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Specific Reduction of Glucose-6-Phosphate Transporter May Contribute to Down-regulation of Hepatic 11β-hydroxysteroid Dehydrogenase Type 1 in Diabetic Mice

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Abstract

Pre-receptor activation of glucocorticoids via 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) has been identified as an important mediator of the metabolic syndrome. Hexose-6-phosphate dehydrogenase (H6PDH) mediates 11β-HSD1 amplifying tissue glucocorticoid production by driving intracellular NADPH exposure to 11β-HSD1 and requires glucose-6-phosphate transporter (G6PT) to maintain its activity. However, the potential effects of G6PT on tissue glucocorticoid production in type 2 diabetes and obesity have not been yet defined. Here, we evaluated the possible role of G6PT antisense oligonucleotides (G6PT ASO) in the pre-receptor metabolism of glucocorticoids as related to glucose homeostasis and insulin tolerance by examining the production of 11β-HSD1 and H6PDH in both male *db/+* and *db/db* mouse liver tissue. We observed that G6PT ASO treatment of *db/db* mice markedly reduced hepatic G6PT mRNA and protein levels and substantially diminished the activation of hepatic 11β-HSD1 and H6PDH. Reduction of G6PT expression was correlated with the suppression of both hepatic gluconeogenic enzymes G6Pase and PEPCK and corresponded to the improvement of hyperglycemia and insulin resistance in *db/db* mice. Addition of G6PT ASO to mouse hepa1-6 cells led to a dose-dependent decrease in 11β-HSD1 production. Knockdown of G6PT with RNA interference also impaired 11β-HSD1 expression and showed comparable effects to H6PDH siRNA on silencing of H6PDH and 11β -HSD1 expression in these intact cells. These findings suggest that G6PT plays an important role in the modulation of pre-receptor activation of glucocorticoids and provides new insights into the role of G6PT in the development of type 2 diabetes.

Keywords

11β-HSD1; H6PDH; G6PT; G6PT ASO; H6PDH siRNA; G6Pase; PEPCK; obesity; Insulin sensitivity; type 2 diabetes

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Introduction

Obesity and type 2 diabetes has reached epidemic prevalence worldwide emphasizing the urgent need for the underlying mechanisms of this disease to lead to new treatment. Glucocorticoids are steroid hormones and chronic elevation of cortisol level is frequently associated with the metabolic syndrome, visceral obesity, and type 2 diabetes (Cushing's syndrome) (Nieman, et al. 1985; Rizza, et al. 1982). Glucocorticoids have many functions in peripheral tissues with key roles in hepatic gluconeogenesis and adipogenic process through activation of steroid receptors (Bamberger, et al. 1996; Friedman, et al. 1993). Tissue glucocorticoid action can be regulated at pre-receptor level in liver and adipose tissues by NADPH-dependent 11β-hydroxysteroid dehydrogenase (11β-HSD1). 11β-HSD1 is located in the endoplasmic reticulum (ER) and its major function is to convert inactive cortisone (11-dehydrocorticosterone in rodents) to active cortisol (corticosterone), therefore amplifying local glucocorticoid action (Bujalska, et al. 1997; Jamieson, et al. 1995). 11β-HSD1 is widely expressed, and over-expression of 11β-HSD1 in liver and adipose tissues is thought to be responsible for induction of metabolic syndrome in mice (Masuzaki, et al. 2001; Paterson, et al. 2004). Similarly, increasing 11β-HSD1 mRNA expression in hepatic and visceral adipose tissue has been associated with metabolic disorders in morbidly obese patients (Baudrand, et al. 2011; Torrecill, et al. 2011). In contrast, 11β-HSD1 knockout mice are resilient to diet-induced insulin resistance (Kotelevtsev, et al. 1997), although a liver-specific 11β-HSD1 knockout mouse showed mild improvement in glucose tolerance without affecting insulin sensitivity (Lavery et al. 2012). Importantly, 11β-HSD1 inhibitors exert benefits in glucose homeostasis and insulin sensitivity in patients with type 2 diabetes as well as in rodent animal models with obesity (Hollis, et al. 2011; Liu, et al. 2008; Wang, et al. 2006). Selective 11β-HSD1 inhibition thus may represent a novel approach to prevent diabetic syndrome and obesity.

11β-HSD1 amplification of tissue cortisol/corticosterone generation within ER is crucially dependent on the production of its cofactor NADPH (Mziaut, et al. 1999; Odermatt, et al. 1999). The supply of NADPH to 11β-HSD1 is ensured by the enzyme hexose-6-phosphate dehydrogenase (H6PDH), which converts glucose-6-phosphate (G6P) and NADP to regenerate NADPH (Atanasov, et al. 2004; Banhegy, et al. 2004; McCormick, et al. 2006). H6PDH is thus likely to be the crucial enzyme supplying NADPH for 11β-HSD1-induced amplification of tissue cortisol/corticosterone production linked to the development of type 2 diabetes and obesity. In the ER lumen, the supply of the metabolic substrate G6P to H6PDH is controlled by a functional membrane ER protein, G6P transporter (G6PT), which shuttles cytosolic G6P across the ER membrane into the ER and therefore drives H6PDH activity (van Schaftingen, et al. 2002; Banhegyi, et al. 1993). G6Pase catalyzes the final step in both the gluconeogenic and glycogenolytic pathways by the hydrolysis of endoluminal G6P to glucose and inorganic phosphate. G6PT is thus a potential candidate supplying G6P for H6PDH-mediated NADPH generation to 11β-HSD1 activity in these target tissues. Two forms of glycogen storage disease (GSD) have been shown to be linked to 11β-HSD1 production. GSD1a is caused by a deficiency of G6Pase-α and results in hypoglycemia with an increase in 11β-HSD1 activity (Lei, et al. 1993; Walker, et al. 2007). In contrast, deficiency of hepatic G6PT leads to GSD type 1b (GSD1b) that results in fasting hypoglycemia with a decrease in 11β-HSD1 production by decreasing G6P availability to H6PDH (Walker, et al. 2007; Gerin, et al. 1997; Hiraiwa, et al. 1999). These studies imply that G6PT expression not only drives G6P hydrolase activity, but also plays a crucial role in maintaining the ability of H6PDH to generate NADPH as a cofactor for 11β-HSD1. However, the potential effects of G6PT on the modulation of H6PDH and 11β-HSD1 mediating tissue glucocorticoid production in type 2 diabetes and obesity have not been yet defined.

