foxo1 (Puigserver et al., 2003; Yoon et al., 2001). And the phenotype of Pgc1α-deficient mice is notable for the absence of significant abnormalities of glucose production (Lin et al., 2004).

The forkhead transcription factor Foxo1 confers insulin responsiveness onto G6pc expression (Nakae et al., 2001) by interacting with Pgc1α (Puigserver et al., 2003). Nonetheless, its involvement in HGP has been evinced exclusively from gain-of-function or partial loss-of-function studies (Nakae et al., 2001, 2002; Puigserver et al., 2003; Samuel et al., 2006), leading to controversy as to whether it is required for either the Pgc1α (Schilling et al., 2006) or the insulin response (Herzig et al., 2001; Yeagley et al., 2001). Although a dominant negative Foxo1, lacking the transactivation domain, reduces gluconeogenesis (Nakae et al., 2001) and reverses hyperglycemia in ob/ob mice (Altomonte et al., 2003), this approach is tainted by potential off-target effects of the mutant Foxo1. To examine this question, we ablated Foxo1 expression in mouse hepatocytes using cre-foxP mutagenesis.
RESULTS

Generation and Analysis of Liver-Specific Foxo1 Knockouts

To ablate Foxo1 in hepatocytes, we intercrossed mice homozygous for a floxed Foxo1 allele (Paik et al., 2007) with α1 antitrypsin-cre mice (henceforth, l-Foxo1 mice). Hepatic Foxo1 mRNA (Figures 1A and 1B) and protein (Figure 1C) levels were decreased by ~90% in l-Foxo1 mice, compared to controls. In contrast, expression of the isoform Foxo3a was unchanged (Figure 1B) as was expression of Foxa2, a related transcription factor that regulates liver metabolism (Wolfrum et al., 2004; Zhang et al., 2005) (Figure 1C).

At birth, l-Foxo1 mice showed a 40% decrease of blood glucose level (Figure 1D), while hepatic glycogen increased (Figure 1E and data not shown). In adult mice, fasting induced glycogenolytic (G6pc) and gluconeogenetic genes (Pck1 and Ppargc1α) 2- to 4-fold. In l-Foxo1 mice, G6pc expression failed to increase with fasting, while the rise of Pck1 and Ppargc1α was curtailed by ~50%, as was expression of two Foxo1 target genes, insulin-like growth factor binding protein-1 (Igfbp1) (Guo et al., 1999), and insulin receptor substrate 2 (Irs2) (Zhang et al., 2001) (Figure 2A). In contrast, Foxo1-independent metabolic pathways, such as those mediated by Srebff1c (Horton et al., 2003) and glucokinase (Gck) (Dentin et al., 2004), were unaffected (Figure 2A). These data indicate that deletion of Foxo1 affected only the expression of Foxo1 target genes and did not result in widespread hepatotoxicity (Table 1). Consistent with the changes in gluconeogenic gene expression, l-Foxo1 mice showed 30% lower blood glucose levels compared with control mice after prolonged (48 hr) fasting (Figure 2B). Notably, 10% of the l-Foxo1 mice developed neuroglycopenia, with seizures that were relieved by glucose administration.

To evaluate the effects of altered gene expression on HGP, we conducted pyruvate challenge tests in 16-week-old mice. Glucose levels were significantly lower in l-Foxo1 mice in response to pyruvate administration (Figure 2C). Moreover, adult l-Foxo1 mice (Table 1) showed reduced glucose excursions during glucose tolerance tests compared with control mice (Figure 2D), while insulin tolerance tests were identical in both groups (Figure 2E).

