Feedback regulation of hepatic gluconeogenesis through modulation of SHP/Nr0b2 gene expression by Sirt1 and FoxO1

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We (12) have recently developed a diabetic mouse model that is deficient in insulin receptor substrates (IRS)-1 and -2 specifically in hepatocytes (DKO IRS1/2). DKO IRS1/2 mice manifest hyperglycemia and severe insulin resistance. Strikingly, inactivation of hepatic FoxO1 largely reverses the diabetic phenotype (12). Since FoxO1 is regulated by Sirt1 through deacetylation (15, 21, 28), we hypothesized that inactivation of Sirt1 in DKO IRS1/2 mice might produce similar outcomes.
to FoxO1 inactivation. However, our results showed that the effect from Sirt1 inactivation was quite different from what we observed in the FoxO1 gene deletion in DKO Irs1/2 mice. Moreover, we have uncovered a novel feedback pathway in the regulation of hepatic gluconeogenesis.

**MATERIALS AND METHODS**

**Animals, blood chemistry, and metabolic analysis.** Irs1, Irs2, and Sirt1 floxed mice were generated as previously described (7, 12, 24). Transgenic mice that carry a Cre coding sequence plus the albumin gene promoter were purchased from the Jackson Laboratory. Irs1 and Irs2 liver-specific double knockout mice (DKO Irs1/2) were generated as described previously (12). To generate Irs1, Irs2, and Sirt1 liver-specific triple knockout mice (TKO Irs1/2:Sirt1), Irs1 and Irs2 double floxed mice were crossed with liver-specific Sirt1 knockout mice (LKOSirt1), and the resultant triple heterozygotes were intercrossed to obtain TKO Irs1/2:Sirt1 mice. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine. Blood glucose levels were measured using a glucose meter (Contour by Bayer) under ad libitum (fed) or overnight 16-h fasting conditions. Serum insulin was measured using a commercial assay kit (ALPCO). Glucose and insulin tolerance tests, fasting, and refeeding were performed as described previously (12). Pyruvate tolerance tests were essentially similar to glucose tolerance tests except 2 g/kg body wt pyruvate solution used for the injection.

**Cell culture and DNA transfection.** Mouse H2.35 cell line was obtained from the American Type Culture Collection (ATCC), and they were maintained in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin, 1 g/l glucose, 200 mM dexamethasone, and 4% fetal bovine serum (FBS). Human Hep G2 cells (ATCC) was cultured in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin, 4.5 g/l glucose, and 10% FBS. Human HEK 293A cell line was purchased from Invitrogen (Carlsbad, CA), and they were maintained in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin, 4.5 g/l glucose, and 10% FBS. DNA transfections were essentially performed using the TurboFect reagent from Fermentas (Glen Burnie, MD). DNA constructs that were used for transfections were cloned into pcDNA3 (Invitrogen) by PCR using the following primers. GFP forward ATGGTGA- CAAAGGCG, reverse TTACCTTGACGGTGTCCATG; mouse Sirt1 forward GCCGACAGGTGGCGCTCG; reverse TTAGTGAT- CTGTGATGGATAGTTTACATCTG; mouse Irs1 forward 5'-GCCAGAGGAGTCGTAATAGC-3', reverse 5'- AGACCCAGGAGCTGGTGT-3'; mouse Irs2 forward 5’-GGTCGCAGGTAGCTGTTTT-3', reverse 5’-GGGCTCTGATGGCTTCT-ACTC-3'; mouse Sirt1 forward 5’-CCCTCAGCAGCTTGTGTGAAT-3', reverse 5’-ACACAGAGGGCTGGGACTC-3'; mouse Pgc-1α forward 5’-TGGAGTGGTGAGGCGAGCAA-3', reverse 5’-GGCTAG- CAATGGTGGCCTCAT-3'; mouse Pck1 forward 5’-ATCATTTTGTT-GGCCGTAG-3', reverse 5’-TGTAGCATTTGCGCCCTGT-3'; mouse G6pc forward 5’-TCGAGAGCTGTTGACACCTC-3', reverse 5’- TACAGGGTGACAGGAAACTC-3'; mouse Gck forward 5’-AGGAAGCAGCGGGCTGGTCC-3', reverse 5’-TCACTGGCTGTTGCTG-3'; mouse Nr0b2 forward GTTCATCATGTTGCTGCA, reverse AGGGATCTTCAAGCTC- CAC; human PPIA forward AGGTCCCAAAGACGACGCAA; reverse GAAGTCACACCTGGGACA; human G6F forward AAGC- GGACCTCAGATTCTG; reverse GAGGAAATGAGCAG- CAAG; human PDK4 forward TGCCCTTGAATGGTCAAGGA, reverse TGCCATTTGCTGCTGCA. Primary hepatocyte preparation and adenoviral transduction. Mouse primary hepatocytes were isolated and cultured as previously described (46). Briefly, primary hepatocytes were isolated from C57BL/6j mice by use of collagenase perfusion under anesthesia. The viability of hepatocytes was assessed by the trypan blue exclusion method. Cells with viability >95% were used for the experiments. The adenoviruses carrying GFP, Sirt1, and FoxO1 coding sequences (with a FLAG tag) were generated using PaDEasy system (Agilent). The adenoviruses carrying GFP (GCACAGGTAAGCTCTCAAAGA), Sirt1 (GCACAGGGCTCC- GAACATT), and FoxO1 (GACGGTGCTACTCTCAAGA) shRNA sequences were generated using BLOCK-iT (Invitrogen). Generally, we used 100 multiplicity of infection (MOI) for overexpression and 600 MOI for shRNA knockdown experiments.