In the current study, we investigated the functional role of G6PT in the phenotype of pre-receptor metabolism of glucocorticoids related to the glucose homeostasis and insulin sensitivity by examining the impact of specific G6PT inhibitor antisense oligonucleotides (ASO) on the expression of G6PT-H6PDH -11β-HSD1 system in the liver tissues of both lean *db/+* and obese *db/db* mice. We also evaluated whether G6PT could directly affect H6PDH expression and subsequently 11β-HSD1 in mouse hepa1-6 cells by using G6PT inhibitor ASO and G6PT siRNA. Finally, we examined the effects of H6PDH siRNA on H6PDH and 11β-HSD1 expression in these intact cells.

Materials and methods

Animal treatment

Male *db/db* (C57BL6/J +/+$Lepr^{db}$) and lean control heterozygous (*db/+*) mice were purchased at 7 weeks of age from Taconic Farms and housed in a room maintained on a 12:12 h light/dark cycle. Animals were allowed *ad libitum* access to tap water and standard laboratory food. The Institutional Animal Care and Use Committee of Charles Drew University approved all animal experiments. 2′-*O*-(2-methoxy) ethyl-modified phosphorothioate antisense oligonucleotides (ASO) specific to the glucose 6-phosphate transporter-1 (G6PT) and control ASO were kindly provided by Dr. Barnes (Isis Pharmaceutical, Inc). Animals were treated with ASO solutions (12.5 mg/kg) twice per week (separated by 3.5 days) via intraperitoneal (i.p) injection for 4 weeks (Sloop, et al. 2007). Four weeks after vehicle or ASO administration, non-fasting blood samples were collected between 0900 and 1000 h and then stored at −80 °C until biochemical assays. Blood samples were collected weekly and assayed for blood glucose levels in the fed state.

Hormonal Assays

Blood glucose levels were determined by the glucose oxidase method. 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).

Intraperitoneal insulin tolerance test

At the end of the four week period after G6PT ASO, animals were fasted for 12 h, and a basal blood sample was taken, followed by an injection of insulin (2 U/kg, ip; Eli Lilly, IN, USA). Blood samples were drawn at different times after insulin injection.

Hepatic Glycogen Assay

Hepatic glycogen production was determined as previously described (Seifter, et al. 1950). Briefly, the liver tissues were homogenized in 30% KOH solution and dissolved at 100°C for 30 min. The glycogen content was measured by treatment with 0.2% anthrone reagent in 95% H_2SO_4 followed by measuring the absorbance at 620 nm.

Mouse hepatic cell culture and treatment

Hepa1–6 cells (mouse hepatocyte cell line) were seeded in six-well culture plates in DMEM medium supplemented with 10% FBS in a humidified 5% CO_2 atmosphere. Cells were treated with G6PT ASO or control using lipofectamine 2000.

Silencing of G6PT and H6PDH siRNA in Hepa1–6 cells

Cells were transfected with the G6PT (SABiosciences siRNA ID KM05106N) and H6PDH siRNA (SABiosciences siRNA ID KM26265), or their negative control siRNA (SABiosciences, Frederick, USA) respectively. In a separate experiment, cells were

transiently transfected with full-length 11β-HSD1 plasmid (generously provided by Dr. Nakagawa, Hamamatsu University), or control. Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, CA), and the cells were incubated for 24–48h.

Measurement of liver microsomal H6PDH and 11β-HSD1 activity

The microsomal pellet was obtained by centrifugation of the supernatant for 1 h at 100,000g. The protein concentrations were determined by Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). The liver microsomes have been permeabilized with 1% Trition-100. H6PDH activity was evaluated by incubation of 20 μg liver microsome protein with 0.5–1 mM NADP and glucosamine-6-phosphate as cofactors at room temperature for 0–20 min as our previous described (Wang, et al. 2011). For 11β-HSD1 reductase activity, the liver microsomes (0.5 mg protein/mL) were incubated with 900 nmol/L unlabeled 11-DHC with 100 nmol/L [^{3}H]11-DHC as tracer in the presence of 0.5 mM NADPH for 10–20 min at 37°C (Liu, *et al*. 2005). Steroids were separated by thin-layer chromatography (TLC) and analyzed by scintillation counting. 11β-HSD1 activity was calculated from the scintillation counting of radioactivity.

RNA extraction and analysis by quantitative real-time RT-PCR

Total RNA was extracted using RNAzol B kit (Invitrogen). Real-time primers for mouse H6PDH (sense: 5′-TGGCTACGGGTTGTTTTTGAA-3′; antisense: 5′-TATACACGGTACATCTCCTCTTCCT-3′), G6PT (sense: 5′-AGGCCTTGTAGGAAG CATTGC-3′; antisense: 5′-TCACCGTTACTCGGAAGAGGAA-3′), 11β-HSD1 (sense: 5′ CCTTGGCCTCATAGACACAGAAAC-3′; antisense: 5′-GGAGTCAAAGGCGATT TGTCAT-3′), and G6Pase (sense: 5′-TTGCTGTGGCTGAAACTTTCAG-3′; antisense: 5′-TCCAAGCGCGAAACCAAA-3′) were designed with Primer express software 2.0 (Applied Biosystems) (Wang, et al. 2011). Amplification of each target cDNA was then performed with SYBR Green Kits in the ABI Prism 7700 System. The expression for a target gene was corrected to the 18S rRNA values.

