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ARTICLE

Tissue-specific dysregulation of hexose-6-phosphate dehydrogenase and glucose-6-phosphate transporter production in *db/db* mice as a model of type 2 diabetes

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Abstract
Aims/hypothesis Tissue-specific amplification of glucocorticoid action through 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) affects the development of the metabolic syndrome. Hexose-6-phosphate dehydrogenase (H6PDH) mediates intracellular NADPH availability for 11β-HSD1 and depends on the glucose-6-phosphate transporter (G6PT). Little is known about the tissue-specific alterations of H6PDH and G6PT and their contributions to local glucocorticoid action in *db*/*db* mice.
Methods We characterised the role of H6PDH and G6PT in pre-receptor metabolism of glucocorticoids by examining the production of the hepatic 11β-HSD1-H6PDH–G6PT system in *db*/*db* mice.
Results We observed that increased production of hepatic H6PDH in *db*/*db* mice was paralleled by upregulation of hepatic G6PT production and responded to elevated circulating levels of corticosterone. Treatment of *db*/*db* mice with the glucocorticoid antagonist RU486 markedly reduced production of both H6PDH and 11β-HSD1 and improved hyperglycaemia and insulin resistance. The reduction of H6PDH and 11β-HSD1 production by RU486 was accompanied by RU486-induced suppression of hepatic *G6pt* (also known as *Slc37a4*) mRNA. Incubation of mouse primary hepatocytes with corticosterone enhanced G6PT and H6PDH production with corresponding activation of 11β-HSD1 and PEPCK: effects that were blocked by RU486. Knockdown of *H6pd* by small interfering RNA showed effects comparable with those of RU486 for attenuating the corticosterone-induced H6PDH production and 11ß-HSD1 reductase activity in these intact cells. Addition of the G6PT inhibitor chlorogenic acid to primary hepatocytes suppressed H6PDH production.
Conclusions/interpretation These findings suggest that increased hepatic H6PDH and G6PT production contribute to 11β-HSD1 upregulation of local glucocorticoid action that may be related to the development of type 2 diabetes.

Keywords 11β-HSD1 · G6PT · G6PT inhibitor · H6PDH · H6PDH siRNA · Insulin resistance · NADPH · Type 2 diabetes

Y. Nakagawa and Y. Wang contributed equally to this study.

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Abbreviations

11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
CA	Chlorogenic acid
11-DHC	11-Dehydrocorticosterone
ER	Endoplasmic reticulum
GC	Glucocorticoid
G6P	Glucose 6-phosphate

G6Pase	Glucose-6-phosphatase
G6PT	Glucose-6-phosphate transporter
GR	Glucocorticoid receptor
H6PDH	Hexose-6-phosphate dehydrogenase
ITT	Insulin tolerance test
TLC	Thin layer chromatography

Introduction

Excess glucocorticoid (GC) production strongly affects the development of type 2 diabetes and obesity via activation of intracellular GC receptor (GR) [1–5]. In rodents, increased GC production promotes hepatic gluconeogenesis and adipocyte differentiation and induces insulin resistance and obesity in genetically obese *db*/*db* and *ob*/*ob* mice [5–8]. However, tissue GC action can also be regulated at the pre-receptor level by the 11β-hydroxysteroid dehydrogenases. Metabolic tissues such as liver and adipose tissue abundantly produces 11β-hydroxysteroid dehydrogenase (11β-HSD1), a resident enzyme of the endoplasmic reticulum (ER) lumen that acts in vivo as an NADPH-dependent reductase to generate active cortisol (corticosterone in rodents) from inactive 11-keto cortisone (11-dehydrocorticosterone) [9–11]. 11β-HSD1 plays an important role in the regulation of tissue GC action [12–15]. Indeed, enhanced 11β-HSD1 could result in the production of excess tissue GCs and induction of GC-receptor-mediated local GC action related to glucose homeostasis, insulin action and adiposity, all of which are associated with the development of type 2 diabetes and visceral obesity [16–18]. Pharmacological evidence further validates the role of 11ß-HSD1 in the control of insulin resistance and obesity as 11ß-HSD1 inhibitors reduced hepatic glucose output and improved insulin sensitivity in obese animal models, as well as in patients with type 2 diabetes [19–23]. These studies implicate the importance of 11ß-HSD1 in the pathogenesis of metabolic syndrome and obesity.

The role of 11ß-HSD1 in the pre-receptor activation of GCs is entirely dependent on the production of its cofactor NADPH in the ER lumen [24, 25]. NADPH is regenerated by hexose-6-phosphate dehydrogenase (H6PDH) [26, 27], a microsomal enzyme located in the lumen of the ER and principally produced in hepatocytes and adipocytes, sites of 11β-HSD1 and GR [28, 29]. In these target tissues, H6PDH uses glucose 6-phosphate (G6P) and NADP to produce NADPH. The supply of G6P to H6PDH is ensured by a functional membrane ER protein, the G6P transporter (G6PT), which specifically transports cytosolic G6P into the ER and therefore regulates H6PDH activity via modulation of G6P level within the ER [30, 31]. The generation of NADPH by H6PDH can be used by NADPH-dependent 11ß-HSD1 reductase activity [32, 33]. H6PDH is thus a potential candidate supplying NADPH for 11β-HSD1-induced amplification of tissue GC production [34–36].

In the present study, we characterised the metabolic phenotype of H6PDH and G6PT in relation to pre-receptor metabolism of GCs by examining the effects of the GC antagonist RU486 on the production of the 11β-HSD1–H6PDH–G6PT system in the liver and adipose tissues of *db*/*db* mice. We also assessed the direct interaction of G6PT and H6PDH in the control of 11β-HSD1 driving tissue GC action in primary cultures of hepatocytes from *db*/*db* mice by using *H6pdh* small interfering (si)RNA and the G6PT inhibitor chlorogenic acid (CA). Finally, we examined the hormonal regulation of H6PDH and G6PT in these intact cells.

