

Dual SGLT1/SGLT2 Inhibitor Phlorizin Ameliorates Non-Alcoholic Fatty Liver Disease and Hepatic Glucose Production in Type 2 Diabetic Mice

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Purpose: NAFLD is a hepatic component of type 2 diabetes mellitus (T2D), in which impaired hepatic glucose production plays an important role. Inhibitors of sodium glucose transporter 2 (SGLT2) reduce glycemia and exert beneficial effects on diabetic complications. Recently, dual SGLT1/2 inhibition has been proposed to be more effective in reducing glycemia. We hypothesized that improving hepatic glucose metabolism induced by SGLT1/2 inhibition could be accompanied by beneficial effects on NAFLD progression.

Methods: Glycemic homeostasis, hepatic glucose production and NAFLD features were investigated in obese T2D mice, treated with SGLT1/2 inhibitor phlorizin for 1 week.

Results: T2D increased glycemia; insulinemia; hepatic expression of phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and glucose transporter 2 (*Slc2a2* gene); hepatocyte nuclear factors 1A/4A/3B-binding activity in *Slc2a2*; endogenous glucose production; liver weight, plasma transaminase concentration as well as hepatic inflammation markers, and induced histological signals of non-alcoholic steatohepatitis (NASH, according to NASH-CRN Pathology Committee System). Phlorizin treatment restored all these parameters (mean NASH score reduced from 5.25 to 2.75 $P < 0.001$); however, plasma transaminase concentration was partially reverted and some hepatic inflammation markers remained unaltered.

Conclusion: NAFLD accompanies altered hepatic glucose metabolism in T2D mice and that greatly ameliorated through short-term treatment with the dual SGLT1/2 inhibitor. This suggests that altered hepatic glucose metabolism participates in T2D-related NAFLD and highlights the pharmacological inhibition of SGLTs as a useful approach not only for controlling glycemia but also for mitigating development and/or progression of NAFLD.

Keywords: GLUT2, PEPCK, G6Pase, phlorizin, NASH

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in Western countries.¹⁻³ NAFLD exhibits a spread pathophysiological spectrum, and the onset of inflammation characterizes the non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and hepatocellular carcinoma.^{2,4-6}

NAFLD is the result of a multi-factorial process, and insulin resistance has been proposed to play a major role in hepatocyte fat accumulation.³ NAFLD is still under-diagnosed, since liver biopsy and histopathological analysis are required.⁷ However, improvements in image analysis systems are facilitating non-invasive diagnostic methods.⁸ Besides, metabolomics is a new tool that can contribute to diagnosing the

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disease and understanding its pathophysiology.⁵ Although disorders in lipid metabolism play an important role in NAFLD development, alterations in the metabolism of amino acids and carbohydrates have also been related to the disease; however, the only glucose metabolism marker that has been related to NAFLD is blood glucose concentration.⁵ Thus, insulin resistance and altered glycemic homeostasis have been associated to NAFLD, which is considered as the hepatic component of obesity-related metabolic syndrome and type 2 diabetes mellitus (T2D),^{8,9} conditions characterized by impaired glucose metabolism.

Hepatocytes play a fundamental role in glycemic homeostasis by promoting glucose influxes to and effluxes from these cells, depending on the fed state and hormonal regulation.¹⁰ For that, glucose transporter 2 (GLUT2, codified by *Slc2a2* gene) and enzymes gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK, codified by *Pck1* gene) and glucose-6-phosphatase (G6Pase, codified by *G6pc* gene) exert a key role.¹⁰ Under insulin-resistant conditions, increased hepatic glucose output is expected, compromising hepatocyte homeostasis and glycemic control.¹¹ However, associations of these disorders with NAFLD have not been clearly demonstrated yet.

In the kidney, glucose reabsorption is mainly performed by a sodium-glucose cotransporter 2 (SGLT2, codified by *Slc5a2* gene), and a residual tubular glucose amount is removed by SGLT1 (codified by *Slc5a1* gene).¹² When glucose filtration rate increases, there is an increase in SGLT2/SGLT1-mediated reabsorption, but these cotransporters saturate, and glycosuria becomes evident.¹¹ For decades, the capacity of phlorizin to inhibit SGLT1/SGLT2 activity inspired researchers to develop a drug that could be administered orally, thus contributing to the diabetes mellitus pharmacopeia.¹³ Firstly, these inhibitors were selectively designed to inhibit only SGLT2, but the limited benefits of these drugs stimulated the development of the dual SGLT1/2 inhibitors.¹³ Nowadays, these inhibitors are considered capable of not only reducing glycemia, but also exerting beneficial effects on the cardiovascular and renal damages induced by T2D.¹³ Additionally, several SGLT2 inhibitors have been proposed to exert beneficial effects upon NAFLD, in both animal models^{14–19} and humans²⁰ with T2D. However, the mechanisms involved in these effects and their possible association with hepatocyte glucose homeostasis are unclear. Besides, effects of the dual SGLT1/2 inhibition in NAFLD have never been investigated.

Once insulin resistance and T2D contribute to the development and/or progression of NAFLD, we hypothesized

that an improvement in glycemic homeostasis and in hepatocyte glucose metabolism induced by SGLT1/2 inhibition could determine beneficial effects on NAFLD progression.

Methods

Animals and Treatments

Obese T2D animals were obtained by neonatal subcutaneous injection of monosodium glutamate (MSG) in male offspring of CD1 mice, during the first 5 days of life (2 mg/g body weight/day), as previously described.²¹ Animals were weaned at 3 weeks and studied at 16 or 17 weeks of life, according to the protocol.

Protocol one: Four-week-old MSG mice were allowed to take standard-diet (T2D) or a high-fat diet (HF-T2D), containing 7% or 35.2% of fat (in terms of g), respectively (Pragsoluções Biotecnológicas, Jaú, SP, Brazil). Analyses were performed 12 weeks later (16-week-old mice) when the animals were obese and had developed T2D.