Immunoblotting analysis

Liver microsomes or total cellular proteins from hepa1-6 cells were prepared using RIPA buffer. G6PT was detected with a polyclonal antibody, H6PDH with a mouse anti-human antibody (Novus Biological, Littleton, CO), 11β-HSD1 with a polyclonal antibody, and G6Pase-α with a polyclonal antibody (Santa Cruz, Biotechnology, INC).

Statistical Analysis

The data shown represent means ± SEM for all of the determinations. Data were compared using an unpaired Student's *t*-tests or ANOVA. A P value < 0.05 was considered statistically significant.

Results

G6PT ASO attenuated the development of type 2 diabetes in db/db mice

To test the efficiency of reducing G6PT expression to treat hyperglycemia and insulin resistance, we targeted the G6PT gene in type 2 diabetic mice using G6PT ASO specific to G6PT. As shown in Table 1, *db/db* mice were more hyperglycemic and obese than *db/+* matched littermates. Animals treated with G6PT ASO for 4 weeks showed a significant reduction in blood glucose levels compared with those of control ASO-treated *db/db* controls ($p < 0.001$). In contrast, plasma corticosterone levels were increased in *db*/*db* mice after G6PT ASO treatment ($p < 0.01$). The plasma insulin levels were significantly reduced after G6PT ASO treatment in *db/db* mice compared with that of control ASO –treated mice

($p < 0.01$). However, the body weight was not significantly changed after G6PT ASO treatment in *db/db* mice compared with controls (Tab 1). Moreover, G6PT ASO did not change weight and the levels of glucose and corticosterone in *db/+* mice compared with their respective controls.

G6PT ASO reduced G6PT expression parallels with the improvement of gluconeogenesis and insulin resistance

At the end of the 4-week treatment period, hepatic G6PT mRNA levels were reduced by 2.2- and 8-fold after treatment with G6PT ASO in both *db+* and *db/db* mice ($p < 0.001$ versus their respective vehicle-treated mice) (Fig. 1A). Western blot analysis showed that G6PT ASO decreased hepatic G6PT protein levels by 1.9-fold ($p < 0.001$) in *db/+* mice and by 2.7-fold ($P < 0.001$) in *db/db* mice (Fig. 1B). Quantitative real-time RT-PCR analysis showed that G6PT ASO resulted in a 33% ($p < 0{\cdot}05$) decrease in hepatic PEPCK mRNA levels in *db/db* mice compared to those of control mice (Fig. 2A), but did not significantly affect hepatic PEPCK mRNA expression in *db/+* mice under fed state. Similarly, G6Pase-α protein levels of liver tissue were decreased by 4-fold after treatment with G6PT ASO in *db/db* mice compared with vehicle-treated controls, but did not affect hepatic G6Pase-α protein levels in *db/+* mice in the fed state (Fig. 2B). In addition, fasting hepatic PEPCK and G6Pase mRNA levels were higher than that of fed *db/db* mice, respectively. G6PT ASO also decreased the fasting hepatic PEPCK and G6Pase expression in *db/db* mice compared fed control mice (data not shown). The hepatic glycogen concentration from G6PT ASO-treated *db/db* mice was significantly increased compared with control mice at 12 h of fasting, but fed G6PT ASO-treated *db/db* mice did not significantly increase hepatic glycogen level compared fed control mice. In contrast, G6PT ASO did not affect hepatic glycogen level in *db/+* mice under both fed and fasted conditions (Fig. 2C). Fasting hyperglycemia continued to improve over time after G6PT ASO treatment in *db/db* mice compared with control ASO-treated mice (Fig. 2D). Moreover, an insulin tolerance test showed that treatment of *db/db* mice with G6PT ASO significantly improved the glucose-lowering effect of insulin in comparison with that observed in *db/db* mice treated with control ASO (data not shown). In addition, the AUC in an insulin tolerance test was increased by 5.5-fold in *db/db* control mice, which was decreased by 1.9-fold in G6PT ASO-treated *db/db* mice compared with control *db/db* mice (Fig. 2E). Additionally, the plasma insulin levels were significantly reduced after G6PT ASO in *db/db* mice compared with that of control mice (Table 1), most likely reflecting a reduced requirement for insulin as a result of improved insulin resistance. However, G6PT ASO did not change the fasting glucose levels and the slope of insulin tolerance curve in *db/+* mice compared with their respective controls.

G6PT ASO effectively suppressed H6PDH and 11β-HSD1 expression

In parallel with the reduction in G6PT gene expression, treatment of *db/db* mice with G6PT ASO significantly reduced hepatic H6PDH activity to 70 % that of control ASO -treated *db/db* mice ($p < 0.01$; Fig. 3A). Similarly, H6PDH protein level was significantly decreased by 3.5-fold in G6PT ASO-treated *db/db* mice ($p < 0.01$) (Fig. 3B). However, G6PT ASO did not significantly affect hepatic H6PDH mRNA levels in *db/+* and *db/db* mice versus respective control mice (Fig. 3C). Moreover, G6PT ASO did not change the hepatic H6PDH activity and protein levels in *db/+* mice (Fig. 3A and 3B). Hepatic 11β-HSD1 activity was reduced by 1.4-fold in *db/+* mice and 2.3-fold in *db/db* mice after G6PT ASO treatment versus respective vehicle-treated mice (Fig. 4A). Furthermore, G6PT ASO decreased 11β-HSD1 protein level by 2-fold in *db/+* mice and by 2.6-fold in *db/db* mice (Fig. 4B). The decrease in protein level was consistent with the results of PCR analysis showing that hepatic 11β-HSD1 mRNA level was significantly reduced in both *db/+* and *db/db* mice after G6PT ASO versus respective control(Fig. 4C).