**Luciferase reporter assay.** Human NR0B2 gene promoter (~606 bp) was cloned by PCR using the following primers: forward ATTC-GTCAAGCTTCTTCAGCA, reverse CAAACACTTGTACG- CAGAAG. Human PDK4 gene promoter (~1322 bp) was cloned using the following primers: forward GTATGACAGGTAATGGGTTTCT, reverse GTGGCTTCTACCCCATG; FoxO1 (GAGGCTGCTACTCTCAAGA) shRNA sequences were generated using BLOCK-iT (Invitrogen). Luciferase reporter system (pGL4.10luc2 and pGL4.74hRluc/TK) was purchased from Promega (Madison, WI). DNA constructs were transfected into mouse H2.35 hepatocytes or HEK 293 cells, and luciferase activity was analyzed using Dual-Luciferase Assay System from Promega.

**ChIP assay.** Chromatin immunoprecipitation (ChIP) assays were performed as previously described (16). Briefly, mouse primary hepatocytes were grown to 90% confluence before they were treated with 1% formaldehyde at 37°C for 15 min. The cross-link reaction was stopped by adding glycine to a final concentration of 125 mM. Chromatin was sonicated to an average size of 150-300 bp. Immunoprecipitation was performed using M2-FLAG antibody (Sigma) following manufacturer’s manual. ChIP DNA was analyzed by real-time PCR using the following primers: Nr0b2 proximal forward 5’-CATGGGAAATGCCATTAAG, Nr0b2 proximal reverse 5’-TCGCTTTTCATCGATGC; Nr0b2 upstream forward 5’-GGTCTGTGGAACCTCAGACT, Nr0b2 upstream reverse 5’-GGTGGTGGACACCTTCAAGA; the internal control Ppia gene promoter forward primer 5’-cgacacaccatacctgaggt, reverse primer 5’-aagccggtgctggagga.

**Statistical analysis.** Data are presented as means ± SE. Significance between two groups was assessed using a two-tailed unpaired Student’s t-test, and P < 0.05 was considered significant.