Protocol two: Standard-diet-fed 16-week-old MSG mice (T2D) were treated or untreated with subcutaneous injections of 0.4 g/kg body weight of phlorizin (CDS000104, Sigma Chemical Co, USA, solution at 0.4 g/mL of propylene glycol) twice a day for 6 days (Phl-T2D).²²

For both protocols, control mice were age-matched, not injected with MSG and fed with standard diet. Experimental procedures were performed in anaesthetized animals (60 mg/kg body weight of sodium pentobarbital, intraperitoneally) from 8:00 to 10:00 a.m., preceded by 4 hrs of food deprivation. Lee's index was used to estimate the obesity degree [body weight (g)^{1/3}/naso-anal length (cm) × 100]. Liver was sampled and stored at -70°C for molecular analysis or immediately processed for histological analysis. Blood was collected by cardiac puncture and plasma was stored for further analyses. All experimental procedures were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo (#082/sheet 105 of book 2), in accordance with the Brazilian College for Animal Experimentation and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Insulin Tolerance Test (ITT)

ITT was performed in 4 hr food-deprived mice at 10:00 a.m., under non-anesthetized conditions. Tail blood was collected at 0 (basal), 5, 10, 20, 30 and 50 min after intraperitoneal injection of regular insulin (0.75 U/kg body weight, Humulin R, Eli Lilly and Co. Indianapolis, USA). Insulin sensitivity

was determined by the glucose disappearance constant (kITT), based on the linear regression of the Napierian logarithm of blood glucose concentration during the test.²¹

Piruvate Tolerance Test (PTT)

PTT was performed in 12 hr food-deprived mice at 8:00 a.m.²¹ Anesthetized animals were injected with pyruvate solution (0.25 g/mL), at a dose of 2 g/kg body weight, intraperitoneally. Glycemia was measured in tail blood at 0 (basal), 10, 20, 30, 40, 60, 90 and 120 min after pyruvate injection. The area under the curve of glycemia was calculated by using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA), and represents the hepatic glucose production.²¹

Liver Glycogen Content

The glycogen content was measured as previously described.²³

Blood Analyses

Blood glucose was measured by a glucometer (ACCU-CHEK[®], Roche, Basel). Plasma concentrations of glucose, triglycerides, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined by colorimetric assay (Labtest Diagnóstica SA, Lagoa Santa, MG, Brasil, and Laborlab, Guarulhos SP, Brasil). Plasma insulin concentration was assessed by ELISA (EMD Millipore Co, MO, USA).

Western Blotting Analyses

Target proteins were analyzed in liver samples by Western blotting as previously described^{21,22,24} and using anti-GLUT2 (#07-1402, EMD Millipore), anti-G6Pase (ab-83690, Santa Cruz Biotechnology), anti-PEPCK (ab-28455, Santa Cruz Biotechnology) and anti-ACTB (A2228, Sigma Aldrich) antibodies. The blots intensity was quantified by densitometry, and results were normalized by the respective ACTB values, considering the mean of control values as one.

Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed in liver samples as previously described.^{21,23} After checking gene stability, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was chosen as the reference gene. Following the RT, the quantitative (q) PCR amplification was performed using the TaqMan[®] PCR master mix kit (Applied Biosystems, ThermoFisher

Scientific, USA), and carried out in a StepOne Plus Instrument (Applied Biosystems Inc., CA, USA). The method of $2^{-\Delta\Delta C_t}$ was adopted for analysis. Identification of the TaqMan[®] assays is presented in [Supplementary Table 1](#).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed in liver samples as previously described.^{24,25} Briefly, double-stranded oligonucleotides containing the binding site of the investigated transcription factors in the *Slc2a2* promoter gene were end-labeled with T4 polynucleotide kinase (Invitrogen, Carlsbad, CA, USA) and γ -³²P ATP (PerkinElmer Life, Waltham, MA, USA). Nuclear proteins were incubated with the labeled oligonucleotide probe in a buffer, for 20 min at room temperature. DNA/protein complexes were electrophoresed on 4% non-denaturing polyacrylamide gel at 4°C. The gel was dried and exposed to a hyperfilm (Amersham Hyperfilm ECL, GE Healthcare Life Sciences) for 2 to 3 days at -80°C. The blots were analyzed by scanner densitometry (ImageScanner III, GE Healthcare, Sweden). Results were expressed as arbitrary units, relative to the mean value of the controls which was set as one. Competition binding experiments were performed by adding 10- to 100-fold molar excess of unlabeled oligonucleotides. Specificity of the transcription factors was checked by incubation with the specific antibodies. Oligonucleotides and antibodies are described in the [Supplementary Table 2](#).

Histological Analysis of Liver

Liver samples were fixed in paraformaldehyde and 5 μ m slices were stained with hematoxylin and eosin for analysis in light microscopy. The analyses were performed on 10 random fields, captured at 20x magnification, using an optical microscope equipped with a digital camera Olympus DP72 (Olympus, Rungis, France). Evaluation of NAFLD was performed based on the NASH-CRN Pathology Committee System,²⁶ as detailed in [Supplementary Table 3](#). The range of total scores varies from zero to 8 and indicates 0–2, absence of NAFLD; 3 and 4, probable presence of NAFLD, ≥ 5 clear presence of NAFLD.

Names of Genes and Proteins

Names and acronyms of genes and proteins used throughout the manuscript are according to the HGNC and Uniprot databases, respectively.

Statistical Analyses

Data were expressed as mean \pm standard error mean (SEM). Comparison of the means, according to the normality of the data distribution (Kolmogorov–Smirnov test), was performed by one-way analysis of variance (ANOVA), followed by Newman-Keuls post-test, or by Kruskal–Wallis test, followed by Dunn post-test. Comparisons were considered statistically significant at $p < 0.05$. Analyses were performed using GraphPad Prism version 7.04 (GraphPad Software Inc., San Diego, USA).

Results

MSG Induces T2D in Mice Regardless of High-Fat Diet

With protocol one, we analyzed the effects of the neonatal MSG treatment (T2D) and the effects of 12-week-treatment of MSG mice with high-fat-diet (HF-T2D) (Table 1). The T2D-mice became obese as observed by the high body weight mass, peri epididymal adipose tissue and Lee's index value; high-fat-diet exacerbated the degree of obesity. As expected for MSG-mice, food intake was decreased, both in g per day and in kcal per day, while high-fat-diet decreased food intake in g per day, but increased it in terms of kcal per day, because of the high energy density of the diet.