The effects of G6PT ASO and G6PT siRNA on G6PT-H6PDH-11β-HSD1 system in mouse hepa1-6 cells

To further explore the functional consequence of G6PT suppression, we treated mouse hepa1-6 cells with G6PT ASO and G6PT siRNA and measured metabolic gene expression. Treatment of mouse hepa1-6 cells with increasing doses of G6PT ASO led to a concentration-dependent decrease in G6PT mRNA expression (Fig. 5A). Real-time RT-PCR analysis also revealed that treatment of hepatocytes with G6PT ASO resulted in a 1.5–1.8 fold decrease in the G6Pase mRNA expression in comparison with that of controls (Fig. 5B). Furthermore, G6PT ASO treatment also decreased G6Pase-α protein expression in a dose-dependent manner in comparison with control levels (Fig. 5C). However, G6PT ASO treatment in these intact cells did not induce significant changes in H6PDH mRNA levels (Fig. 6A), but decreased the H6PDH protein expression by 4.0–4.2 fold in comparison with control levels (Fig. 6B). Similarly, in hepa1-6 cells, treatment with G6PT ASO decreased 11β-HSD1 mRNA by 1.2–1.5 fold in comparison with control levels (Fig. 6C). Consequently, the protein levels of 11β-HSD1 were decreased by 1.6–3.2 fold in these intact cells after G6PT ASO treatment (Fig. 6D).

The effects of G6PT siRNA and H6PDH siRNA on H6PDH and 11β-HSD1 expression in mouse hepa1-6 cells

G6PT siRNA treatment resulted in a 1.7-fold decrease in H6PDH mRNA level in mouse hepa1-6 cells compared to negative control siRNA-treated cells (Fig. 7A). In agreement with reduction of H6PDH, G6PT siRNA treatment also reduced 11β-HSD1 mRNA expression by 2.5- fold ($p < 0.001$) in these intact cells compared to that of control cells (Fig. 7A). In addition, G6PT siRNA silenced H6PDH and 11β-HSD1 protein expression in these cells compared to negative controls (Fig. 7B), indicating that reduction of G6PT is responsible for inhibition of H6PDH *in* mouse hepatocytes.

Furthermore, 11β-HSD1 mRNA level was determined in intact hepatocytes treated with H6PDH siRNA for 48h. As shown in Fig. 7C, H6PDH mRNA levels were markedly reduced in hepa1-6 cells transfected with H6PDH siRNA in comparison to cells treated with negative siRNA control ($p < 0.001$). Similarly, H6PDH siRNA reduced 11β-HSD1 mRNA by 48% in these intact cells comparison with control levels ($p < 0.01$; Fig. 7C), indicating that suppression of H6PDH by siRNA exerted comparable effects to G6PT siRNA in reducing H6PDH and 11β-HSD1 expression. In contrast, plasmid encoding 11β-HSD1 transfection induced G6PT mRNA expression by 1.4-fold in hepa1-6 cells compared with control vector ($P < 0.001$; Fig. 7D). These data support a concept of an inter-relationship between 11β-HSD1 and G6PT within hepatocytes.

Discussion

In the present study, we demonstrated that the important role of G6PT inactivation in the control of tissue glucocorticoids production. We found that specific G6PT ASO reduced hepatic G6PT mRNA and its protein expression leading to decrease in 11β-HSD1 activity and gene expression, and improved hyperglycemia and insulin resistance in *db/db* mice. In support of these findings, our *in vitro* data demonstrated a similar reduction in 11β-HSD1 gene expression in mouse hepa 1-6 cells following treatment with G6PT ASO and G6PT siRNA. Our results indicate that specific reduction of G6PT expression caused down-regulation of hepatic 11β-HSD1 gene transcription, thereby reducing intracellular corticosterone regeneration. Our findings are also indirectly supported by a recent study demonstrating that the G6PT inhibitor chlorogenic acid leads to inhibition of 11β-HSD1 *in vitro* in normal rat liver microsomes (McCormick, et al. 2006), although they did not study the role of G6PT inhibitors *in vivo* animals. Our findings imply that specific G6PT

inhibition may represent a potential metabolic pathway to reduce local GC reactivation by diminishing 11β-HSD1.