Methods

Animals Male C57BL/KsJ-obese (*db*/*db*) mice and their lean littermates (*db*/+) were purchased at 10 weeks of age from Taconic Farms (Hudson, NY, USA) and housed in a room illuminated daily from 07:00 to 19:00 hours (12 h light/dark cycle) with free access to water and standard laboratory chow. RU486 (25 mg/kg) or vehicle was given by intraperitoneal injection twice each day (at 07:00 and 19:00 hours) for 3 days or 3 weeks as previously described by Friedman et al. and Liu et al. [5, 15]. Body weight and food intake were recorded daily. All animal experiments were approved by the Charles Drew University Institutional Animal Care and Use Committee.

Insulin tolerance test For the insulin tolerance test (ITT), animals were fasted for 12 h, and blood samples were drawn at different times following insulin injection (2 U/kg i. p.; Novolin R; Eli Lilly, Indianapolis, IN, USA).

Biochemical assays Blood glucose levels were determined by the glucose oxidase method [36]. Plasma corticosterone levels were determined by RIA using mouse corticosterone as a standard (ICN Biomedicals, Costa Mesa, CA, USA). Plasma insulin levels were measured by RIA using rat insulin as a standard (Crystal Chemicals, Chicago, IL, USA).

Cell culture and treatment Primary hepatocytes were isolated from male *db*/*db* mice [21] and seeded onto collagen-coated dishes in DMEM/F-12 medium with 10% FBS at 37°C for 4 h. Cells were then washed with PBS, and the medium was changed to DMEM/F-12 medium without FCS. After 12 h, cells were treated with corticosterone $\left(1 \times 10^{-7} - 1 \times 10^{-5} \text{mol/l}\right)$ or insulin (1×10^{-5} mol/l) in the presence or absence of RU486 (1×10^{-6} mol/l) for 48 or 72 h. The G6PT inhibitor CA (0–200 μmol/l) was added to

Table 1 Body weight, blood glucose, plasma insulin, lipid and corticosterone

Variable	*db*/+ + vehicle	*db*/+ + RU486	*db/db* + vehicle	*db/db* + RU486
n	7	7	8	8
Body weight (g)	31±1.5	31.5±1.8	55±4.3***	48.8±3.6
Glucose (mmol/l/)	8.2±0.5	7.3±0.6	30±2.3***	11.7±1.2 †††
Insulin (pmol/l)	2.8±0.5	2.4±0.4	17.2±2.9***	15±3.1
Cholesterol (mmol/l)	1.6±0.1	57±5.7	110±7**	94±12
Triacylglycerols (mmol/l)	1.5±0.2	1.4±0.13	2.5±0.25**	2.1±0.2
Corticosterone (nmol/l)	122±25	295±34**	469±110**	870±121†††

Data are mean ± SEM from seven to eight mice per group
**$p<0.01$, ***$p<0.001$ vs *db*/+ vehicle
†††$p<0.001$ vs *db/db* + vehicle

primary cultures of hepatocytes for 48 h. This pharmacological dose of CA is similar to doses previously used in mouse 3T3-L1 pre-adipocytes and glioma cells [37, 38].

siRNA experiments in primary cultures of hepatocytes Cells were transfected with the *H6pd* siRNA (SABiosciences siRNA ID KM26265; SABiosciences, Frederick, MD, USA), or negative control siRNA (SABiosciences) using lipofectamine 2000 transfection agent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After 4 h, cells were treated with vehicle or corticosterone (1×10^{-6} mol/l) for an additional 48 h.

Microsomal enzymatic activity assays The liver microsomal pellet was obtained and 11ß-HSD1 activity was evaluated by addition of 0.4–1 mmol/l NADPH and 900 nmol/l unlabelled 11-dehydrocorticosterone (11-DHC) with 100 nmol/l 11-[^{3}H]DHC as tracer to microsomes in Krebs–Ringer buffer solution at 37°C for 10–30 min [36, 39]. The liver microsomes (1 mg/ml protein) were permeabilised with 1% Triton-100 to allow the free access of the cofactor to the intraluminal enzyme. Steroids were separated by thin-layer chromatography (TLC) and analysed by scintillation counting. The percentage of interconversion of [^{3}H]corticosterone and 11-[^{3}H]DHC was calculated from the radioactivity in each fraction. Reductase activity of 11ß-HSD1 was also evaluated by immunoassay of the corticosterone produced from 11-DHC using a sensitive corticosterone ELISA kit.

H6PDH enzyme activity was determined by spectrophotometric measurement of NADPH production in the presence of G6P and NADP using absorbance at 340 nm with a spectrophotometer (Ultrospec 2100, Amersham Biosciences) [36, 40]. Protein, 50 μg, from liver microsomes was incubated with 0.5–5 mmol/l G6P, 1–5 mmol/l NADP and 100 mmol/l glycine buffer solution at 22°C for 0–5 min. Specific activities were calculated and expressed as micro-