Table 1 General Characteristics of Control (C), Obese Type 2 Diabetes Mellitus (T2D) and Obese High-Fat-Fed T2D (HF-T2D) Mice

	C	T2D	HF-T2D
Body weight (g)	44.9 \pm 1.45	51.3 \pm 1.80*	53.1 \pm 2.50*
Food intake (g/day)	6.99 \pm 0.14	6.09 \pm 0.24**	5.22 \pm 0.08***###
Food intake (kcal/day)	26.9 \pm 0.53	23.4 \pm 0.92**	26.8 \pm 0.52###
Lee's index	33.8 \pm 0.35	37.1 \pm 0.48***	39.5 \pm 0.55***###
Adipose tissue (g)	1.50 \pm 0.08	2.10 \pm 0.11**	2.71 \pm 0.15***###
Plasma glucose (mM)	10.0 \pm 0.51	15.7 \pm 0.72**	18.3 \pm 1.78***
Plasma insulin (pM)	24.6 \pm 4.99	83.9 \pm 20.3*	97.9 \pm 18.5*
Plasma triglycerides (mM)	1.15 \pm 0.81	1.48 \pm 0.057**	1.51 \pm 0.061**
Plasma AST	42.4 \pm 15.5	166.8 \pm 20.3**	164.1 \pm 29.3**
Plasma ALT	44.5 \pm 11.1	174.2 \pm 26.5***	145.4 \pm 22.1**
HOMA-IR	1.62 \pm 0.12	8.1 \pm 0.79***	9.11 \pm 0.14***
kITT (%/min)	1.59 \pm 0.13	0.86 \pm 0.12**	0.61 \pm 0.14***

Notes: Lee's index: [body weight (g)^{1/3} + naso-anal length (cm)] \times 100; adipose tissue: peri epididymal fat pad; Data are means \pm EPM of 7–16. Data of plasma insulin were compared by Kruskal–Wallis test, followed by Dunn post-test; other data were compared by ANOVA, followed by Newman-Keuls post-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs C; ### $P < 0.01$ vs T2D.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostatic model assessment of insulin resistance; kITT, glucose decay constant during insulin tolerance test.

Despite the different obesity degrees, both T2D and HF-T2D mice showed similar metabolic-hormonal alterations (Table 1), such as significant increases in plasma glucose, insulin and triglycerides. Furthermore, T2D and HF-T2D presented very high indexes of HOMA-IR and decreased indexes of glucose decay during insulin tolerance test (kITT), clearly revealing their insulin-resistant state. Increased plasma concentrations of the hepatic enzymes AST and ALT were also observed in both T2D mice, indicating some grade of liver injury.

T2D Increases Expression of Hepatic Glucose Production Markers

Figure 1A shows that, regardless of the diet, T2D mice presented higher *Slc2a2* (70% and 80%), *Pck1* (170%) and *G6pc* (112% and 140%) mRNA contents ($P < 0.01$ to $P < 0.001$). Similarly, the respective proteins (Figure 1B and C) also increased ($P < 0.05$ to 0.01), although the magnitude of the increase is slightly smaller, suggesting some posttranscriptional regulation. Additionally, disturbed glucose metabolism was revealed by the 240% and 270% increase ($P < 0.001$) in hepatic glycogen content observed in T2D and HF-T2D mice, respectively (Figure 1D).

T2D Increases Transcriptional Activity of HNF1A, HNF3B, HNF4A and NFKB Upon *Slc2a2* Gene

To investigate transcriptional mechanisms potentially involved in the regulation of the expression of genes related to glucose hepatic metabolism in T2D, we have chosen *Slc2a2* gene, related to the final and limiting step of glucose influx/efflux. Analysis of the transcription factors of *Slc2a2* gene revealed that expression of *Hnf1a*, *Hnf4a* and *Foxa2* mRNAs (Supplementary Figure 1A) as well as *Rela* mRNA (Figure 2B) increased ($P < 0.05$ to $P < 0.001$) by 70% to 170% in both T2D and HF-T2D mice.

Thus, the binding activity of these transcriptional factors to their specific binding site in the *Slc2a2* promoter region was analyzed by EMSA, after confirming the specificity of the nuclear protein binding (Supplementary Figure 2). Figure 1E shows representative images of the specific binding, and Figure 1F shows that the binding activity in the *Slc2a2* promoter region increased ($P < 0.05$ to $P < 0.001$) by ~60% (HNF1A and HNF4A) to ~150% (HNF3B and NFKB) in both T2D and HF-T2D mice.