The current study also showed that G6PT ASO-induced suppression of 11β-HSD1 expression paralleled with the decrease of hepatic G6Pase protein and PEPCK mRNA expression in *db/db* mice with reduced hyperglycemia and AUC glucose levels suggesting that G6PT ASO –mediated suppression of hepatic 11β-HSD1 production may be involved in the amelioration of hepatic glucose homeostasis in diabetic mice. Consistently, the pyruvate tolerance test showed that G6PT ASO almost completely normalized the elevation of hepatic glucose production in *db/db* mice (data not shown). This is supported by recent reports that G6PT ASO reduction of G6PT expression and improves hyperglycemia in diabetic *ob/ob* mice, although the effects of G6PT ASO on glucocorticoid metabolism has not been previously measured (Sloop, et al. 2007). The current study is also consistent with recent reports that inactivation of 11β-HSD1 was associated with a reduction of the key gluconeogenic enzyme PEPCK production, a glucocorticoid-responsive target gene in liver (Kotelevtsev, et al. 1997; Hanson, et al. 1997). Similarly, pharmacological inhibition of hepatic corticosterone production in type 2 diabetic mice improved metabolic syndrome with the suppression of G6Pase mRNA expression (Wang, et al. 2006). In addition, G6PT ASO-mediated reduction of G6Pase itself could promote hepatic glycogen synthesis by decreasing the hydrolysis of G6P to release glucose into the bloodstream that could also contribute to the improvement of hyperglycemia and insulin sensitivity in *db/db* mice. Although, gene deletion of G6PT results in the excess glycogen storage and causes profound fasting hypoglycemia in mice, the current study showed that the G6PT ASO-stimulated fasted and fed hepatic glycogen synthesis and did not cause hypoglycemia in *db/db* mice. In contrast, G6PT ASO did not affect hepatic glycogen level in *db/+* mice under both fed and fasted conditions. This may due to G6PT ASO-reduced hepatic G6PT protein by 60% and improved hyperglycemia in *db/db* mice, which is different to the result from the complete loss of G6PT in knockout mice. This is in agreement with previous observations that G6PT ASO did not cause excess hepatic glycogen accumulation and fasted hypoglycemia and avoids complications of GSD in *ob/ob* mice (Sloop, et al. 2007). Our data suggest that some of the beneficial metabolic effects of G6PT ASO may be partly due to down-regulation of hepatic 11β-HSD1 through endogenous inactivation of local GC generation linked to the improvement of diabetic phenotype in *db/db* mice. In addition, our data also show that G6PT ASO -mediated reduction of hepatic 11β-HSD1 did not affect glucose homeostasis and AUC levels in lean *db/+* mice, consistent with a liver-specific 11BHSD1 knockout mouse model had a slight metabolic phenotype (Lavery, et al. 2012).

It is known that H6PDH can effectively drive the endoluminal pentose phosphate pathway that generates ER NADPH (Atanasov, et al. 2004; Banhegyi, et al. 2004). The NADPH within the lumen of the ER is important for in vivo function of 11β-HSD1-mediated local glucocorticoid production. Here, we observed that G6PT ASO reversed the elevation of hepatic H6PDH activity and NADPH production and prevented activation of 11β-HSD1 in *db/db* mice, further validates that H6PDH is key for in vivo activation of 11β-HSD1 (Uckaya, et al. 2008). However, G6PT ASO did not significantly affect hepatic H6PDH mRNA levels *in vivo*, indicating that inactivation of hepatic H6PDH activity may be independent of the changes in its mRNA expression, because a dose-dependent reduction of H6PDH protein expression were also observed without affecting its mRNA levels *in vitro* in mouse hepa1-6 cells after G6PT ASO treatment. Thus, the observed reduction of hepatic H6PDH activity may be at post transcriptional level. Reduction of hepatic H6PDH activity by G6PT ASO could decrease the ability of H6PDH utilizing NADP to regenerate NADPH, thereby attenuating the intraluminal NADPH exposure to 11β-HSD1 linked to the reduction of 11β-HSD1 and the improvement of metabolic parameters. Moreover, we also observed that suppression of H6PDH by siRNA decreased 11β-HSD1 production in intact

hepatocytes. These data support our hypothesis that suppression of hepatic 11β-HSD1 expression may be an additional mechanism of the G6PTASO-mediated reduction of H6PDH. Our findings are also supported by earlier reports that impairment of H6PDH production is responsible for reduction of 11β-HSD1 in the liver (Draper, et al. 2003; Lavery, et al. 2006).

A host of mechanisms may be accountable for the G6PT inhibitor ASO mediated suppression of endogenous H6PDH activity. Indeed, G6PT is known to be required to maintain H6PDH metabolic substrate G6P availability within ER lumen (McCormick, et al. 2006). We and other groups have reported that mouse liver tissue express a functional G6PT and H6PDH complex and responds to 11β-HSD1 activity (Chou, et al. 2002; Walker, et al. 2007; Wang, et al. 2011). Moreover, a recent study also demonstrated that G6PT inhibitor S3483 decreased G6P –induced H6PDH expression and resulted in the inhibition of reductase activity of 11β-HSD1 in rat liver microsomes (Banhegyi, et al. 1998). Thus, the observed the specific reduction of G6PT production after G6PT ASO treatment could result in low G6P concentration in the ER lumen that not only inactivates the ER of G6P hydrolase activity, but also probably impairs H6PDH function through a G6P-mediated mechanism in liver. Thus, the suppression of hepatic H6PDH by specific G6PT ASO or siRNA may be mediated, at least in part**,** from their ability to inhibit the supply of G6P to H6PDH through interfering with G6PT expression. These findings are consistent with a recent study showing that reduction of G6PT after RU486 treatment lowered hepatic G6P levels with corresponding suppression of H6PDH in diabetic mice (Nakagawa, et al. 2011). These findings indicate that the beneficial actions of G6PT ASO on the phenotype of diabetes in *db/db* mice may not be limited to specific reduction of G6PT/G6Pase couple but may also be associated with the endogenous impairment of H6PDH activity leading to downregulation of 11β-HSD1 amplifying hepatic GC action. These findings raise the possibility that that tissue-specific modulation of G6PT expression may lead to new ways to target 11β-HSD1 and treat metabolic syndrome.

In conclusion, we showed that some of antidiabetic actions of G6PT ASO in type 2 diabetes in *db/db* mice may be associated with down-regulation of 11β-HSD1 in the liver. Our data suggest that selective G6PT inhibition may represent a novel approach for the reduction of endogenous tissue glucocorticoid production by diminishing 11β-HSD1. However, further studies are needed to investigate on the beneficial effects of specific G6PT inhibitors on the metabolic control in rodent models and patients with type 2 diabetes.