**RESULTS**

**Inactivation of hepatic Sirt1 in vivo.** To examine the role of hepatic Sirt1 in glucose homeostasis in vivo, we set out to...
TKO_{irs1/2:Sirt1} mice, we performed the following several tests. Pyruvate tolerance tests showed that inactivation of Sirt1 improved tolerance to exogenous pyruvate injection by 13 and 26% in LKO_{Sirt1} and TKO_{irs1/2:Sirt1} mice relative to controls, respectively (Fig. 2A), suggesting that hepatic gluconeogenesis may be decreased in the absence of Sirt1. Glucose tolerance tests

Fig. 1. Sirt1, Irs1, and Irs2 (A and B) protein levels were analyzed in control, LKO_{Sirt1}, and TKO_{irs1/2:Sirt1} mice by immunoblot analysis. Blood glucose levels were measured in ad libitum-fed 6-wk-old (C) and overnight fasted 2-mo-old (D) control loxp, LKO_{Sirt1}, DKO_{irs1/2}, and TKO_{irs1/2:Sirt1} mice (n = 8–10), respectively. Values are presented as means ± SE.

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Fig. 2. A: pyruvate tolerance tests (PTT) were performed on 4-mo-old control and knockout mice (n = 6–8), and data are presented as areas under curve (AUC, artificial units). B: glucose tolerance tests (GTT) were performed on 2-mo-old control and knockout mice (n = 6–9). C: insulin tolerance tests (ITT) were performed on 2-mo-old control and knockout mice (n = 6–8). Values are presented as means ± SE. *P < 0.05 indicates a significant difference between DKO_{irs1/2} and TKO_{irs1/2:Sirt1} mice.
showed that LKO<sup>Sirt1</sup> mice had slight glucose intolerance compared with control mice, whereas TKO<sup>Ins1/2:Sirt1</sup> mice had moderate improvement in glucose tolerance relative to DKO<sup>Ins1/2</sup> mice (Fig. 2B). However, insulin tolerance tests (ITT) revealed no significant difference between LKO<sup>Sirt1</sup> and control mice, and DKO<sup>Ins1/2</sup> and TKO<sup>Ins1/2:Sirt1</sup> mice as well (Fig. 2C). In line with the ITT results, blood insulin levels were not significantly different between LKO<sup>Sirt1</sup> and control mice and remained high in TKO<sup>Ins1/2:Sirt1</sup> mice relative to DKO<sup>Ins1/2</sup> mice (Supplementary Fig. S2). The persistent systemic insulin resistance might partly explain the glucose intolerance in the TKO<sup>Ins1/2:Sirt1</sup> mice.

**Hepatic deficiency in Sirt1 and gluconeogenic gene expression.**

To elucidate the molecular events in glucose metabolism in livers of LKO<sup>Sirt1</sup> and TKO<sup>Ins1/2:Sirt1</sup> mice, we analyzed expression of several genes involved in gluconeogenesis and glycolysis in livers from overnight fasted and 4-h refed mice. Hepatic deficiency of Sirt1 alone did not cause any significant changes in expression of gluconeogenic genes of Pgc1<sub>a</sub>, Pck1, G6pc, and Pdk4 and the glycolytic gene Gck (Fig. 3, A–E). Consistent with the previous report (12), hepatic deficiency of Irs1 and Irs2 led to elevated expression of Pgc1<sub>a</sub>, G6pc, and Pck1 but diminished Gck gene expression; however, inactiva-