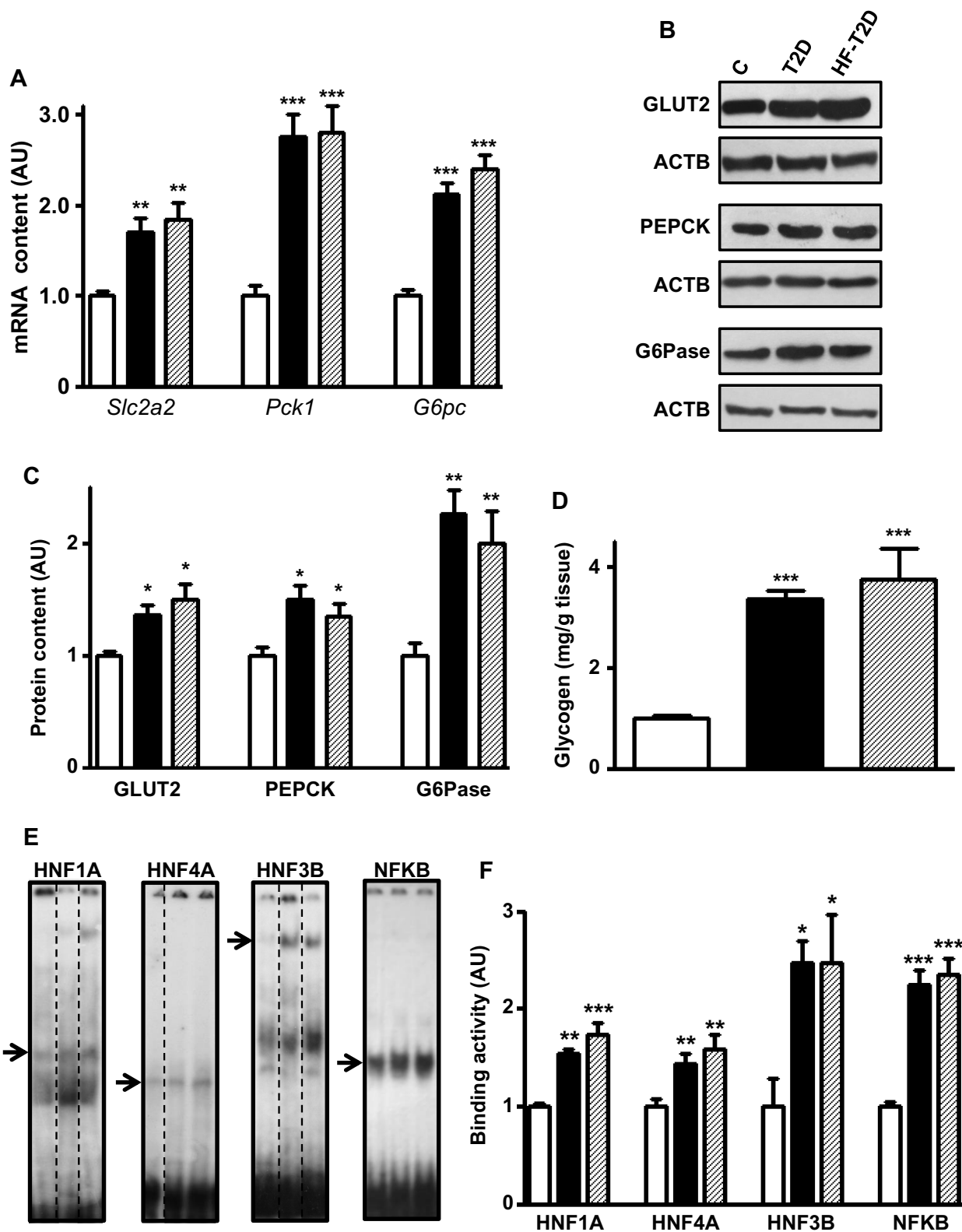


Figure 1 Type 2 diabetes (T2D) effects upon hepatic glucose production markers. Liver samples from control (C, white bars), type 2 diabetic (T2D, black bars) and high-fat-fed T2D (HF-T2D, dashed bars) mice were analyzed. (A), *Slc2a2*, *Pck1* and *G6pc* mRNA; (B and C) GLUT2, PEPCK and G6Pase protein; (D) glycogen content; (E and F) HNF1A, HNF4A, HNF3B and NFKB binding activity in the *Slc2a2* gene promoter: DNA/protein binding activity was evaluated by electrophoretic mobility shift assay (EMSA). In (E), dashed lines indicate that the lanes were not contiguously placed in the gel; for each transcription factor, the sequence of the lanes corresponds to the sequence of the results shown in the graphs; the arrows indicate the target protein/DNA complex. Data are expressed as mean \pm SEM of 14 (GLUT2), 4 (EMSA for NFKB) or 5 to 10 animals (other results), and were analyzed by Kruskal–Wallis followed by the Dun post-test (*Slc2a2* mRNA and GLUT2) or one-way ANOVA followed by the Newman-Keuls post-test (other results). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs C.