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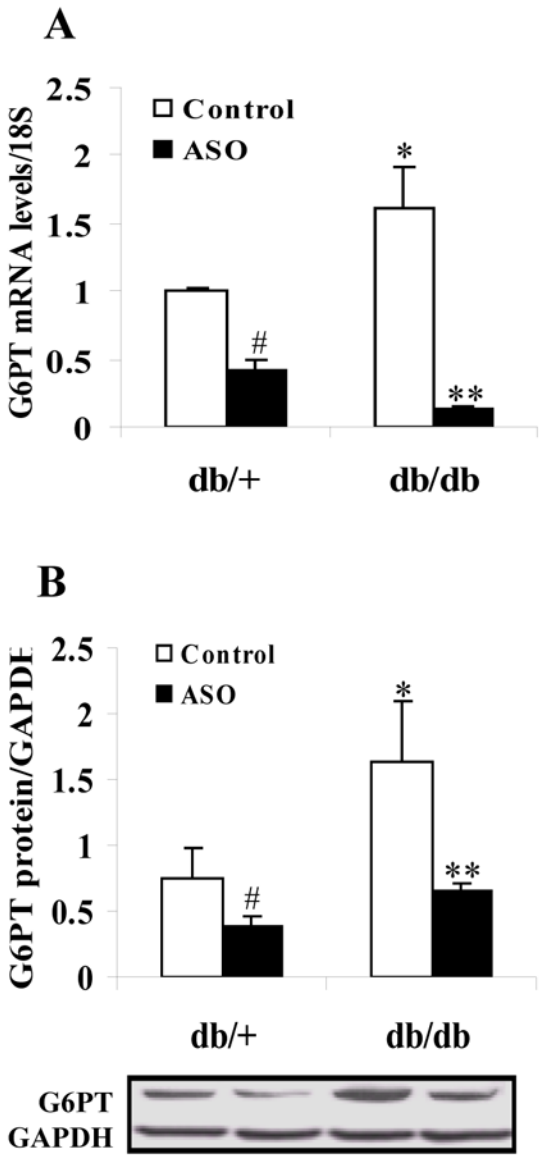

Fig. 1.

G6PT mRNA and protein expression in liver of *db*/+ and *db/db* mice treated with ASO control or G6PT-ASO. **A.** Relative expression of mRNA levels was measured by RT-PCR and normalized to 18S. **B.** Expression and relative quantification of G6PT protein levels expressed relative to the amount of GAPDH. Values are mean ± SEM of six mice per group. #p<0.001 vs. *db*/+ controls; *p<0.01 vs. *db/+ c*ontrols; **p <0.001 vs. *db/db* control mice.

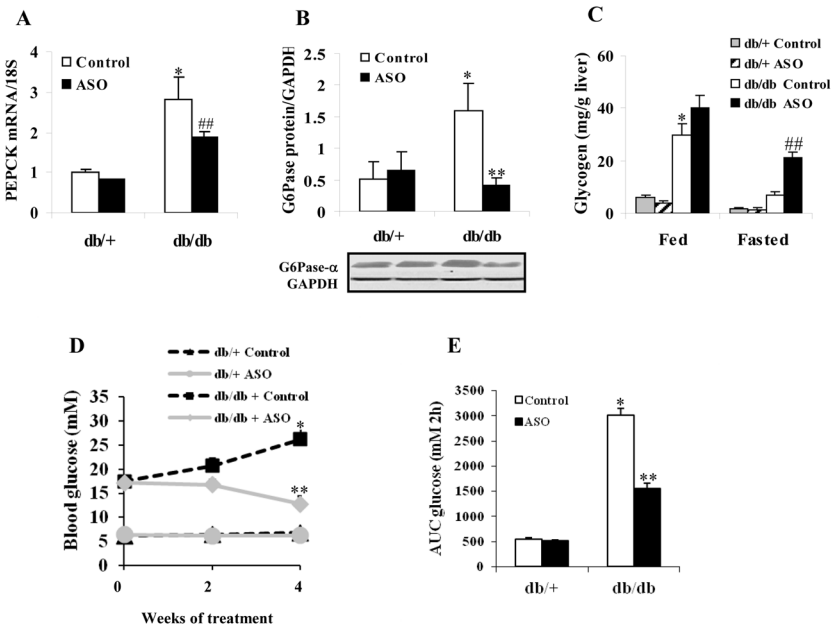

Fig. 2.

Gluconeogenic PEPCK mRNA (**A**) and G6Pase-a protein (**B**) expression in the liver of *db/+* and *db/db* mice treated with G6PT ASO in the fed condition. Glycogen content (**C**) is measured in liver tissues from ASO-treated mice in the fed condition and after a 12 h fast. Fasting blood glucose (**D**) and the glucose (**E**) area under the curve (AUC) during insulin tolerance test. Values are mean ± SEM of eight mice per group. **p*<0.01 vs. *db/+* controls; ##*p*<0.05 vs. *db/db* controls; ***p*<0.01 vs. *db/db* control. ## *p*<0.01 vs. fasting control.

