Original Article

MOLECULAR METABOLISM

E2F1 promotes hepatic gluconeogenesis and contributes to hyperglycemia during diabetes

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ABSTRACT

Objective: Aberrant hepatic glucose production contributes to the development of hyperglycemia and is a hallmark of type 2 diabetes. In a recent study, we showed that the transcription factor E2F1, a component of the cell cycle machinery, contributes to hepatic steatosis through the transcriptional regulation of key lipogenic enzymes. Here, we investigate if E2F1 contributes to hyperglycemia by regulating hepatic gluconeogenesis.

Methods: We use different genetic models to investigate if E2F1 regulates gluconeogenesis in primary hepatocytes and *in vivo*. We study the impact of depleting E2F1 or inhibiting E2F1 activity in diabetic mouse models to evaluate if this transcription factor contributes to hyperglycemia during insulin resistance. We analyze E2F1 mRNA levels in the livers of human diabetic patients to assess the relevance of E2F1 in human pathophysiology.

Results: Lack of E2F1 impaired gluconeogenesis in primary hepatocytes. Conversely, E2F1 overexpression increased glucose production in hepatocytes and in mice. Several genetic models showed that the canonical CDK4-RB1-E2F1 pathway is directly involved in this regulation. E2F1 mRNA levels were increased in the livers from human diabetic patients and correlated with the expression of the gluconeogenic enzyme Pck1. Genetic invalidation or pharmacological inhibition of E2F1 improved glucose homeostasis in diabetic mouse models.

Conclusions: Our study unveils that the transcription factor E2F1 contributes to mammalian glucose homeostasis by directly controlling hepatic gluconeogenesis. Together with our previous finding that E2F1 promotes hepatic steatosis, the data presented here show that E2F1 contributes to both hyperlipidemia and hyperglycemia in diabetes, suggesting that specifically targeting E2F1 in the liver could be an interesting strategy for therapies against type 2 diabetes.

Keywords Gluconeogenesis; E2F1; Liver metabolism; Hyperglycemia; Diabetes; Cell cycle regulators

1. INTRODUCTION

Sustaining blood glucose levels within a relatively narrow range is essential for maintaining global homeostasis. This status is achieved through a complex balance between glucose production and utilization by different tissues. These processes are dynamically regulated by hormonal and nutritional signals [1]. Hepatic glucose production, which comprises glycogenolysis and gluconeogenesis (*de novo* production of glucose from non-carbohydrate substrates), is critical for adaptation to fasting conditions and represents up to 80% of total endogenous glucose production [2]. Hepatic gluconeogenesis is largely controlled by substrate flux and by transcriptional regulation of the key rate-limiting enzymes phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose-6-phosphatase (G6PC), which catalyze the first committed steps and the terminal steps of gluconeogenesis, respectively. Several transcription factors and coactivators, control the expression of these gluconeogenic enzymes in response to hormones, especially insulin and glucagon [1]. Aberrant regulation of hepatic glucose production is a major contributor to the hyperglycemia observed in type 2 diabetes and the insulin-resistant state. Indeed, clinical suppression of hepatic glucose production to lower glycemia is one of the main targets of diabetes treatment [2]. In normal subjects, hepatic gluconeogenesis and lipogenesis are mutually exclusive events. In contrast, during diabetes, the liver exhibits abnormally high levels of gluconeogenesis

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Abbreviations: PCK1, Phosphoenolpyruvate carboxykinase 1; G6PC, glucose-6-phosphatase G6PC; PTT, Pyruvate tolerance test; CDK4, cyclin—cyclin-dependent kinase 4; PKA, protein kinase A; cAMP, Cyclic adenosine monophosphate; PPARGC1A, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; FOXO1, Forkhead box protein O1; CREB, cAMP response element-binding protein; HFD, high fat diet; FAO, fatty acid oxidation

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as well as high levels of cholesterol and triglyceride synthesis, resulting in both dyslipidemia and hyperglycemia. This highlights the apparent paradox of selective insulin resistance in the liver [3]. Although some explanations have been proposed, this phenomenon is not yet fully understood [4,5].