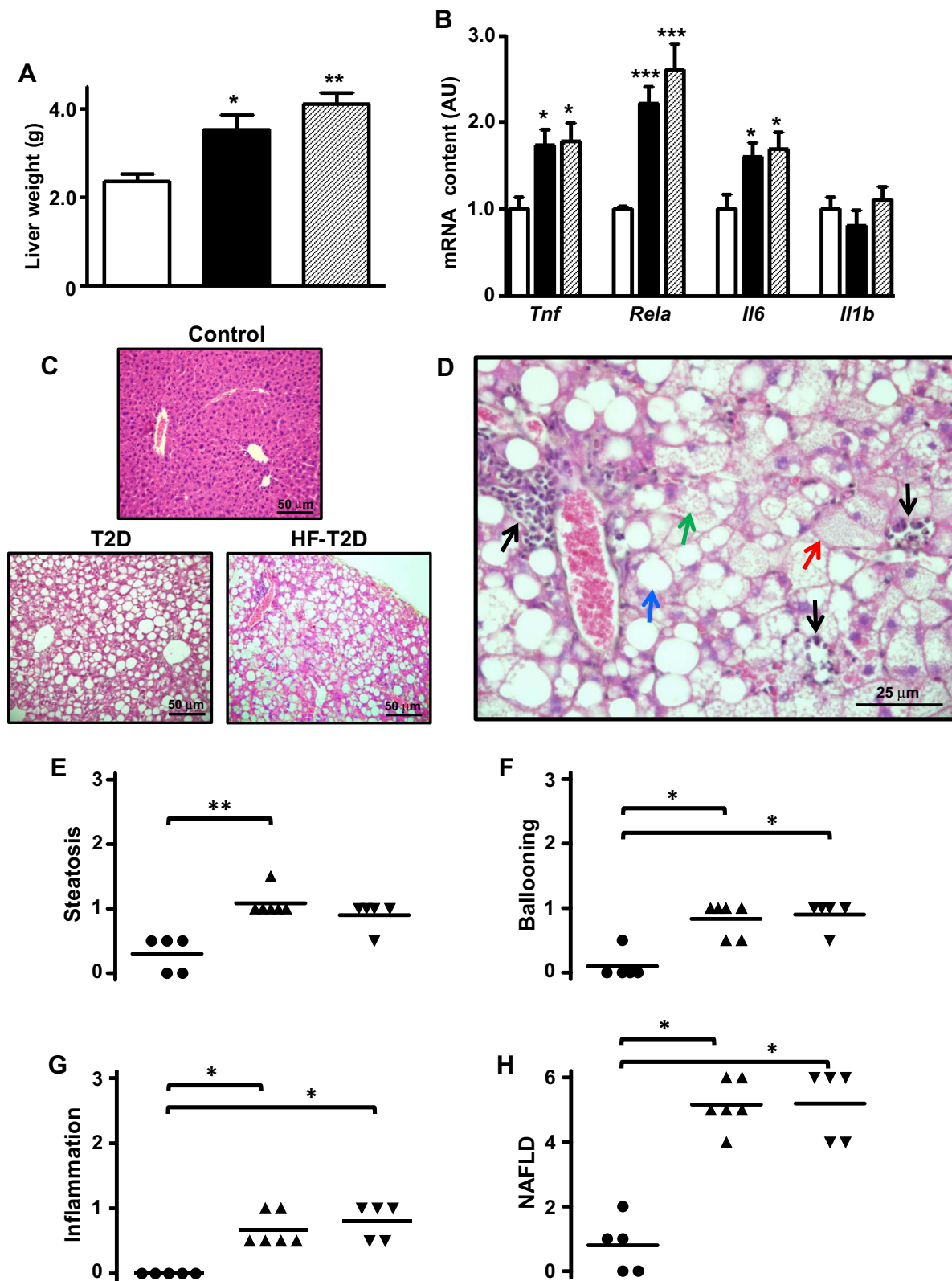


Figure 2 Type 2 diabetes (T2D) induces severe NAFLD. Liver from control (C, white bars, circles), type 2 diabetic (T2D, black bars, up triangles) and high-fat-fed T2D (HF-T2D, dashed bars, down triangles) mice were analyzed. (A), liver weight; (B) mRNA expression of inflammatory markers *Tnf*, *Rela*, *Il6* and *Il1b*; (C) hematoxylin-eosin stained liver tissue revealing evident steatosis in T2 mice (20X magnification); (D) increased magnification (40X) of hematoxylin-eosin stained liver tissue from T2D mice revealing the presence of micro-steatosis (green arrow), macro-steatosis (red arrow), ballooning (blue arrow) and inflammatory foci (black arrows); (E-G) steatosis, ballooning and inflammation grade; (H) NAFLD score. Data are expressed as mean \pm SEM of 10 to 16 (A) or 6 to 8 animals (B). In (E-H), data are the means and the individual values of 5 animals. The means were analyzed by one-way ANOVA followed by the Newman-Keuls post-test (A, B) or Kruskal-Wallis followed by the Dun post-test (E-H). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs (C).

T2D Induces Severe NAFLD in Mice

Increased liver mass was observed in both T2D (50%, $P<0.05$) and HF-T2D (74%, $P<0.01$) mice (Figure 2A). Hepatic proinflammatory activity (Figure 2B) is suggested by increased expression of *Tnf*, *Il6* and *Rela* mRNAs in both T2D and HF-T2D mice (by 70% to 120%, $P<0.05$ to $P<0.001$). Histological examination (Figure 2C) revealed extensive hepatic alterations in T2D mice; Figure 2D shows in detail the main features of NAFLD such as micro- and macro-steatosis, hepatocyte ballooning and several extensive inflammatory foci. No signals of fibrosis were observed in liver from T2D mice. Quantification of the NAFLD components is shown in Figure 2E–G. The final mean scores (Figure 2H) for NAFLD in T2D (5.17 ± 0.31) and in HF-T2D (5.2 ± 0.49) were significantly higher ($P<0.05$) than the mean value of controls (1.0 ± 0.45) and indicated the development of NASH in T2D mice.

Phlorizin Treatment Reverses the Metabolic Derangements of T2D Mice

With protocol two, we analyzed the effects of 6-day phlorizin treatment in standard-fed T2D mice (Table 2). Phlorizin treatment did not alter the obesity degree; however, it reversed hyperglycemia, hyperinsulinemia, hypertriglyceridemia and decreased the HOMA-IR value. Besides, increased plasmatic AST and ALT concentrations were partially reversed (47% and 51% reduction, respectively), though remaining higher than the values observed in control mice (Table 2).

Table 2 General Characteristics of Control (C), Obese Type 2 Diabetes Mellitus (T2D) and Phlorizin-Treated T2D (Phi-T2D) Mice

	C	T2D	Phi-T2D
Body weight (g)	43.8 ± 1.3	50.3 ± 1.6**	50.9 ± 1.6**
Plasma glucose (mM)	9.94 ± 0.75	16.3 ± 0.62**	10.4 ± 0.83###
Plasma insulin (pM)	23.9 ± 4.81	74.8 ± 20.0**	31.9 ± 4.55###
Plasma triglycerides (mM)	1.15 ± 0.07	1.51 ± 0.06**	1.25 ± 0.05###
Plasma AST	40.0 ± 4.9	164.0 ± 8.7***	105.4 ± 4.7***###
Plasma ALT	45.1 ± 5.1	151.3 ± 13.2***	87.5 ± 12.8###
HOMA-IR	1.46 ± 0.02	7.32 ± 0.08***	1.99 ± 0.03###

Notes: Data are means ± EPM of 5–10 animals. Data of plasma insulin were compared by Kruskal–Wallis test, followed by Dunn post-test; other data were compared by ANOVA, followed by Newman-Keuls post-test. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs C; ### $P<0.01$ and #### $P<0.001$ vs T2D.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostatic model assessment of insulin resistance.

Phlorizin Treatment Restores Alterations in Hepatic Glucose Production Markers Observed in T2D Mice

Phlorizin treatment of T2D mice reversed the increased hepatic expression of *Slc2a2*, *Pck1* and *G6pc* mRNA contents (Figure 3B) and their respective proteins GLUT2, PEPCK and G6Pase (Figure 3C and D) ($P<0.05$ to $P<0.001$ vs T2D), suggesting a reduction in hepatic glucose production and efflux. The increased hepatic glycogen content observed in T2D was partially restored by phlorizin (Figure 3A).

A pyruvate tolerance test (PTT) was performed to confirm the functional role of these markers in hepatic glucose production. In response to the pyruvate injection (Figure 3E), the increase in blood glucose was clearly higher in T2D mice, and that was reversed by phlorizin treatment. The area under the curve of glycemia during the 0 to 120 min of the PTT (Figure 3F) was ~2-fold-increased in T2D mice ($P<0.001$ vs C), and that was completely restored by phlorizin treatment ($P<0.001$ vs T2D).

Finally, the analysis of *Slc2a2* gene transcription factors revealed that expression of *Hnf1a*, *Hnf4a* and *Foxa2* mRNAs (Supplementary Figure 1B) as well as *Rela* mRNA (Figure 4D) decreased ($P<0.05$ to $P<0.001$) in Phi-T2D mice as compared to T2D mice. Additionally, the binding activity of transcriptional factors HNF1A, HNF4A, HNF3B and NFkB into the promoter region of *Slc2a2* gene (Figure 4A and B), which was increased in T2D, was reversed by phlorizin treatment, explaining the mechanism through which *Slc2a2* mRNA was reversed.