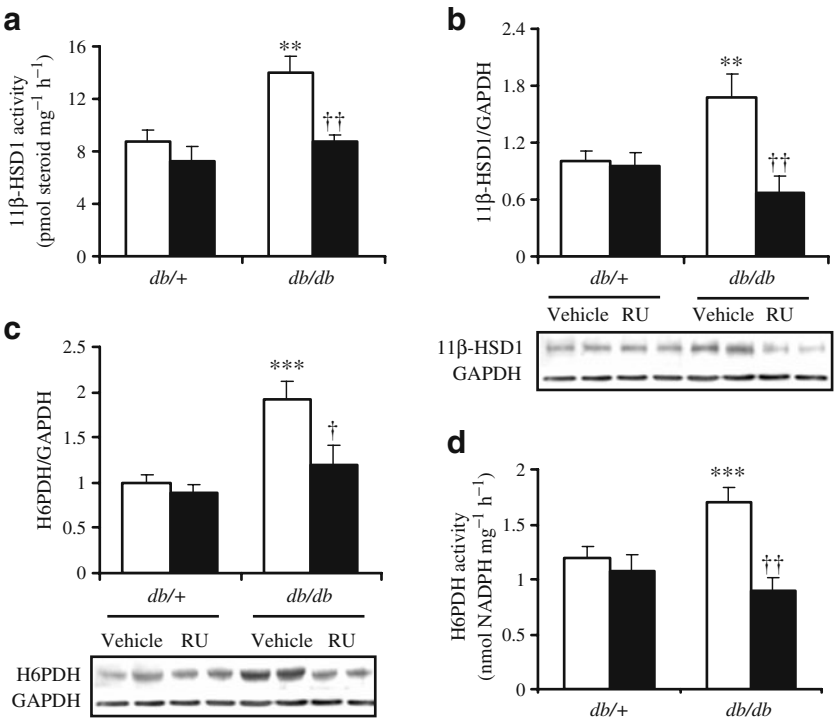

Fig. 1 11β-HSD1 and H6PDH activity and protein levels in liver microsomes of lean *db*/+ and obese *db/db* mice treated with vehicle or RU486. **a** 11β-HSD1 reductase activity was measured in mouse liver microsomes using 11-DHC as substrate in the presence of NADPH. Production and relative quantification of 11β-HSD1 (**b**) and H6PDH (**c**) protein was done relative to the amount of GAPDH. **d** H6PDH activity was measured in liver microsomes on the basis of NADPH formation using 2 mmol/l G6P as substrate in the presence of NAD. Data are mean ± SEM from seven to eight mice (per group). **$p<0.01$, ***$p<0.001$ vs *db*/+ controls; †$p<0.05$, ††$p<0.01$ vs *db/db* controls. White bars, vehicle; black bars, RU486

moles NADPH production per minute per milligram protein. The protein concentration was measured by the Bradford assay (Bio-Rad Protein Assay Kit, Bio-Rad, Hercules, CA, USA).

11β-HSD1 and H6PDH activity in primary hepatocytes Cells were incubated with 2 nmol/l 11-[^{3}H]DHC with 18 nmol/l unlabelled 11-DHC for 10–30 min [41]. Steroids were extracted from 1.0 ml of culture medium with ethyl acetate and separated by TLC. Enzyme activity levels were determined by counting the radioactivity. For the H6PDH activity in vitro, protein extracts, 20 μg, from primary hepatocytes were incubated with 2 mmol/l G6P as substrate in 100 μl total volume of glycine buffer (pH 10.0) with 0.5 mmol/l NADP as a cofactor. The changes in absorbance at 340 nm were measured during 25 min at 5 min intervals [36].

RNA extraction and real-time quantitative PCR analysis Total RNA was extracted using a single-step extraction method (RNAzol B, Invitrogen). cDNA synthesis from 2.0 μg RNA was performed using high capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time primers were designed with Primer express software 2.0 (Applied Biosystems) and are listed in the Electronic supplementary material (ESM) Table 1. Amplification of each target cDNA was then performed with SYBR Green I Master Kits in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the protocols recommended by the manufacturer. All reactions were carried out using the following cycling parameters: 55°C for 2 min and 95°C for 10 min, following by 40 cycles of 95°C for 15 s and 60°C for 1 min. Threshold cycle (C_t) readings for each of the unknown samples were then used to calculate the amount of target genes and were normalised to the signal of 18S rRNA. Data analysis is based on the ΔC_t method.

G6P uptake measurement Microsomal G6P uptake measurements were performed according to protocols previously published by Hiraiwa et al. [42]. Briefly, 50–100 μg of microsomes were incubated in 50 mmol/l sodium cacodylate buffer containing 250 mmol/l sucrose (pH 6.5) in the presence of 0.2–1 mmol/l G6P plus [U-^{14}C]G6P (American Radiolabeled Chemicals, St Louis, MO, USA) at 22°C for 3 min. The reaction was stopped by filtering through a