E2F transcription factors are the downstream effectors of distinct signaling cascades that regulate the expression of genes involved in cellular homeostasis. In proliferating cells, E2F target genes include effectors of DNA replication, mitosis, DNA repair, and apoptosis [6]. E2Fs exist either as heterodimers associated with dimerization partner (DP) proteins or within larger complexes, including members of the retinoblastoma family of proteins (pRBs). In general, the association of E2Fs with pRB family members induces the repression of their target genes. When phosphorylated by active cyclin–cyclin-dependent kinase (CDK) complexes, pRBs are released, enabling E2Fs to drive transcriptional activation [6].

We have previously demonstrated that E2F1, the first member of the E2F family to be described and studied extensively, has important metabolic functions beyond the control of the cell cycle in non-proliferating cells [7–9]. Studies of the retinoblastoma protein RB1 further support a major role of E2F1 in metabolism [10]. In recent studies, we showed that E2F1 is essential for controlling liver metabolism, regulating cholesterol uptake and promoting lipid synthesis through transcriptional regulation of key lipogenic enzymes [11,12]. We also highlighted that widely used animal models of non alcoholic fatty liver disease (NAFLD) had increased levels of *E2f1* expression and activity in the liver [12]. Here, we demonstrate that E2F1 contributes to mammalian glucose homeostasis through the control of hepatic glucose production. Importantly, our results indicate that E2F1 is critical for inducing hyperglycemia in type 2 diabetes. This suggests that reducing E2F1 activity could protect against obesity-induced hyperglycemia.

2. MATERIALS AND METHODS

Animal experiments. C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). *E2f1*$^{-/-}$ (B6; 129S4-*E2f1*tm1Meg/J) mice and *db*/+ mice (Janvier Labs) were crossed to obtain *db/db; E2f1*$^{+/-}$ mice. *E2F1*$^{tg/+}$ mice harboring the Rosa26-loxP-LacZ-LoxP-E2F1 conditional expression cassette were obtained from Ulrike Ziebold (MDC, Berlin, Germany) [13]. *E2F1*$^{tg/+}$ mice were crossed with *Alb-cre*$^{+}$ mice to obtain *E2F1*$^{tg/+}$ *Alb-Cre*$^{+}$ mice and their control littermates *E2F1*$^{tg/+}$ *Alb-Cre*$^{-}$ mice. *E2f1*$^{fl/fl}$ mice (Taconic Biosciences, NY, USA) were crossed with *Alb-cre*$^{+}$ mice to obtain *E2f1*$^{fl/fl}$ *Alb-Cre*$^{+}$ (*E2f1*-LKO) and their control littermates *E2f1*$^{fl/fl}$ *Alb-Cre*$^{-}$ mice. Mice were housed under a 12-hour light/dark cycle and sacrificed as indicated. For high fat diet (HFD) experiments, mice were fed during 12 or 16 weeks, as indicated, with a HFD (2127, Kliba Nafag or TD06414, Envigo). For pharmacological inhibition of E2F, during the last 10 days of the HFD, mice were gavaged daily with 40 mg/kg of E2F inhibitor HLM006474 (324461, Merck-Millipore) or vehicle. For Pyruvate tolerance test (PTT), mice were fasted overnight and injected with pyruvate (1 g/kg). For insulin tolerance test (ITT), mice were fasted for 6 h and injected with insulin 0.75 U/kg (Actrapid, Novonordisk). Blood glucose was determined using a glucometer and glucose strips (Accu-Chek Aviva, Roche). Serum levels of lactate dehydrogenase, ALT and AST were measured by the Mouse Metabolic Facility of the University of Lausanne. All animal experiments were performed according to Swiss animal welfare laws and were approved by the Canton of Vaud SCAV (authorization VD2627 and VD3046).