Phlorizin Treatment Ameliorates NAFLD in T2D Mice

Hepatic mRNA expression (Figure 4D) of inflammatory genes *Tnf* and *Il6* remained unaltered, while *Rela* mRNA expression decreased in response to phlorizin treatment ($P<0.05$ vs T2D); however, it remained significantly higher as compared to control mice (87%, $P<0.001$ vs C).

The increased liver mass presented in T2D mice was reversed by phlorizin treatment (Figure 4C). Histological analysis (Figure 4E) revealed a noticeable amelioration of T2D-induced NAFLD. Hepatic steatosis (Figure 4F) and hepatocyte ballooning (Figure 4G) presented in T2D mice were reversed by phlorizin treatment ($P<0.01$ vs T2D), but tissue inflammation was not (Figure 4H). The mean NAFLD score (Figure 4I) decreased after phlorizin treatment (from 5.25 to 2.75, $P<0.001$), indicating an

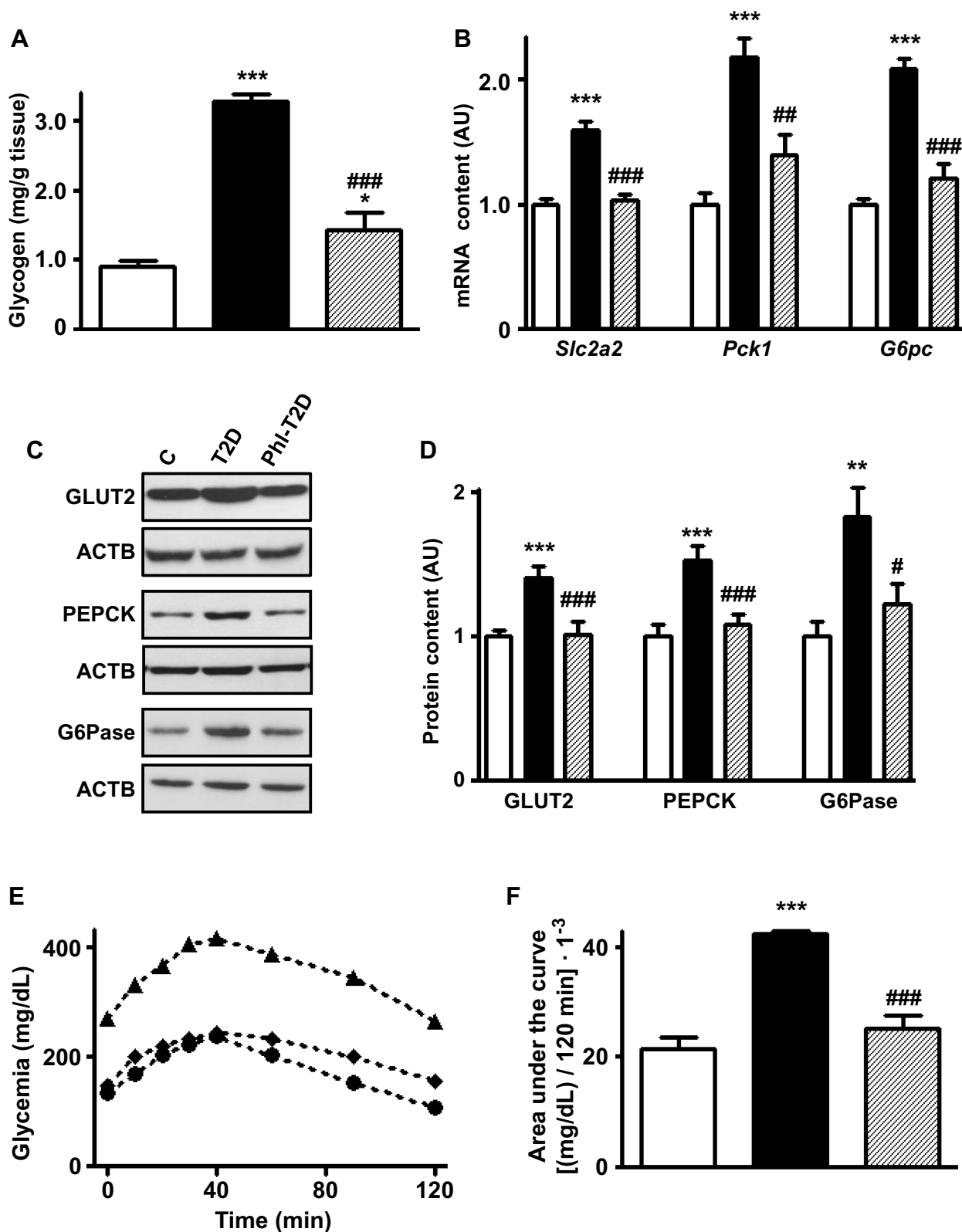


Figure 3 Phlorizin treatment restores type 2 diabetes-induced alterations in hepatic glucose production markers. Liver samples from control (C, white bars, circles), type 2 diabetes (T2D, black bars, up triangles) and phlorizin-treated T2D (PhI-T2D, dashed bars, diamonds) mice were analyzed: (A), glycogen content; (B) *Slc2a2*, *Pck1* and *G6pc* mRNA; (C and D) GLUT2, PEPCK and G6Pase protein; (D) glycogen content; (E and F) hepatic glucose production during the pyruvate tolerance test: (E) zero to 120 min blood glucose concentration; (F) area under the curve of the blood glucose variation during the test. Data are expressed as mean \pm SEM of 5 (A), 6 to 7 (B), 6 to 10 (D) or 4 to 5 (E and F) animals. The means were compared by one-way ANOVA followed by the Newman-Keuls post-test. * $P < 0.05$ and *** $P < 0.001$ vs C; ### $P < 0.01$ and #### $P < 0.001$ vs T2D.