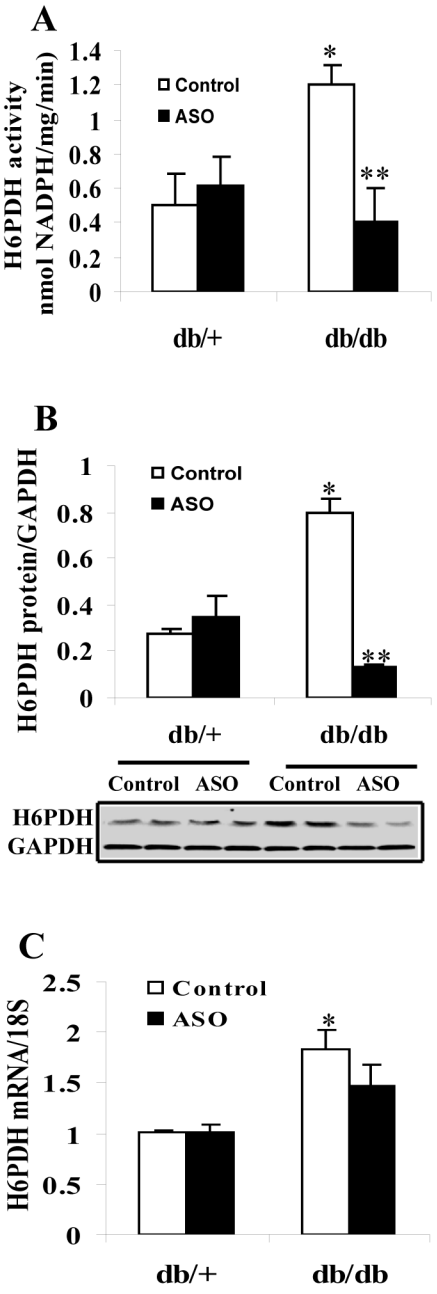

Fig. 3.

H6PDH activity (**A**) and protein (**B**) and mRNA (**C**) in liver of *db*/+ and *db/db* mice treated with ASO control or G6PT-ASO for 4 weeks. *A.* H6PDH activity was measured in liver microsomes on the basis of NADPH formation using 2 mM G6P as substrate in the presence of NADP. *B.* Expression and relative quantification of G6PT protein levels expressed relative to the amount of GAPDH. *C.* Relative expression of mRNA levels was measured by RT-PCR and normalised to 18S. Values are mean±SEM of seven mice per group. *p <0.01 vs. *db*/+ controls; **p<0.01 vs. *db/db* mice.

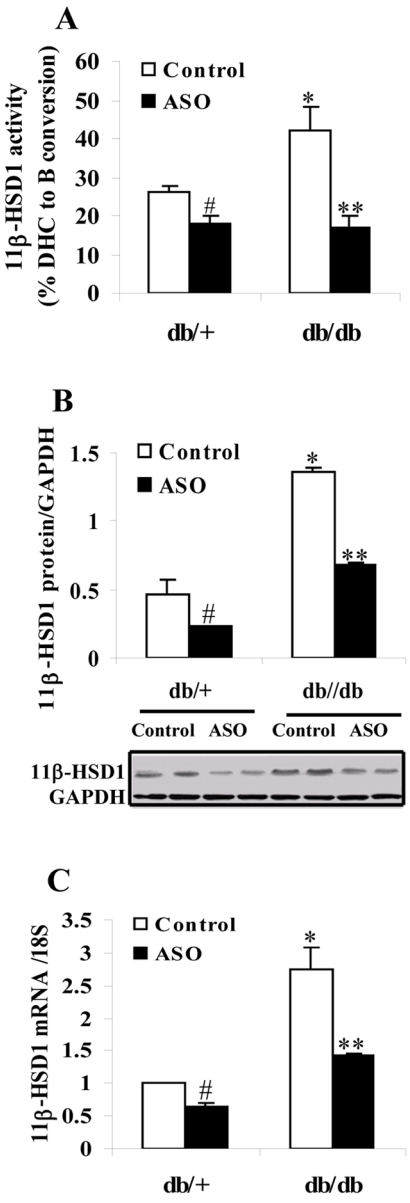

Fig. 4.

The alterations of 11β-HSD1 activity and gene expression in the liver of *db/+* and *db/db* mice treated with G6PT-ASO or ASO control. **A.** 11β-HSD1 reductase activity was measured in mouse liver microsomes using 11-DHC as substrate in the presence of NADPH. The relative expression levels of protein (**B**) and mRNA (**C**) were normalized with GAPDH and 18S reference gene, respectively. Values are mean ± SEM of six mice per group. *p<0.01 vs. *db/+* controls; **p<0.01 vs. *db/db* controls; #p<0.01 vs. *db/+* control.

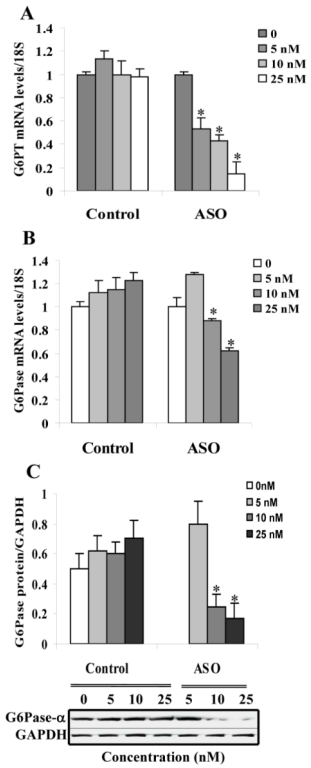

Fig. 5.

The effects of G6PT-ASO on G6PT and G6Pase gene expression in mouse hepa 1-6 cells. Cells were transfected with different concentrations of G6PT ASO or ASO control for 4 h, and incubated for an additional 16-24h. **A** and **B**. G6PT and G6Pase mRNA were measured by real-time RT-PCR. **C.** G6Pase-a protein levels are expressed relative to the amount of GAPDH. Values are the mean ± SEM of three separate culture preparations. *p<0.01 vs. respective control ASO-treated cells.