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**Fig. 3.** Hepatic mRNA levels for Pgc1<sub>a</sub> (A), Pck1 (B), G6pc (C), Pdk4 (D), and Gck (E) genes were analyzed in control loxp, LKO<sup>Sirt1</sup>, DKO<sup>Ins1/2</sup>, and TKO<sup>Ins1/2:Sirt1</sup> mice by real-time RT-PCR after being fasted overnight for 16 h with free access to water or refed ad libitum for another 4 h immediately after fasting. Values are presented as means ± SE; n = 3. Protein levels for Pgc-1<sub>a</sub>, Pck1, Pdk-4, Gck, and the internal control actinin (F) were also analyzed in the above fasted and refed liver lysates.
tion of Sirt1 in the DKOIRS1/2 mice resulted in 57 and 51% decreases in Pgc1a and Pck1 gene expression after refeeding, respectively (Fig. 3, A and B). Interestingly, Pdk4 gene expression still responded to food cue even in the livers of DKO1SIRT1/2 and TKOIRS1/2:SIRT1 mice (Fig. 3D), suggesting that pathways independent of IRS1/2 and Sirt1 might be involved in feeding-mediated Pdk4 gene regulation. Sirt1 inactivation did not improve Gck gene expression under severe deficiency of insulin signaling in TKOIRS1/2:SIRT1 livers (Fig. 3D). Protein levels for Pgc-1a, Pck1, Pdk-4, and Gck were generally consistent with their corresponding mRNA levels described above, and it was notable that Pdk-4 protein diminished much faster in the liver of that LKO[SIRT1 than that in the control mice in response to feeding (Fig. 3F). Together, these results suggest that hepatic Sirt1 may be involved in dysregulated hepatic gluconeogenesis in the diabetic DKOIRS1/2 mice through modulation of some of the gluconeogenic genes.

Nr0b2 gene is downregulated in the Sirt1-deficient liver. Since inactivation of Sirt1 has less dramatic impact on gluconeogenesis than FoxO1 does as previously reported (12, 26), we thought that there might be a negative feedback mechanism in the Sirt1-FoxO1 pathway. It has been reported that SHP/Nr0b2 can inhibit the transcriptional activity of FoxO1 on the G6pc gene promoter (47). To test whether Sirt1 regulates Nr0b2, we first analyzed the Nr0b2 gene expression in the liver of Sirt1-deficient mice. Indeed, mRNA levels of the Nr0b2 gene were decreased by 47% in LKO[SIRT1 livers relative to controls (Fig. 4A). Compared with controls, DKOIRS1/2 livers had a 48% increase in the Nr0b2 gene expression, and this was normalized in TKO IRS1/2:SIRT1 livers (Fig. 4A). Consistently,

Fig. 4. Nr0b2 mRNA (A) and protein levels (B) were analyzed in the liver of control loxp, LKO[SIRT1, DKOIRS1/2, and TKOIRS1/2:SIRT1 mice by real-time RT-PCR (Values are presented as means ± SE; n = 3) and immunoblotting, respectively. Immunoblot analysis of Sirt1, FoxO1, and Nr0b2 proteins (C and E) and real-time RT-PCR analysis of Nr0b2 mRNA (D) in mouse primary hepatocytes infected with adenoviruses carrying control GFP-, Sirt1-, and Foxo1-overexpressing or shRNA constructs. Values are presented as means ± SE; n = 3.

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Nr0b2 protein levels were also decreased in the liver of LKO<sup>Sirt1</sup> and TKO<sup>fx1/2:Sirt1</sup> mice relative to the controls (Fig. 4B). To further verify this observation, we overexpressed or knocked down Sirt1 and FoxO1 in mouse primary hepatocytes (Fig. 4C). Overexpression of Sirt1 and FoxO1 upregulated the Nr0b2 gene expression by 40 and 98% at mRNA levels, respectively, and knockdown of Sirt1 and FoxO1 suppressed the Nr0b2 gene expression by 35 and 64%, respectively (Fig. 4D). The regulation of Nr0b2 gene by Sirt1 and FoxO1 was also confirmed by immunoblot analysis (Fig. 4E). These results suggest that the Nr0b2 gene might be a target gene of Sirt1 and FoxO1.