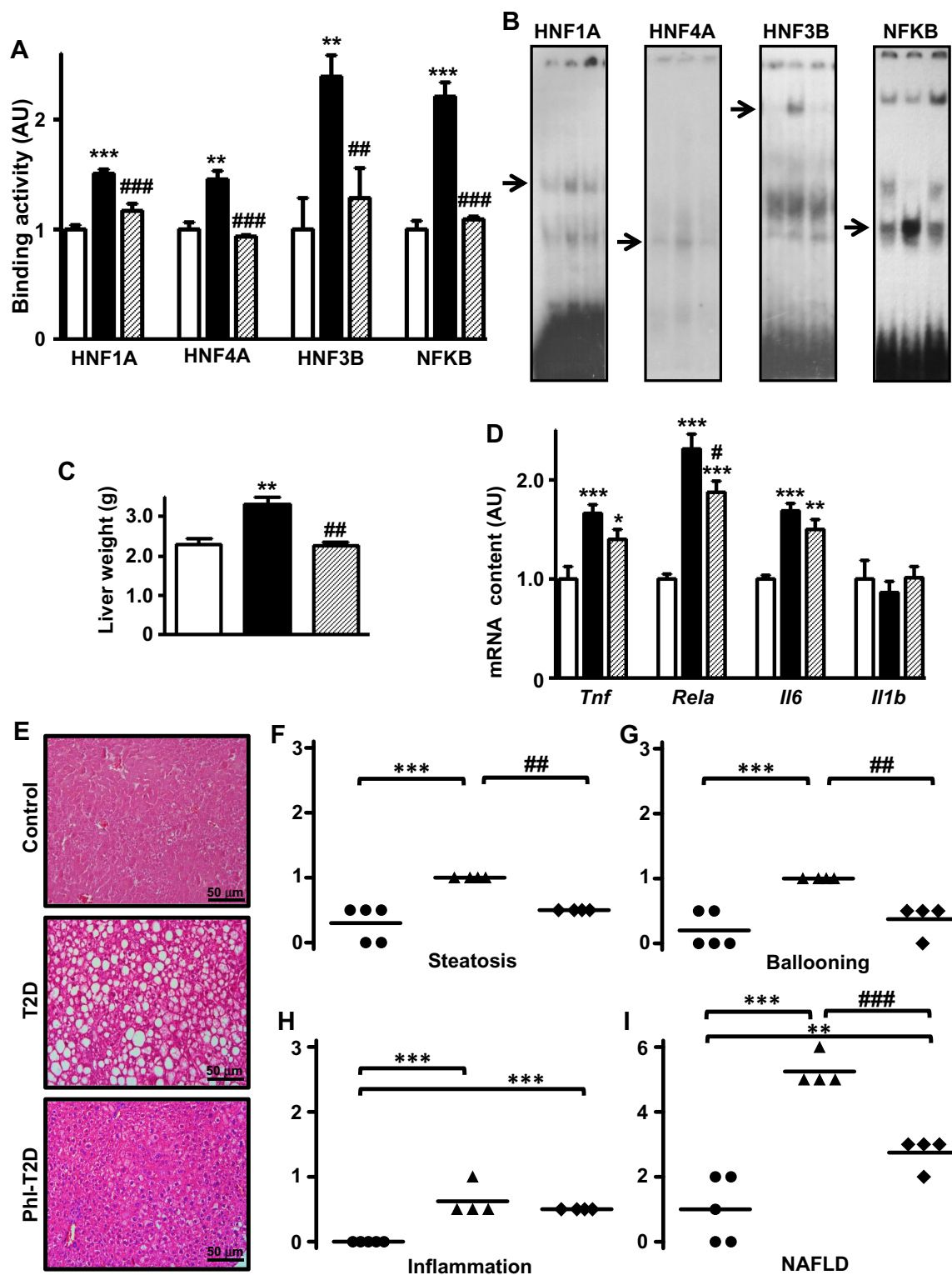


Figure 4 Phlorizin treatment ameliorates type 2 diabetes-induced NAFLD. Liver samples from control (C, white bars, circles), type 2 diabetes (T2D, black bars, up triangles) and phlorizin-treated T2D (Phl-T2D, dashed bars, diamonds) mice were analyzed: (A and B) HNF1A, HNF4A, HNF3B and NFKB binding activity in the *Slc2a2* gene promoter measured by electrophoretic mobility shift assay (EMSA). In (B), for each transcription factor, the sequence of the lanes corresponds to the sequence of the results shown in the graphs; the arrows indicate the target protein/DNA complex. (C), liver weight; (D) mRNA expression of inflammatory markers (*Tnf*, *Rela*, *Il6* and *Il1b*); (E) hematoxylin-eosin stained liver tissue (20X magnification); (F–H) steatosis, ballooning and inflammation grade; (I) NAFLD score. Data are expressed as mean \pm SEM of 4 to 7 (A and D) or 5 to 9 (C) animals. In (F–I), data are the means and the individual values of 4 or 5 animals. The means were compared by Kruskal–Wallis, followed by the Dun post-test (HNF4A EMSA and *Tnf* mRNA) or one-way ANOVA, followed by the Newman-Keuls post-test (other results). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs C; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs T2D.

amelioration of NASH, although it remained suggestive of NASH: 3 animals showed score=3 (suggestive of NASH) and one animal showed score=2 (not NASH).

Discussion

The present study investigated the effect of the dual SGLT1/2 inhibitor phlorizin in diabetes-related NAFLD, as well as the possible participation of glucose hepatic metabolism in this process, using the MSG-induced T2D mice model. Half a century ago, the neonatal administration of MSG to mice was described as capable of inducing obesity in adult life.²⁷ Later on, it was demonstrated that adult MSG mice develop insulin resistance with severe hyperglycemia, thus characterizing an obese T2D experimental model.^{21,28-34} Interestingly, the glycemic impairment occurs concurrently with hyperinsulinemia in T2D MSG-mice,^{21,29,31,34} a profile not observed in T2D humans, in whom loss of glycemic control appears when plasma insulin concentrations begin to decrease. This difference is a consequence of the high replication capacity of beta cells in young mice in response to increased insulin requirements.³⁵ Furthermore, liver glycogenesis was observed in T2D, indicating that, despite the insulin resistance, hyperglycemia and hyperinsulinemia, together, guarantee increased glycogen storage.³⁶

More recently, it has been reported that MSG-mice might develop NAFLD^{30,32,33,37} and these animals have been proposed as an useful model for the study of NAFLD.³⁸ However, in these studies, NAFLD has been related to aging^{32,37} or to a high-fat (HF) diet,³⁰ highlighting that HF-diet is capable of inducing NAFLD per se in C67BL/6J mice, without impairment of glycemic control.³⁹ Because of that, we initially investigated T2D-mice receiving or not a HF-diet, and the results revealed that the HF-diet did not alter the metabolic/hormonal impairment and the liver injury, indicating that the MSG treatment was sufficient to induce T2D and NAFLD/NASH.