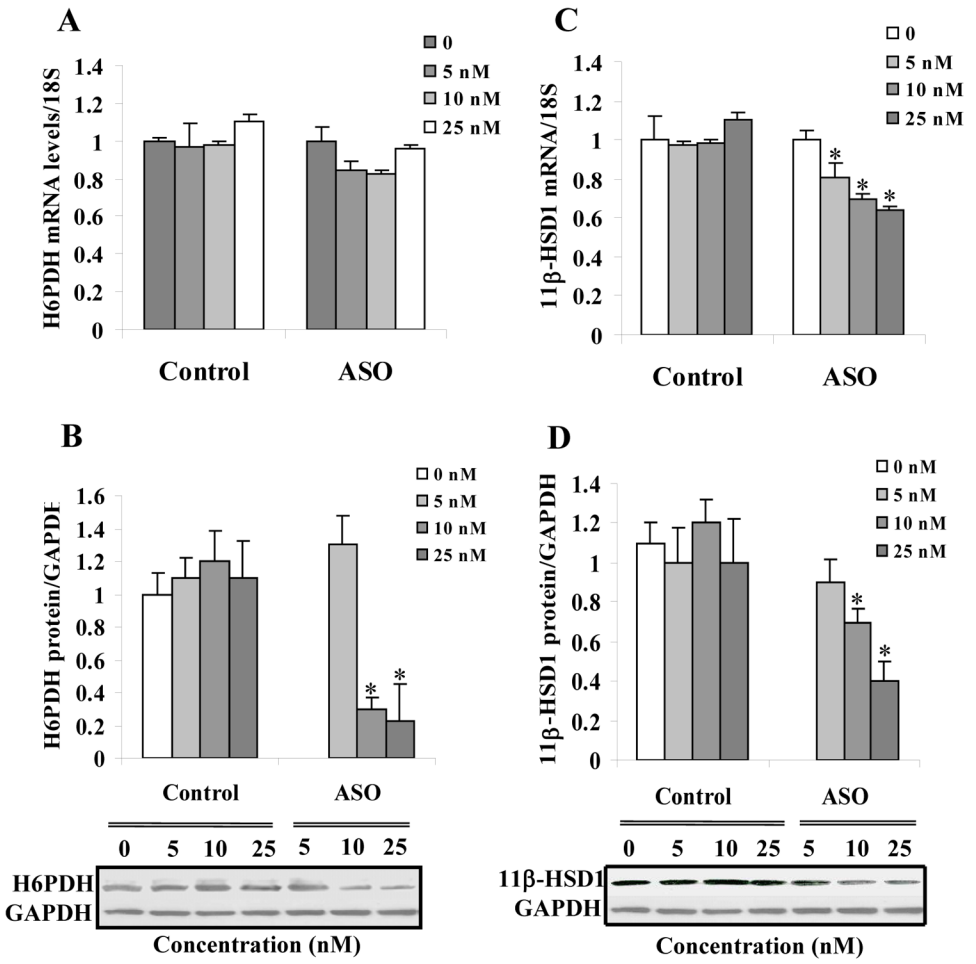

Fig. 6.

Effects of G6PT-ASO on H6PDH and 11β-HSD1 production in mouse hepa 1-6 cells. Cells were transfected with different concentrations of G6PT ASO or ASO control (0–25 nmol/L) for 4 h, and incubated for an additional 20 h. A and C. H6PDH and 11β-HSD1 mRNA were measured by real-time RT-PCR. B and D. H6PDH and 11β-HSD1 protein were measured by Western blot. Values are the mean ± SEM of three separate culture preparations. *p<0.01 vs. respective ASO control -treated cells.

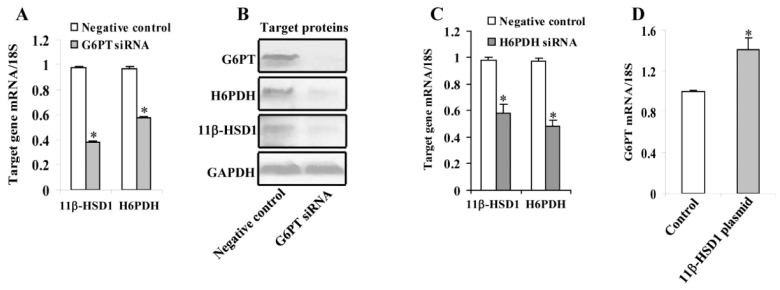

Fig. 7.

The effects of G6PT and H6PDH siRNA on H6PDH and 11β-HSD1 in mouse hepa 1-6 cell. Mouse hepatocytes hepa1-6 cells were transfected with either siRNA or negative control siRNA for 48 h. A and C. H6PDH and 11β-HSD1 mRNA were measured by real-time RT-PCR. B. H6PDH, 11β-HSD1 and G6PT protein were measured by Western blot. D. G6PT mRNA was measured after mouse 11β-HSD1 plasmid transfection. Values are expressed as mean ± SEM of three separate culture preparations. ***$p < 0.01$** vs. respective negative control-treated cells.

Table 1

Metabolic parameter in *db/+* and *db/db* mice treated with G6PT ASO

Metabolic parameter	Lean *db/+*mice		Obese *db/db* mice	
	Control ASO	G6PT ASO	Control ASO	G6PT ASO
N	7	7	8	8
Glucose (mg/dl/)	144 ± 12	135 ± 16.9	570 ± 90 *	252 ± 25.2 **
Body weight (g)	28.4 ± 1.7	29.2 ± 1.38	47 ± 0.86 *	45.7 ± 3.1
Corticosterone (ng/ml)	37 ± 4.4	53 ± 4.8	272 ± 46 *	320 ± 57
Insulin (pg/ml)	15 ± 2.1	11 ± 3.3	122 ± 12 *	88 ± 8.6 **

Data are mean ± SD of sevento eightmice per group.

* *P*< 0.001 vs. *db/+*control ASO;

** P < 0.01 vs. *db/+*vehicle;

P < 0.001 vs. *db/db* + control ASO.