FoxO1 interacts with the Nr0b2 gene promoter. To further investigate the Nr0b2 gene regulation, we first performed an in silico analysis of mammalian Nr0b2 gene promoters and found that there are four conserved putative FoxO1 binding sites (insulin-responsive element, IRE) nearby the previously characterized farnesoid X receptor response element (FXRE) in the proximal promoters of human, mouse, and rat Nr0b2 genes (Fig. 5, A and B). Luciferase reporter assays confirmed that the proximal promoter of human NR0B2 gene could be activated by FXR, FoxO1, and Sirt1 (Fig. 5C). Interestingly, Sirt1 and FXR had additive effects on top of the FoxO1 induction of the NR0B2 gene promoter (Fig. 5C). To examine whether Sirt1 and FoxO1 are associated with the Nr0b2 gene promoter in the chromatin context, we performed ChIP analysis in mouse primary hepatocytes. Indeed, the DNA fragments flanking the conserved IREs (from −300 to −400 bp in mouse Nr0b2 gene promoter) were enriched six- and threefold by FoxO1 and Sirt1 immunoprecipitation, respectively, whereas no specific enrichment was observed in an upstream putative IRE (near −1 kb) (Fig. 5D). These results further suggest that FoxO1 and Sirt1 may regulate the Nr0b2 gene through an interaction with its promoter.

Nr0b2 inhibits Sirt1 and FoxO1-activated gluconeogenesis. Previously, Nr0b2 has been shown to mediate bile acid inhibition of FoxO1 activity in the regulation of G6pc gene expression (47). To test whether Nr0b2 also represses other gluconeogenic genes, we generated a human <i>PDK4</i> promoter reporter construct. As previously reported (22), FoxO1 acti-
verted the human PDK-4 promoter 2.7-fold relative to GFP control (Fig. 6A). Notably, Sirt1 also induced the promoter activity threefold. In contrast, Nr0b2 suppressed the basal or FoxO1- and Sirt1-activated promoter activity by 42, 56, and 41%, respectively. FoxO1 and Sirt1 also exhibited an additive effect on the activation of the PDK4 promoter; however, this was repressed 38% by Nr0b2. To verify the luciferase reporter results, we analyzed endogenous G6PC and PDK4 gene expression in Hep G2 cells after transfection of relevant DNA plasmids (Fig. 6D). As expected, cotransfection of Nr0b2 with Sirt1, FoxO1, or both led to 40, 77, and 78% reduction of G6PC mRNAs, respectively (Fig. 6B). Consistent with the PDK4 promoter assay results, Sirt1 and FoxO1 individually or collectively induced expression of the PDK4 gene, and their activation was again suppressed 31, 43, and 31% by Nr0b2, respectively (Fig. 6C). These data demonstrate that Nr0b2 can negatively regulate FoxO1- and Sirt1-mediated gluconeogenic gene expression.

**DISCUSSION**

As an NAD+-dependent deacetylase, Sirt1 has been suggested to play critical roles in nutrient and energy homeostasis (17, 45, 53). However, the role of Sirt1 in glucose homeostasis is not totally clear (2, 3, 13–15, 25, 27, 28, 30, 39, 40, 51). Recently, it has been reported that Sirt1 activators such as SRT1720 can improve type 2 diabetes by increasing systemic insulin sensitivity and mitochondrial capacity and by lowering hepatic glucose production in the Zucker fa/fa obese rat model (27); however, the specificity of SRT1720 is still a matter of debate (8, 32). Systemic overexpression of Sirt1 in mice has been shown to have protective effects against diabetes and insulin resistance-induced inflammation (2, 34). The increase in circulated adiponectin levels is thought to be part of the anti-diabetes mechanism, whereas induction of antioxidant proteins including MnSOD (manganese superoxide dismutase) and NRF1 (nuclear respiratory factor 1) and downregulation of NF-κB (nuclear factor of κ light polypeptide gene enhancer in B-cells) may contribute to lower inflammation in the Sirt1 transgenic mice (2, 34). In the present study, we have used liver-specific knockout mouse models to address hepatic functions of Sirt1 in glucose homeostasis. Although inactivation of Sirt1 alone in the liver does not cause any significant changes in expression of gluconeogenic genes and blood glucose levels in LKO<sub>Sirt1</sub> mice, pyruvate tolerance is slightly improved, which is consistent with the previous reports by downregulation of Sirt1 using specific shRNA or antisense oligonucleotides (13, 40). Remarkably, Sirt1 inactivation in the DKO<sub>Irs1/2</sub> liver significantly improves hyperglycemia in those mice. Whereas glucose tolerance is only moderately improved, pyruvate tolerance is significantly improved, possibly due to attenuated gluconeogenesis. This phenotype is consistent with gene expression profiles, because expression of gluconeogenic genes is decreased in TKO<sub>Irs1/2:Sirt1</sub> mice compared with DKO<sub>Irs1/2</sub> mice, although glucokinase gene expression is still suppressed. With regard to glucose tolerance, because inactivation of hepatic Sirt1 cannot improve systemic insulin resistance and impaired hepatic glucose utilization, TKO<sub>Irs1/2:Sirt1</sub> mice remain largely glucose intolerant.