Cell culture. HepG2 cells were obtained from the American Type Culture Collection (ATCC). *E2F1*$^{-/-}$ HepG2 cells were described here [11]. Primary hepatocytes were isolated as previously described [14]. Primary hepatocytes were isolated from C57Bl/6J mice, *E2f1*$^{-/-}$ mice (B6; 129S4-*E2f1*^{tm1Meg}/J), *E2F1*$^{tg/+}$ crossed with *Abl-Cre*$^{+}$ mice, *Rb1*$^{fl/fl}$ mice (B6,129-*Rb1*^{tm3Tyj}/J) crossed with *Abl-Cre*$^{+}$ mice, *Cdk4*$^{-/-}$ mice, and *Cdk4*$^{R24C/R24C}$ mice [15] and their respective control mice. C57Bl/6J hepatocytes were not used as controls for mutant hepatocytes. Mouse hepatocytes were isolated, cultured, and infected with adenoviruses Ad-GFP and Ad-E2F1, as previously described [12,14]. For mRNA expression experiments, primary hepatocytes were treated for 3 h with 10 μM Forskolin (Cat. F6886, Sigma-Aldrich) or vehicle (DMSO). For glucose production experiments, hepatocytes were rinsed twice with PBS and cultured in media without glucose, glutamine, and phenol red (Cat. A14430, Life Technologies) supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, and forskolin (10 μM). After 6 h, media was collected for glucose quantification (Cat. GAGO20, Sigma-Aldrich) and proteins were quantified for normalization.

Real-time quantitative PCR analysis. Total mRNA was extracted from 20 to 30 mg of mouse liver or cultured cells using an RNeasy kit (Cat: 74106, Qiagen) according to the manufacturer's protocol. One microgram of the RNA was subsequently reverse-transcribed and quantified via real-time quantitative PCR using an ABI 7900HT instrument. Gene expressions were determined using the delta Ct method or the standard curve method normalized housekeeping gene ribosomal protein S9 (RS9) levels. The complete list of primers is presented in Supplemental Table 2.

Seahorse analyses. Mitochondrial function was determined with an XF-24 extracellular flux analyzer (Seahorse Bioscience). Oxygen consumption Rate (OCR) was measured in control and mutant hepatocytes that were seeded in an XF 24-well cell culture microplate 16 h before the experiment. OCR was expressed as pmol of O2 per minute and was normalized by protein content. Just before the experiment the cells were washed, and the hepatocyte culture medium was replaced with KHB containing 2.5 mM Glucose and 1.5 mM of carnitine. After measuring baseline OCR as an indication of basal respiration, OCR was measured after an acute injection of 300 μM of palmitate coupled to BSA.

ChIP experiment. ChIP was performed as previously described [16]. The sheared DNA was immunoprecipitated by an E2F1 antibody (Cat. 3742, Cell Signaling Technology) coupled to magnetic beads. The immunoprecipitated chromatin was washed, reverse cross-linked at 65°C overnight and purified using minielute columns (Cat. 28006, Qiagen). Finally, E2F1 DNA binding was quantified via real-time quantitative PCR using an ABI 7900HT instrument.

Immunoblotting. Total protein extract and immunoblot analysis were performed as previously described [14]. The following antibodies were used: E2F1 (Cat. 3742, Cell Signaling Technology), Ser^{780} phosphorylated RB (Cat. 8180, Cell Signaling Technology), RB (Cat.C-15 Santa Cruz Biotechnology), CREB (Cat. 9197, Cell Signaling Technology), Ser^{133} phosphorylated CREB (Cat. 9198, Cell Signaling Technology), and beta-ACTIN (Cat. A2066, Sigma-Aldrich).

Human samples. All obese patients included in this study are from the ABOS study (Atlas Biologique de l'Obésité Sévère). This study also included a group of lean and normoglycemic control patients that had surgery for benign and non-inflammatory pathologies. Clinical data were collected at Centre Hospitalier Régional Universitaire de Lille. The protocol concerning the use of biopsies from patients follows French regulations and was approved by the Institutional Ethical Committee of the University of Lille and the Centre Hospitalier Régional Universitaire de Lille (ClinGov NCT 01129297). All patients gave written and