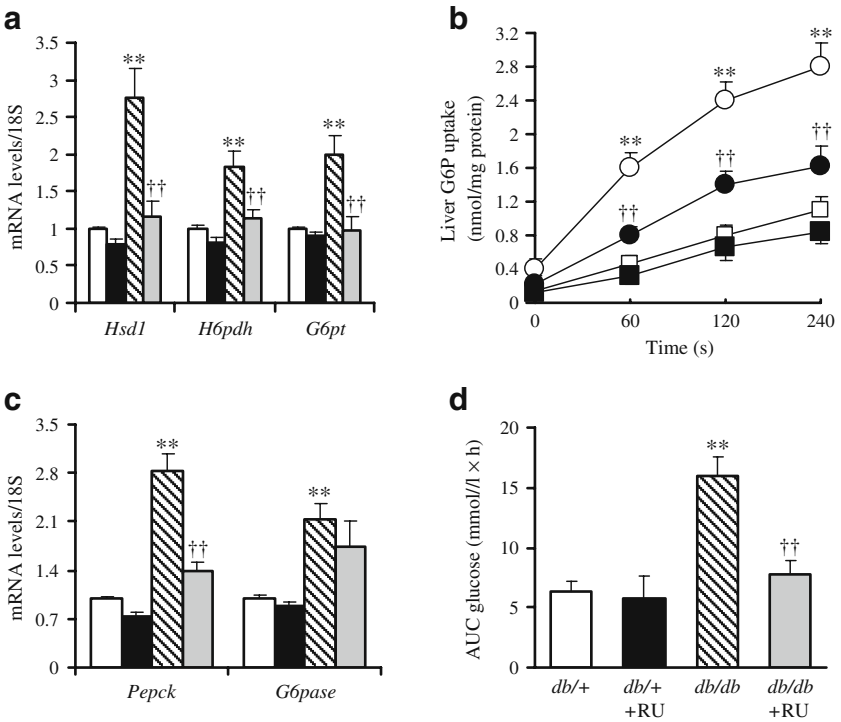

Fig. 2 **a** Quantitative real-time RT-PCR analysis demonstrating the relative alterations of *11β-Hsd1*, *H6pd* and *G6pt* mRNA expression in the livers of *db*/+ and *db*/*db* mice treated with vehicle or RU486. White bars, *db*/+; black bars, *db*/++RU486; hatched bars, *db*/*db*; grey bars, *db*/*db*+RU486. **b** Uptake of [U-^{14}C]G6P into the liver microsomes of lean *db*/+ and *db*/*db* mice treated with vehicle or RU486. White squares, *db*/+; black squares, *db*/++RU486; white circles, *db*/*db*; black circles, *db*/*db*+RU486. **c** Expression levels of *Pepck* and *G6Pase* mRNA in the liver of *db*/+ and *db*/*db* mice treated with vehicle or RU486. White bars, *db*/+; black bars, *db*/++RU486; hatched bars, *db*/*db*; grey bars, *db*/*db*+RU486. **d** The effects of RU486 on AUC of plasma glucose level in ITT. Data are mean ± SEM from seven to eight mice per group. $^{**}p<0.01$ vs *db*/+ controls; $^{\dagger\dagger}p<0.01$ vs *db*/*db* controls

nitrocellulose filter and samples were quickly washed with an ice-cold buffer containing 50 mmol/l TRIS–HCl, pH 7.4 and 250 mmol/l sucrose. Microsomes were subsequently permeabilised with 0.2% deoxycholate to abolish intravesicular G6P uptake. The radioactivity associated with microsomes retained by filters was measured by liquid-scintillation counting.

Western blot analysis Protein, 25 μg samples from liver microsomes or 50 μg total cellular proteins from primary hepatocytes, was analysed by SDS-PAGE as described by Liu et al. [36]. Membranes were incubated with a polyclonal anti-11β-HSD1 antibody (1:1,500; Affinity Bioreagents, Rockford, IL, USA) and mouse anti-human H6PDH antibody (1:2,000; Novus Biological, Littleton, CO, USA). The 11β-HSD1 and H6PDH protein signal was quantified with the use of the Eagle Eye II Quantitation System (Stratagene, La Jolla, CA, USA).

Statistical analyses All data are expressed as the mean± SEM. The normality of the distribution of data was established using the Wilks–Shapiro test, and outcome measures between groups were compared by Student's *t* test. To compare multiple groups, one-way ANOVA used. If ANOVA reveals significant differences, then individual group comparisons were performed by using the Newman–Keul's post hoc test. The differences among groups were considered significant at $p<0.05$.

Results

Characterisation of H6PDH and G6pt levels in db/db mice *db*/*db* mice had higher body weight and plasma levels of insulin, corticosterone and blood glucose than matched littermates (Table 1). Plasma levels of cholesterol and triacylglycerols in *db*/*db* mice were significantly higher than those in lean mice (Table 1). 11β-HSD1 reductase activity in liver microsomes of *db*/*db* mice was significantly increased to 47% over that of *db*/+ controls ($p<0.01$; Fig. 1a). Western blot analysis revealed the 11β-HSD1 protein production in the liver of *db*/*db* mice was increased 1.7-fold over that of *db*/+ controls ($p<0.01$; Fig. 1b). H6PDH activity and protein production in the liver of *db*/*db* mice were increased 1.57- and 1.9-fold, respectively, compared with *db*/+ control mice ($p<0.001$; Fig. 1 c, d). Real-time RT-PCR analysis revealed that hepatic *11β-Hsd1* (also known as *Hsd11b1*) mRNA levels were increased 2.8-fold in *db/db* mice and level was positively correlated with *H6pd* mRNA levels ($p<0.01$), which increased to 1.8-fold in liver over that of *db*/+ animals ($p<0.01$; Fig. 2a). Similarly, the hepatic *G6pt* (also known as *Slc37a4*) mRNA levels in *db*/*db* mice were significantly increased by 1.9-fold over that of *db*/+ controls ($p<0.01$; Fig. 2a). Moreover, the [U-^{14}C]G6P uptake in hepatic microsomes of *db*/*db* mice was higher than that of *db*/+ controls ($p<0.01$; Fig. 2b). The levels of hepatic *Pepck* and *G6Pase* mRNA in *db/db* mice were higher than the respective in *db*/+ mice ($p<0.01$; Fig. 2c). AUC analysis showed that the glucose levels in *db*/*db* mice were significantly higher than those in vehicle-treated *db*/+ animals (Fig. 2d). In addition, subcutaneous and epididymal adipose *11β-Hsd1* mRNA levels were higher than those of lean controls (Fig. 3a, b). Moreover, *H6pd* mRNA levels were also markedly increased in subcutaneous fat and in epididymal fat in *db/db* mice compared with lean levels ($p<0.05$), respectively. However, there was no difference in *G6pt* mRNA levels in subcutaneous and epididymal fat between *db/db* mice and lean controls (Fig. 3a, b).

The effects of RU486 treatment on the phenotypes of type 2 diabetes in db/db mice As shown in Table 1, RU486 compared with vehicle treatment for 3 weeks lowered blood glucose levels with no significant changes in body weight and levels of plasma insulin and lipids in *db*/*db* mice.