Although Sirt1 regulates the activities of several key factors involved in hepatic gluconeogenesis, including FoxO1, PGC-1α, STAT3, CRTC2, and HNF-4α (15, 25, 30, 39, 40, 51), the phenotypes of LKO<sub>Sirt1</sub> and TKO<sub>Irs1/2:Sirt1</sub> mice are surprisingly moderate. In contrast, deletion of FoxO1 in the liver of DKO<sub>Irs1/2</sub> mice largely normalizes insulin-regulated gene expression and systemic glucose (12). By identifying the Nr0b2 gene, which feeds back onto Sirt1 and FoxO1 to modulate their activity.
gene as a target gene of Sirt1/FoxO1, now we understand better about the difference in the regulation of hepatic gluconeogenesis by Sirt1 and FoxO1 (Fig. 6E). In addition to regulation through the Sirt1-FXR pathway (20), the Sirt1-FoxO1 pathway also plays an important role in the regulation of the Nr0b2 gene expression. As an orphan nuclear receptor, Nr0b2 has been shown to inhibit numerous nuclear receptors and transcription factors, including HNF-4α, CREB, and FoxO1 (4, 5, 23, 33, 47). Inactivation of hepatic Sirt1 not only attenuates the functions of PGC-1α and FoxO1 due to their hyperacetylation but may also dampen Nr0b2-mediated negative feedback on transcriptional activities of HNF-4α, CREB, and FoxO1 due to decreased gene expression of Nr0b2. This may partly explain why LKO Sirt1 mice can still maintain normal glycemia, but we cannot rule out that adaptation might have developed due to chronic Sirt1 inactivation, because acute knockdown of Sirt1 in mouse liver lowers both fasting and fed glucose levels (40). In the case of DKO Nr0b2 mice, expression of gluconeogenic genes is highly elevated, and inactivation of Sirt1 might have a greater impact on gluconeogenesis in TKO Nr0b2:Sirt1 mice than in LKO Sirt1 because both mRNA and protein levels of PGC-1α and Pck1 are decreased in TKO Nr0b2:Sirt1 mice. Since Nr0b2 mRNA and protein levels are higher in TKO Nr0b2:Sirt1 than those in LKO Sirt1 liver, feedback inhibition on gluconeogenesis by Nr0b2 might be greater as well. Consistently, Sirt1 knockdown in the liver by specific antisense oligonucleotides lowers hepatic glucose production only in diabetic but not in normal rats (13). However, in the case of FoxO1 knockout, because Nr0b2 can no longer impact FoxO1-mediated gluconeogenesis, hepatic gluconeogenesis is remarkably decreased (12, 47). In addition, unlike downregulation of gluconeogenic genes, inactivation of Sirt1 in the DKO Nr0b2 liver does not alter gck gene expression as FoxO1 does (12), although knockdown of Sirt1 can upregulate Gck gene expression in mouse liver (40), suggesting that insulin plays a predominant role in the regulation of Gck gene, partly through FoxO1.

In summary, hepatic Sirt1 normally modulates hepatic glucose production during fasting through balanced regulation of gluconeogenic genes. One of the mechanisms that confer feedback regulation of hepatic gluconeogenesis is through Sirt1- and FoxO1-controlled Nr0b2 gene expression. This feedback mechanism ensures gluconeogenesis under control.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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