Decreased Hepatic Glucose Production in l-Foxo1 Mice

In hyperinsulinemic euglycemic clamps, l-Foxo1 mice required a 30% increase of the glucose infusion rate to maintain euglycemia, consistent with a state of heightened insulin sensitivity (Figure 2F). Despite this, HGP in l-Foxo1 was > 50% lower than control mice (Figure 2G), while peripheral glucose uptake was unaffected (Figure 2H). The combination of increased glucose infusion and reduced HGP, in the absence of changes in peripheral glucose uptake, indicates that glucose is primarily incorporated into hepatic glycogen. To analyze changes in hepatic glucose fluxes, we measured glucose cycling,
Figure 2. Metabolic Effects of Hepatic Foxo1 Ablation in Adult Mice

(A) Expression of hepatic genes in fasted and fed 16-week-old l-Foxo1 and littermate control mice (n = 6–9 mice per group).

(B) Blood glucose levels in 48-hr-fasted animals. * = p < 0.001, control versus l-Foxo1 mice.

(C) Pyruvate challenge.

(D and E) Intraperitoneal glucose tolerance (D) in 16-week-old; and insulin tolerance tests (E) in 12-week-old l-Foxo1 and littermate controls (n = 7–10 mice per group).

(F–K) Glucose clamps. Glucose infusion rates (F), glucose production (G), glucose disappearance (H), glucose cycling (I), glycogenolysis (J), and gluconeogenesis (K) during hyperinsulinemic euglycemic clamps in 10-week-old l-Foxo1 and littermate controls (n = 9 mice per group). I, PAS staining of liver sections and hepatic glycogen content in 14-week-old l-Foxo1 and littermate controls (n = 8 mice per group). All data are presented as means ± SEM. One asterisk indicates p < 0.05; two asterisks p < 0.01.

(L and M) Measurements of hepatic glycogen content and PAS staining of liver sections.
glycogenolysis, and gluconeogenesis. Glucose cycling, i.e., the amount of glucose-6-phosphate that is dephosphorylated and released without being further metabolized (Vranic, 1992), decreased by >60% in l-Foxo1 mice, consistent with reduced flux through G6pc (Figure 2I). Glycogenolysis and gluconeogenesis were both decreased by 50% (Figure 2J and 2K), consistent with the gene expression data (Figure 2A). Moreover, fasting hepatic glycogen content in l-Foxo1 mice increased 6-fold (Figure 2L). These results indicate that Foxo1 ablation in liver suppresses HGP by decreasing both glycogenolysis and gluconeogenesis.

Foxo1 Is Required for Pgc1α-Dependent Induction of Pck1 and G6pc

Pgc1α mediates HGP during fast (Herzig et al., 2001; Yoon et al., 2001) in a Torc2-dependent manner (Koo et al., 2005). It has been disputed (Schilling et al., 2006) whether Foxo1 is required for the Pgc1α response (Puigserver et al., 2003). To address this question, we analyzed Pgc1α’s ability to regulate glucogenic genes in hepatocytes lacking Foxo1. In hepatocytes derived from control mice, transduction of Pgc1α increased G6pc and Pck1 expression 2,500- and 80-fold, respectively (Figure 3, empty bars). Insulin partly inhibited Pgc1α-induced gene expression. In Foxo1-deficient hepatocytes, Pgc1α induction of either gene decreased by >95% (Figure 3, full bars). In contrast, Pgc1α induction of Cycs and Atp5b genes, which are not regulated by Foxo1, was preserved. These data indicate that Foxo1 is required for Pgc1α induction of hepatic glucogenetic genes.