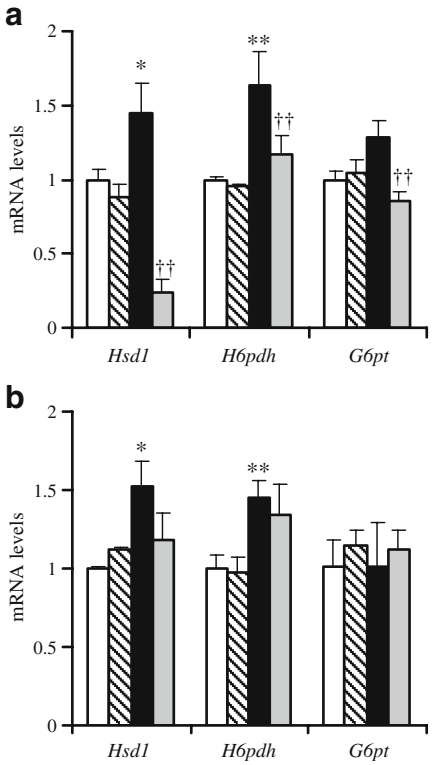

Fig. 3 Adipose *11β-Hsd1*, *H6pd* and *G6pt* mRNA expression in (**a**) subcutaneous and (**b**) epididymal fat of lean *db*/+ and obese *db*/*db* mice treated with vehicle or RU486. Relative expression of mRNA levels was measured by RT-PCR and normalised to 18S rRNA expression. *$p<0.05$, **$p<0.01$ vs *db*/+ control; $^{\dagger\dagger}p<0.01$ vs *db*/*db* controls. White bars, *db*/+; hatched bars, *db*/+ + RU486; black bars, *db*/*db*; grey bars, *db*/*db*+RU486

However, plasma corticosterone levels were increased in *db*/*db* mice after RU486 treatment ($p<0.001$). In contrast, hepatic 11β-HSD1 reductase activity was reduced 1.6-fold in RU486-treated *db*/*db* mice (Fig. 1a). Western blot analysis revealed that 11β-HSD1 protein production was decreased 2.5-fold in RU486-treated *db*/*db* mice ($p<0.01$ vs *db*/*db* controls; Fig. 1b). Similarly, treatment of *db*/*db* mice with RU486 for 3 weeks significantly reduced hepatic H6PDH activity to 45% that of vehicle-treated *db*/*db* mice ($p<0.01$; Fig. 1d); this reduction in enzyme activity occurred in parallel with decreased hepatic H6PDH protein production in RU486-treated *db*/*db* mice (Fig. 1c). Real-time RT-PCR analysis showed that in *db*/*db* mice, RU486 reduced hepatic *11β-Hsd1* and *H6pd* mRNA expression, respectively, to the levels of vehicle-treated *db*/+ mice (Fig. 2a). In parallel with the decrease in *H6pd* mRNA levels, hepatic *G6pt* mRNA expression was decreased by twofold in *db*/*db* mice after RU486 treatment (Fig. 2a). Consistent with decreased *G6pt* expression, RU486 reduced [U-^{14}C]G6P uptake in the liver microsomes, although it did not restore G6P uptake levels to those of *db*/+ controls (Fig. 2b). Moreover, RU486 also significantly reduced hepatic *Pepck* mRNA levels, but did not exert significant effects on the hepatic *G6Pase* (also known as *G6pc*) mRNA expression in *db*/*db* mice (Fig. 2c). The AUC glucose levels were reduced in *db*/*db* mice after RU486 treatment (Fig. 2d). RU486 treatment for 3 weeks also reduced the *G6pt* mRNA level with simultaneous reduction in H6PDH and 11β-HSD1 expression in subcutaneous fat of *db*/*db* mice as compared with controls (Fig. 3a), but did not alter epididymal fat G6PT, H6PDH and 11β-HSD1 expression (Fig. 3b). However, RU486 did not exert significant effects on *11β-Hsd1*, *H6pd* and *G6pt* mRNA expression in adipose tissues from *db*/+ mice (Fig. 3a, b). In addition, a shorter treatment of *db*/*db* mice with RU486 for 3 days also significantly reduced the expression of *11β-Hsd1*, *H6pd* and *G6pt* in the liver and improved glucose levels, but did not change the slope of insulin tolerance curve (data not shown), indicating that blocking the action of GC with RU486 leads to changes in gene expression that are independent of improvement in insulin sensitivity.

H6PDH and G6PT abundance in primary cultures of hepatocytes To confirm our in vivo observations, we