The involvement of impaired hepatic lipid metabolism in the development of NAFLD is well known⁵ and a clear association between NAFLD and insulin resistance in T2D is also well demonstrated, suggesting that altered hepatic glucose metabolism may also be related to NAFLD physiopathology.^{8,40,41} However, whether insulin resistance is a cause³ or a consequence⁴¹ of NAFLD is a great scientific challenge, and probably both hypotheses are true, comprising a vicious circle. Importantly, high intracellular glucose concentration is a powerful inducer of de novo lipogenesis in isolated hepatocytes,⁴² and that highlights impaired glucose

metabolism as an important mechanism in NAFLD pathogenesis. We observed NAFLD/NASH in obese T2D-mice with severely impaired glycemic homeostasis and hepatic glucose metabolism, reinforcing the role of altered hepatic glucose metabolism in the NAFLD pathogenesis of T2D subjects.

In the present study, we reported, for the first time, the impairment of hepatic glucose metabolism in T2D-mice with NAFLD. Altogether, the data demonstrated augmented hepatic glucose production in T2D-mice, a phenomenon in which the increased hepatocyte glucose efflux depends on upregulated GLUT2 expression. Considering that, we investigated molecular mechanisms involved in the transcription of *Slc2a2* gene, and we detected increased activity of the *Slc2a2* enhancers HNF1A, HNF3B, HNF4A and NFKB. Importantly, these transcriptional factors have also been related to gluconeogenic *Pck1* gene, which can be activated by high intracellular glucose concentration.⁴³ Thus, the activation of the transcriptional factors reported here in T2D-mice must be playing a fundamental role not only in the upregulation of GLUT2, guaranteeing increased hepatic outflow, but also in the production of hepatocyte glucose.

The development of SGLT2 inhibitors, as well as dual SGLT1/2 inhibitors, has expanded the pharmacopeia for T2D, not only regarding glycemic control, but also focusing on the prevention and/or treatment of diabetic complications.¹³ Concerning diabetes-associated liver disease, SGLT2 inhibitors such as ipragliflozin,^{14,15,18,20} remogliflozin etabonate,¹⁷ empagliflozin¹⁶ and luseogliflozin¹⁹ have been reported to be able to prevent and/or to alleviate the development of NAFLD in several experimental models of NAFLD. Besides, ipragliflozin²⁰ and dapagliflozin⁴⁴ were also reported to ameliorate steatosis-associated hepatic enzymatic markers in T2D subjects with NAFLD. However, these studies investigated neither the effects of a dual SGLT1/2 inhibitor nor the NAFLD relationship with hepatic glucose metabolism.

We chose to investigate phlorizin due to its dual SGLT1/2-inhibitor effect, and we conducted a 6-day-treatment since this period of time has already been reported as sufficient to reduce the expression of *Slc2a2*/GLUT2 in liver and kidney of T1D rats.^{22,24} In the present study, phlorizin treatment reversed the metabolic hormonal characteristics of T2D, as well as the markers of increased hepatic glucose production.

The reduction observed in hepatic glucose production following phlorizin treatment is in contrast with that

reported in T2D subjects, in whom empagliflozin⁴⁵ and dapagliflozin⁴⁶ increased hepatic glucose production. Such increase has been supposed to be the result of 1) the increased glucagon secretion induced by the SGLT2 inhibition, and 2) the fact that alpha cells could express SGLT1/2 transporters.⁴⁷ However, experimental conditions cannot be compared: 1) a dual SGLT1/2 inhibitor was used in the present study while selective SGLT2 inhibitors were used in the studies in T2D subjects; 2) the pyruvate tolerance test used here reflects gluconeogenesis activity only, while the radioactive glucose used in the studies with T2D subjects also reflects the glycogenolysis processes, and 3) in those studies, endogenous glucose production was evaluated during an euglycemic hyperinsulinemic clamp⁴⁶ and after mixed meal ingestion,⁴⁵ conditions not appropriate for analysis of hepatic glucose production. Besides, an elegant and well conducted study demonstrates, undeniably, that alpha cells neither express SGLTs nor change glucagon secretion in response to SGLTs inhibition, including the dual inhibitor phlorizin used in our study.⁴⁸

The improvement in glucose metabolism was accompanied by amelioration of NAFLD, as shown by the reversal of steatosis and ballooning, which has been related to the reduction in the expression of hepatic lipogenic enzymes.⁴⁹ However, the phlorizin-induced recovery of NAFLD was not complete since signals of liver inflammation remained present. However, it is important to highlight that the current treatment was very short (6 days) and we cannot exclude the possibility that a long-term treatment could completely revert liver inflammation.

Interestingly, also in HFD-induced NAFLD, a 4-week treatment with the selective SGLT2 inhibitor remogliflozin did not reverse NAFLD, and a partial reversion was observed only when food intake was restricted.¹⁷ These results underline the strong potency of the dual SGLT1/2 inhibitor used in the present study. Furthermore, in rats exposed to a choline-deficient L-amino acid-deficient diet, a model of NAFLD without T2D, ipragliflozin prevented hepatic triglyceride accumulation and fibrosis development, without affecting liver inflammation and plasmatic AST and ALT concentrations.¹⁴ These findings suggest that inflammatory activity is the most resistant component of NAFLD.

Conclusions

This study demonstrated, in T2D mice, that increased hepatic glucose production accompanies NASH, suggesting its

participation in the T2D-related NAFLD development. Importantly, the study revealed that short-term treatment with the dual SGLT1/2 inhibitor phlorizin restored glycemic control and hepatic glucose metabolism, and greatly ameliorated NASH, reinforcing that altered hepatic glucose metabolism participates in T2D-related NAFLD. The present results highlight that the pharmacological inhibition of SGLTs in subjects with T2D may be a useful approach not only for improving glycemic control but also for mitigating the development and/or progression of NAFLD.

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Disclosure

The authors report no conflicts of interest in this work.

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