We next assessed the effect of Foxo1 inactivation in primary hepatocytes, using a short-hairpin (sh) RNA adenovirus to reduce Foxo1 expression by 90% (Figure 4A). We examined gene expression induced by cAMP (G6pc), dexamethasone (Igfbp1), or both (Pck1 and Ppargc1α). In all instances, Foxo1 ablation reduced the hormonal response by ~50% to ~70% (Figure 4B), indicating that Foxo1 plays a role in both cAMP and glucocorticoid induction of hepatic gene expression. Consistent with these results, Foxo1 shRNA curtailed basal and prevented cAMP-induced glucose release in the medium (Figure 4C). Moreover, expression of Foxo1 target genes in the insulin signaling pathway was also decreased by ~50%, with a commensurate decrease of insulin-induced phosphorylation (Figure 4D and 4E) (Gershman et al., 2007; Ide et al., 2004; Matsumoto et al., 2006; Puig and Tjian, 2005; Zhang et al., 2001). These changes in gene expression attenuated insulin signaling in hepatocytes expressing Foxo1 shRNA (Figure 4E).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>l-Foxo1</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.5 ± 0.7</td>
<td>28.0 ± 1.3</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>2.41 ± 0.28</td>
<td>2.81 ± 0.44</td>
</tr>
<tr>
<td>Fat mass/BW (%)</td>
<td>8.68 ± 2.79</td>
<td>10.10 ± 1.39</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>22.6 ± 0.5</td>
<td>22.6 ± 1.2</td>
</tr>
<tr>
<td>Lean mass/BW (%)</td>
<td>82.48 ± 0.90</td>
<td>80.78 ± 1.03</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.25 ± 0.07</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>Liver/BW (%)</td>
<td>4.28 ± 0.14</td>
<td>4.47 ± 0.11</td>
</tr>
<tr>
<td>Fed Glucose (mg/dl)</td>
<td>177 ± 15</td>
<td>161 ± 17</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>127 ± 9</td>
<td>131 ± 16</td>
</tr>
<tr>
<td>Fed Insulin (ng/ml)</td>
<td>1.04 ± 0.10</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.45 ± 0.11</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>54 ± 5</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>102 ± 5</td>
<td>115 ± 6</td>
</tr>
<tr>
<td>NEFA (mEq/l)</td>
<td>0.59 ± 0.03</td>
<td>0.65 ± 0.07</td>
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<tr>
<td>β-Hydroxybutyrate (mM)</td>
<td>0.69 ± 0.08</td>
<td>0.89 ± 0.17</td>
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<tr>
<td>Fed Liver triglyceride (mg/g)</td>
<td>15.9 ± 1.8</td>
<td>18.6 ± 1.3</td>
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<tr>
<td>Liver triglyceride (mg/g)</td>
<td>66.7 ± 4.7</td>
<td>72.5 ± 4.8</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g)</td>
<td>5.8 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>Fed Liver glycogen (mg/g)</td>
<td>20.86 ± 3.56</td>
<td>16.83 ± 3.50</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>27 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>3 ± 1</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td>γ-GTP (IU/l)</td>
<td>9 ± 3</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>23 ± 1</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>20.9 ± 3.6</td>
<td>16.8 ± 3.5</td>
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All data, unless otherwise indicated, were obtained in overnight-fasted animals. No difference achieved statistical significance.
We also studied the cAMP response in primary hepatocytes from l-Foxo1 mice. We observed a 50% decrease in G6pc induction and a 30% decrease in Ppargc1a induction, whereas Pck1 expression was unaffected (Figure 4F). Glucose release in the medium decreased by ~25% (Figure 4G). The difference between the knockdown and knockout hepatocytes indicates that adaptive mechanisms (e.g., Foxo3) can compensate for chronic, but not for acute, Foxo1 ablation.
Figure 5. Hepatic Foxo1 Ablation in Insr⁻/⁻ Mice
(A) Metabolic parameters in WT control, Insr⁻/⁻ and Insr⁻/⁻::l-Foxo1 mice (Dko) (n = 7 mice for each group).
(B) Kaplan-Meier plots of Insr⁻/⁻ (solid line) and Dko mice (dotted line).
(C) Appearance of the liver, PAS, and oil red O staining of liver sections in Insr⁻/⁻ and Dko mice. The liver is outlined for comparison.
(D) Hepatic gene expression in fed mice (n = 6–8 mice for each group). Data in (A)–(D) were obtained at postnatal day 2.
(E) Metabolic parameters in 14-week-old Dko (filled bars) and littermate controls (open bars) (n = 7 mice for each group). Data are means ± SEM.
(F) Necroscopic analyses of 14-week-old Dko and control mice.
(G) H&E staining of pancreatic sections demonstrating islet hyperplasia.
(H) PAS and oil red O staining of liver sections.
Genetic Epistasis of Foxo1 and Insulin Receptor