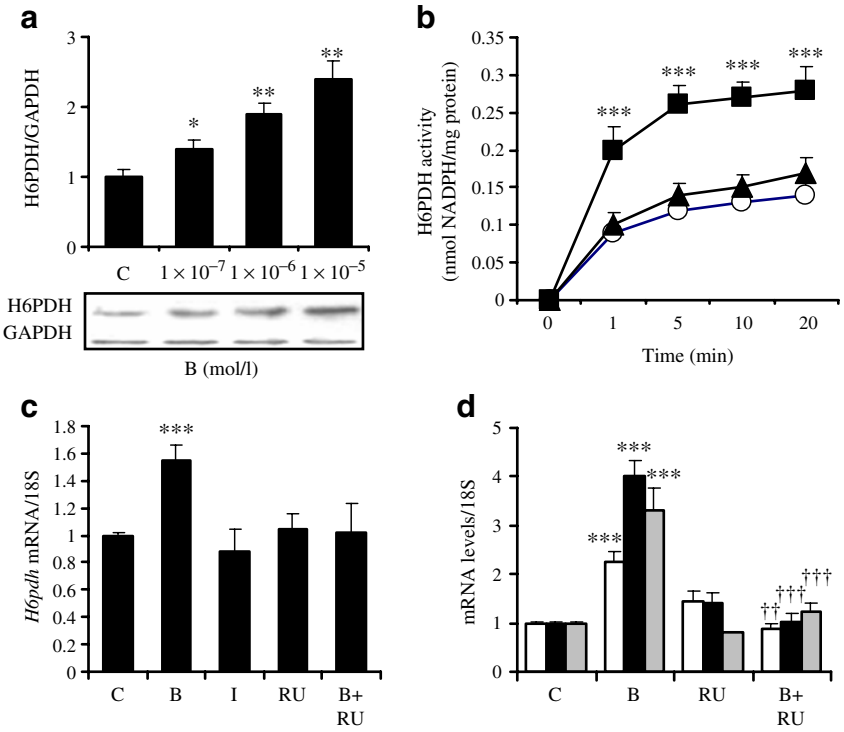

Fig. 4 Effects of corticosterone and insulin on primary cultures of *db*/*db* mouse hepatocytes. Levels of H6PDH production (**a**) and activity (**b**) in cells treated with corticosterone for 48 h. In (**b**), white circles, control; black squares, corticosterone; black triangles, corticosterone + RU486. The levels of *H6pd*, *11β-Hsd1*, *G6Pase* and *Pepck* mRNA expression (**c**, **d**) in cells treated with corticosterone (1×10^{-6}mol/l) or insulin (1×10^{-6}mol/l) in the presence or absence of RU486 (1×10^{-6}mol/l) for 48 h. In (**d**), white bars, *11β-Hsd1*; black bars, *G6Pase*; grey bars, *Pepck*. Production and relative quantification of H6PDH protein levels are expressed relative to the amount of GAPDH. Relative H6PDH activity was measured on the basis of NADPH production at 5 min intervals for 20 min. Values are the mean± SEM from three separate culture preparations. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs corticosterone-treated hepatocytes. C, control; B, corticosterone; I, insulin; RU, RU486

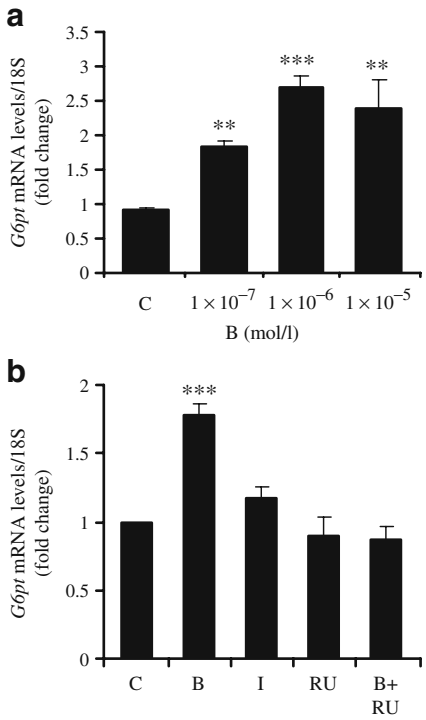

Fig. 5 **a** Effect of different doses of corticosterone on *G6pt* mRNA expression in primary cultures of hepatocytes from *db/db* mice. **b** Hepatocytes were incubated with corticosterone (10^{-6} mol/l) or insulin (10^{-6} mol/l) for 48 h in the absence or present of RU486. Expression and relative quantification of *G6pt* mRNA levels expressed relative to the amount of 18S in vehicle-treated cells. Values are the mean±SEM from three separate culture preparations. $^{**}p<0.01$, $^{***}p<0.001$ vs vehicle-treated cells. C, control; B, corticosterone; I, insulin; RU, RU486

investigated the effects of exogenous corticosterone and insulin on G6PT and H6PDH in primary hepatocytes from *db/db* mice. Western blot analysis showed that treatment of hepatocytes with increasing doses of corticosterone led to a concentration-dependent induction of H6PDH protein production (Fig. 4a). A concentration of corticosterone (1×10^{-6} mol/l), similar to that occurring in *db/db* mice in vivo, significantly increased NADPH production in the presence of G6P as a substrate (Fig. 4b). The increase in H6PDH activity was consistent with real-time RT-PCR analyses, which showed that corticosterone increased *H6pd* mRNA levels 1.6-fold in primary hepatocytes compared with vehicle ($p<0.001$; Fig. 4c). In contrast, treatment of hepatocytes with both corticosterone and RU486 (10^{-6} mol/l) for 48 h failed to increase H6PDH activity (Fig. 4b) or *H6pdh* mRNA levels (Fig. 4c). Moreover, co-treatment with corticosterone and RU486 also blocked the corticosterone-induced changes in *11β-Hsd1*, *Pepck* and *G6Pase* mRNA expression (Fig. 4d) in these intact cells. However, no changes in *H6pdh* mRNA expression were observed after 48 h in cells exposed to a relatively high concentration of insulin (1×10^{-6} mol/l; Fig. 4c). Extending our findings on H6PDH activity, corticosterone-induced expression of *G6pt* mRNA in primary hepatocytes was reduced significantly by RU486 (Fig. 5a, b).

The effects of H6PDH siRNA and G6PT inhibitor (CA) on H6PDH and 11β-HSD1 As shown in Fig. 6, H6PDH protein and *H6pd* mRNA levels were markedly reduced in intact hepatocytes transfected with *H6pd* siRNA in comparison to cells transfected with siRNA control, respectively ($p<0.001$, Fig. 6a, b). Similarly, in comparison with control levels, 11β-HSD1 reductase activity in primary hepatocytes after transfection with *H6pdh* siRNA was decreased by 44% ($p<0.01$; Fig. 6c). Furthermore, *H6pd* siRNA attenuated the corticosterone-mediated increase in H6PDH protein and 11β-HSD1 reductase activity in these intact cells in comparison with control levels (Fig. 6), indicating that suppression of H6PDH by siRNA exerted effects comparable with those of

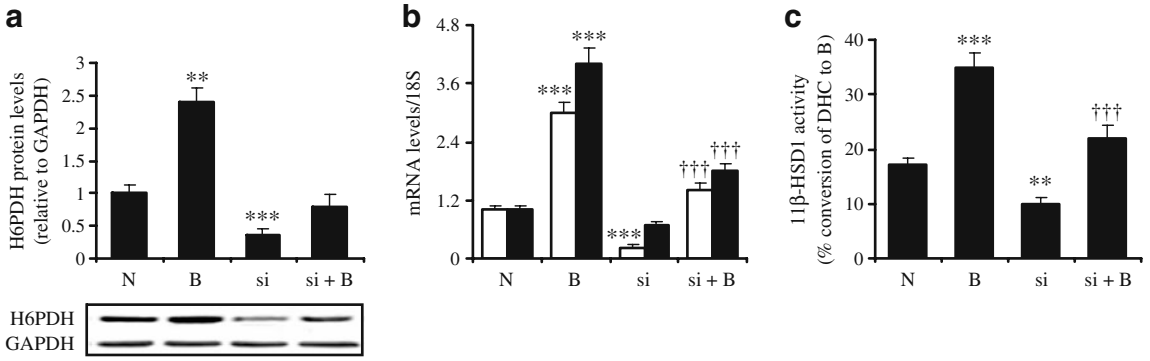

Fig. 6 Suppression of *H6pd* expression by *H6pd* siRNA decreased 11β-HSD1 reductase activity and attenuated the effects of corticosterone on H6PDH and 11β-HSD1 in primary culture of hepatocytes. Cells were transfected with either *H6pd* siRNA (si) or negative control siRNA (N) and cultured with or without corticosterone (**b**; 1×10^{-6} mol/l) for 48 h. **a** Western blots showing effect of *H6pd* siRNA inhibition on H6PDH protein in cells. **b** *H6pd* and *11β-Hsd1* mRNAs were measured by real-time RT-PCR. White bars, *H6pd;* black bars, *11β-Hsd1*. **c** 11ß-HSD1 reductase activity was expressed as the percentage of [^{3}H]DHC converted to [^{3}H]corticosterone (**b**) in medium from hepatocytes. Values are the mean±SEM from three separate culture preparations. $^{**}p<0.01$, $^{***}p<0.001$ vs negative control; $^{†††}p<0.001$ vs corticosterone-treated hepatocytes

RU486 on reducing the corticosterone-induced H6PDH levels and 11ß-HSD1 activity.

The effects of the G6PT inhibitor CA on H6PDH and 11β-HSD1 are shown in Fig. 7. CA significantly reduced H6PDH protein production in primary hepatocytes compared with vehicle treatment for 48 h (Fig. 7a). Consequently, the mRNA levels of *H6pd* were decreased by 1.6-fold in these intact cells after CA treatment (Fig. 7b). In agreement with reduction of H6PDH, CA treatment also reduced *11β-Hsd1* mRNA expression as compared with that of controls ($p<0.01$; Fig. 7b).

Discussion

We found that the phenotype of type 2 diabetes in *db*/*db* mice was associated with the induction of hepatic H6PDH activity and gene expression. H6PDH activity and protein expression were elevated in liver microsomes from *db*/*db* mice, and this was accompanied by the induction of hepatic 11ß-HSD1 reductase activity. This elevated production of hepatic H6PDH corresponded with increased hepatic *Pepck* and *G6pase* mRNA levels, high blood glucose and insulin resistance, suggesting that elevated hepatic H6PDH levels may contribute to the development of type 2 diabetes in our animal model. Induction of hepatic *H6pd* expression could increase the generation of the crucial co-factor NADPH and thus enhance 11ß-HSD1 upregulation of local GC production, which would lead to gluconeogenesis linked to the development of hyperglycaemia in diabetic animals [15, 21]. Moreover, *H6pd* and *11ß-Hsd1* mRNA levels were also increased in the adipose fat of *db*/*db* mice. Our present results are consistent with recent reports that adipose *H6pd* and *11ß-Hsd1* mRNA were increased in patients with type 2 diabetes [43]. To our knowledge, hepatic H6PDH has not been measured in patients with type 2 diabetes. Our findings are also supported by earlier reports that increased hepatic 11ß-HSD1 production may contribute to the pathogenesis of type 2 diabetes in *db*/*db* mice [21, 44].

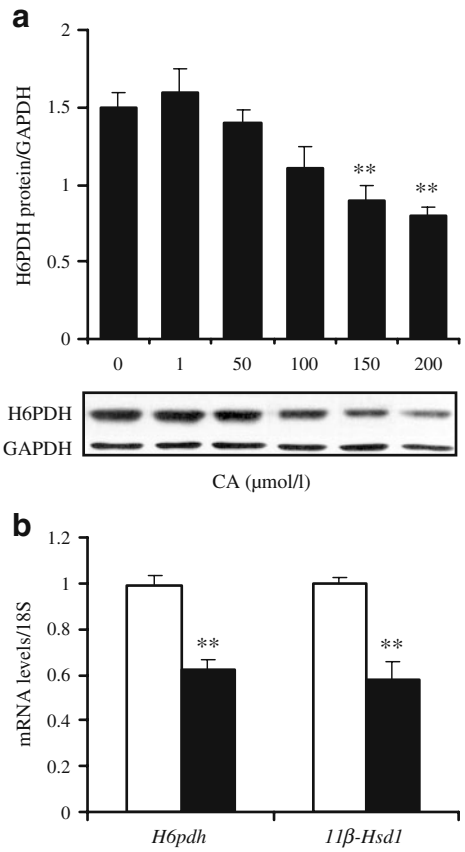

Fig. 7 The effects of CA on H6PDH and 11β-HSD1 in primary cultures of hepatocytes from *db/db* mice. **a** Primary hepatocytes were incubated with increased concentrations of the G6PT inhibitor CA (0–200 μmol/l) for 48 h and H6PDH protein levels were determined by western blot analysis. **b** Hepatocytes were incubated with CA (150 μmol/l) for 48 h. *H6pdh* and *11β-Hsd1* mRNA were measured by real-time RT-PCR. Values are the mean±SEM from three separate culture preparations. $^{**}p<0.01$ vs vehicle-treated hepatocytes. White bars, vehicle; black bars, CA