In diabetes, fasting hyperglycemia reflects an altered balance between the insulin and glucagon effects on HGP (Cherrington, 1999). Foxo1 gain-of-function, dominant negative inhibition, and generalized haploinsufficiency (Nakae et al., 2001, 2002) have shown impairment and potentiation, respectively, of insulin action on HGP. But these results are neither unique to Foxo1 (Hall et al., 2000; Inoue et al., 2004; Zhang et al., 2005) nor conclusive when glucogenetic genes are first induced (Girard et al., 2000; Inoue et al., 2004; Zhang et al., 2005) nor conclusive. We now show that hepatic Foxo1 ablation results in the following: (1) 40% reduction of glucose levels at birth, and (2) 30% reduction of glucose levels in adult mice (Francisco et al., 2001, 2002) have shown impairment and potentiation, respectively, of insulin action on HGP. But these results are neither unique to Foxo1 (Hall et al., 2000; Inoue et al., 2004; Zhang et al., 2005) nor conclusive when glucogenetic genes are first induced (Girard et al., 2000; Inoue et al., 2004; Zhang et al., 2005) nor conclusive.

Our results provide the hitherto missing, critical test of the hypothesis that Foxo1 is required for hormonal regulation of HGP. We now show that hepatic Foxo1 ablation results in the following: (1) 40% reduction of glucose levels at birth, when glucogenetic genes are first induced (Girard et al., 1992); (2) 30% reduction of glucose levels in adult mice after prolonged (48 hr) fasting; (3) fasting-induced neuroglycopenia in 10% of I-Foxo1 mice; (4) blunted induction of glucogenetic genes with fasting; and (5) 50% decrease of glycogenolysis and gluconeogenesis during hyperinsulinemic euglycemic clamps. The data show a surprising role for Foxo1 as the shared element coordinating HGP control by cAMP and insulin. Although we have previously shown that Foxo1 plays a role in insulin inhibition of cAMP-induced glucose production (Nakae et al., 2001; Puigserver et al., 2003), the present data show that it is required for cAMP induction of HGP, thus expanding its role. Finally, the reversal of the metabolic abnormalities of Insr-deficient mice by hepatic Foxo1 ablation, which virtually phenocopies transgenic restoration of insulin signaling in liver (Okamoto et al., 2004), underscores the comprehensive role of Foxo1 in insulin signaling.

The important role of Foxo1 in HGP can explain the effects observed in vivo following genetic ablation of other transcription factors and coregulators that participate in this process (Matsumoto and Accili, 2006). For example, despite the well-established role of Pgc1α in the fasting response, ablation of Pgc1α does not affect gluconeogenesis in vivo (Lin et al., 2004). This phenotype has been ascribed to the compensatory increase of c/Ebαβ. But, based on the present data, it is likely to result from preserved Foxo1 function in Pgc1α-deficient hepatocytes.

HGP is a complex process and is regulated both directly and indirectly (Obici et al., 2002) via transcriptional and posttranscriptional mechanism, in response to hormones, nutrients, and other cues. Because of these complexities and attendant compensatory mechanisms, it is difficult to determine the relative contribution of individual transcription factors to this process in vivo. In this regard, residual HGP in Foxo1-deficient liver likely reflects the contribution of Foxo3 to this process, or that mediated by c/Ebαβ (Wang et al., 1995), NR4 (Pei et al., 2006), STAT3 (Inoue et al., 2004), Fxr (Ma et al., 2006), and Foxa2 (Zhang et al., 2005).

The selective and limited effect on HGP, brought about by Foxo1 loss of function in otherwise healthy animals, supports therapeutic modalities for diabetes treatment, based on Foxo1 inhibition. In this regard, it should also point to the increase in Gck levels in Dko mice as a potential added therapeutic benefit of inhibiting Foxo1 in insulin-resistant states. Finally, the absence of tumor-promoting effects in Foxo1 knockouts (Paik et al., 2007; Tothova et al., 2007) allays concerns regarding potential, long-term, adverse effects of this approach (Accili and Arden, 2004).