In *db*/*db* mice, excess corticosterone production has been shown to be crucial for the development of hyperglycaemia, insulin resistance and obesity, and adrenalectomy reverses these changes [6–8]. In the present study, we observed that the induction of hepatic H6PDH activity and *H6pd* gene expression was associated with the elevated circulating levels of corticosterone. This was validated using isolated hepatocytes from *db*/*db* mice in which corticosterone increased H6PDH activity at the transcriptional level. Increased H6PDH activity could then provide the cofactor NADPH to 11ß-HSD1, suggesting that GCs could positively promote their own pre-receptor metabolism through activation of NADPH availability to 11β-HSD1 reductase activity. Our results are consistent with those of earlier reports that cortisone increased H6PDH activity leading to 11ß-HSD1 amplifying cortisol production in rat liver microsomal fractions [34]. These data support our suggestion that the induction of hepatic H6PDH production may, in part, result from excess circulating corticosterone action.

It is well known that pharmacological blockade of GR by RU486 antagonises hypercortisolaemia-related hyperglycaemia and insulin resistance in *db*/*db* mice as well as in patients with Cushing's syndrome [5, 8]. In the present study, we found that treatment of *db*/*db* mice with RU486 markedly reduced production of hepatic H6PDH and 11β-HSD1 and improved hyperglycaemia and insulin resistance. Moreover, we observed that RU486 treatment reversed corticosterone-induced production of H6PDH activity and NADPH and prevented activation of 11β-HSD1 and PEPCK production, consistent with a recent study reporting that the dexamethasone-induced upregulation of *H6pd* mRNA can be blocked by RU486 in mouse 3T3-L1

adipocytes [45]. Moreover, we also observed that suppression of *H6pd* by siRNA decreased 11β-HSD1 reductase activity and showed effects comparable with those of RU486 on blocking the corticosterone-induced H6PDH and 11ß-HSD1 in intact hepatocytes. Reduction of hepatic H6PDH expression by RU486 could decrease the ability of H6PDH utilising NADP to regenerate NADPH, thereby limiting the intraluminal NADPH exposure to 11β-HSD1 linked to the reduction of 11β-HSD1 and the improvement of glycaemic variables. This is in agreement with a recent study reporting that the role of RU486 in glycaemic homeostasis is associated with reduction of hepatic 11β-HSD1 levels in type 2 diabetic mice [46].

In addition, the current study also observed a positive relationship between hepatic H6PDH activity and G6PT abundance in *db*/*db* mice and *db*/+ controls. Indeed, earlier studies showed that H6PDH requires G6P transport to maintain its metabolic substrate G6P availability within the ER lumen [30, 47, 48]. We observed that the induction of H6PDH production is associated with an elevation of *G6pt* mRNA levels with increased hepatic intraluminal G6P uptake. In contrast, decreased hepatic G6PT abundance after RU486 treatment reduced the intraluminal hepatic G6P uptake with corresponding suppression of H6PDH activity. In agreement with the reduction of H6PDH levels, the current study also showed that the G6PT inhibitor CA, at doses used in vitro [37, 38], markedly reduced H6PDH levels and led to the suppression of 11β-HSD1 in intact mouse hepatocytes, indicating that G6PT is required to maintain H6PDH levels. Our findings are consistent with recent studies reporting that G6P availability in the ER lumen was reduced, leading to decreased H6PDH and 11β-HSD1 activity in mice with global deletion of G6PT [48]. This is also indirectly supported by a recent study demonstrating that the G6PT inhibitor S3483 (derived from CA) decreased G6P-induced H6PDH levels and resulted in the inhibition of reductase activity of 11β-HSD1 in rat liver microsomes [33, 49]. These data support our hypothesis that suppression of G6PT production in hepatocytes may be an additional mechanism of the RU486-mediated reduction of H6PDH production. Moreover, we also observed that RU486 prevented corticosterone-mediated induction of G6PT production in mouse hepatocytes, suggesting that endogenous GCs could exert positive effects on G6PT production in liver that is likely to occur through a GR-mediated mechanism. These findings are supported by a recent study which showed that there are three GC response elements present in the promoter region of the *G6pt* gene [50]. This is also in agreement with previous studies showing that the GR signalling pathway was required for the regulation of 11β-HSD1 and PEPCK production by GCs [15, 51].

In summary, we showed that increased hepatic H6PDH activity and G6PT expression may contribute to 11β-HSD1 upregulation of local GC action linked to the development of type 2 diabetes. We also found that the induction of hepatic G6PT and H6PDH production may be mediated, at least in part, through the action of elevated circulating GCs. The benefits of RU486 in *db*/*db* mice may be associated with the endogenous inactivation of 11β-HSD1 amplifying tissue GC action through reducing production of H6PDH and G6PT. These findings raise the possibility that tissue-specific modulation of H6PDH and G6PT production may be a new strategy to be coupled with the investigation of 11β-HSD1 as a therapeutic target in the metabolic syndrome.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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