EXPERIMENTAL PROCEDURES

Mice

We generated l-Foxo1 mice using Foxo1lox/lox mice (Paik et al., 2007) and α1-antitrypsin-cre transgenic mice. The latter were generated using a construct in which a NLS-containing cre is driven by the 1.2 kb liver-specific α1-antitrypsin promoter (Shen et al., 1989). We used ROSA26-eGFP reporter mice to assess specificity of transgene expression. The line employed herein yielded recombination in liver only and was already fully active at P0. Insrfl/fl mice have been described (Accili et al., 1996). The Columbia University Institutional Animal Care and Utilization Committee approved all experiments.

Chemicals and Antibodies

Insulin, pCPT-cAMP, and dexamethasone are from Sigma; phospho-Ser172Akt and Akt antibodies are from Cell Signaling; Foxo1 (H-128) and insulin receptor polyclonal (C-19) and monoclonal (29B4) antibodies are from Santa Cruz Biotechnology; lrs1, lrs2, p85, and phospho-tyrosine (4G10 and PY20) antibodies are from Upstate Biotechnology and BD Biosciences, respectively; and acitin antibodies (JLA20) are from Calbiochem. The anti-Foxa2 antisemur has been described (Sasaki and Hogan, 1994).
Metabolic Studies
We measured blood or plasma glucose using a glucose monitor (One Touch Ultra, Lifescan) or glucose oxidase reagent (Teco diagnostics); insulin levels were measured by ELISA (Mercodia), plasma β-hydroxybutyrate, triglyceride (Pointe scientific), cholesterol, and nonesterified fatty acids by colorimetric assays (Cholesterol E and NEFA C, Wako Pure Chemicals). Body composition, insulin, glucose tolerance tests (Nakae et al., 2002), pyruvate tests (Miyake et al., 2002), and hepatic glycogen measurements have been described (Nakae et al., 2002). We extracted hepatic triglycerides and cholesterol using 2-propanol and chloroform/methanol, respectively, and determined levels by colorimetric assay as described above.

Histological Analyses
We fixed livers and pancreas in 10% formaldehyde for HE and PAS staining. We used frozen livers for oil red O staining to evaluate hepatic lipid content.

Hyperinsulinemic Euglycemic Clamp
Studies were performed in conscious, unrestrained, catheterized mice for 90 min as previously described (Pocai et al., 2005).

RNA and Protein Analysis
We used standard RNA extraction and quantitation procedures (Matsumoto et al., 2006). Primer sequences are available on request. Immunoprecipitation and western blotting have been described (Matsumoto et al., 2006). We prepared hepatic nuclear extracts using Celllytic NuCLEAR Extraction Kit (Sigma).

Cell Culture
Primary mouse hepatocytes were isolated from 8- to 12-week-old male mice as described previously (Matsumoto et al., 2002). Cells were incubated in serum-free Medium 199 overnight before addition of hormones.

Adenovirus Infection
Adenoviruses encoding PGC1α, control shRNA, or Foxo1 shRNA have been described (Matsumoto et al., 2006; Puigserver et al., 2003). Primary hepatocytes were transduced 2 days after plating. RNA and protein isolation or glucose production were carried out 2 days after transduction. pCPT-cAMP, dexamethasone, and insulin were added for 6 hr. To analyze protein phosphorylation, we treated cells with insulin (10 nM) for 2 min.

Glucose Production Assay
We cultured primary hepatocytes in Medium 199 supplemented with 5% FBS or 0.5 mM pCPT-cAMP and measured glucose production in glucose- and phenol red-free DMEM (pH 7.4) supplemented with 5% FBS or 0.5 mM pCPT-cAMP and measured glucose production.

Statistical Analysis
All results are presented as mean ± SEM. P value was calculated by unpaired Student’s or Welch’s t tests, as appropriate.

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Foxo1 and Hepatic Glucose Production


