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Mice lacking the proton channel Hv1 exhibit sex-specific differences in glucose homeostasis

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Sex as a physiologic factor has a strong association with the features of metabolic syndrome. Our previous study showed that loss of the voltage-gated proton channel Hv1 inhibits insulin secretion and leads to hyperglycemia and glucose intolerance in male mice. However, there are significant differences in blood glucose between male and female Hv1-knockout (KO) mice. Here, we investigated the differences in glucose metabolism and insulin sensitivity between male and female KO mice and how sex steroids contribute to these differences. We found that the fasting blood glucose in female KO mice was visibly lower than that in male KO mice, which was accompanied by hypotestosteronemia. KO mice in both sexes exhibited higher expression of gluconeogenesis-related genes in liver compared with WT mice. Also, the livers from KO males displayed a decrease in glycolysis-related gene expression and an increase in gluconeogenesis-related gene expression compared with KO females. Furthermore, exogenous testosterone supplementation decreased blood glucose levels in male KO mice, as well as enhancing insulin signaling. Taken together, our data demonstrate that knockout of Hv1 results in higher blood glucose levels in male than female mice, despite a decreased insulin secretion in both sexes. This sex-related difference in glucose homeostasis is associated with the glucose metabolism in liver tissue, likely due to the physiological levels of testosterone in KO male mice.

In order to maintain glucose homeostasis, insulin secretion by pancreatic β-cells in mammals is in proportion to increasing concentrations of glucose. Insulin can reduce blood glucose level by stimulating the utilization of glucose in peripheral tissues and inhibiting the production of hepatic glucose (1). Type 1 and type 2 diabetes are both characterized by uncontrolled hyperglycemia, and maintenance of blood glucose within a physiological range is critical for the prevention of diabetes-related complications (2). It is now clear that gender has an important influence on the pathogenesis of metabolic diseases, such as type 2 diabetes. The first manifestation of dimorphism is related to the diabetes epidemic, with a male predominance reported in humans and in most animal models, while females are usually not affected by metabolic disorders caused by diet (3). Therefore, recent guidelines emphasize the need to consider this gender difference in preclinical (cell and animal models) to clinical studies and avoid traditional male dominance when using these methods (4).

The liver plays a great role in maintaining homeostasis in glucose, lipid, and protein. In diabetes, hyperglycemia is partly attributed to abnormally increased hepatic gluconeogenesis (5). Indeed, loss or reduction of insulin action in the liver leads to dysregulation of glucose production, and lipogenesis, consequently resulting in dyslipidemia (6). The maintenance of glucose homeostasis partly depends on hepatic glucose production through gluconeogenesis and glycogenolysis (1), which is regulated by several key enzymes, such as phosphoenolpyruvate carboxykinase-1 (Pck1), glucose-6-phosphatase (G6pc), and glycogen phosphorylase (Pygl). These genes are transcriptionally regulated by FoxO1 (forkhead box O1), a key transcription factor (7, 8). FoxO1 is suppressed by insulin through the activation of Akt, also known as Protein Kinase B, *via* the insulin receptor substrate 1 (IRS1) and IRS2-associated phosphoinositide 3-kinase (PI3K) (9). On the other hand, glycolytic enzymes such as glucokinase (Gck) and pyruvate kinase liver and red blood cell (Pklr) could reduce blood glucose.

Testosterone is the most important biologically relevant form of androgens in humans (10). Low plasma testosterone concentrations, in men with hypogonadism, are associated with an increased risk of type 2 diabetes and vascular disease, and supplementation of testosterone significantly improves glycolipid homeostasis (11). It has been reported that testosterone treatment to T2DM animals showed reduced hepatic glucose output (12). Testosterone treatment inactivates GSK3α, but not relying on the PI3K/Akt pathway, inhibits FoxO1 through the interaction of androgen receptor with FoxO1 and downregulating PEPCK, thereby suppressing the gluconeogenesis pathway and achieving better glucose homeostasis (12).

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Sex-dependent glucose homeostasis

Our previous work showed that the voltage-gated proton channel Hv1 is expressed in pancreatic β-cells and regulates insulin secretion as a positive regulator (13, 14). Loss of Hv1 inhibits insulin secretion in both first and second phase, which contributes to glucose intolerance and hyperglycemia in male mice (14). After treated with high-fat diet, male mice lacking Hv1 displayed diet-induced obesity, along with tissue inflammation and hepatic steatosis (15). Surprisingly, we found that fasting blood glucose in female mice lacking Hv1 was significantly lower than that in Hv1-deleted male mice, suggesting a sex dimorphism in glucose homeostasis between Hv1-deleted mice. Therefore, here, we investigated the difference in glucose homeostasis between Hv1-deleted male and female mice. Our data demonstrated that the sex-related difference in glucose metabolism is due to the deficiency of physiological testosterone in male mice. Exogenous testosterone supplementation could decrease the blood glucose in Hv1-deleted male mice, along with enhanced insulin signaling in liver tissues. The finding suggested the possibility of more effective sex-specific therapies for obesity, diabetes, and metabolic syndrome.

Results

Knockout of Hv1 results in higher blood glucose levels in males than female mice

When experimenting with a mouse model of obesity induced by high-fat diet, we found that KO male mice are more likely to develop excessive obesity caused by fat accumulation than WT mice (15). At the same time, we unexpectedly found that the high-fat challenge had little effect on KO female mice. Therefore, we noticed that there is an obvious sex dimorphism in glucose and lipid metabolism in KO mice. Firstly, glucose and insulin levels were measured respectively in KO male and female mice in fasted state and the WT males and females as controls. The body weight of the male mice was obviously higher than that of the female mice in both WT and KO groups (Fig. 1, *A* and *B*), and the blood glucose levels in fasted state were markedly high in KO males (9.66 ± 1.05 mM, n = 12, $p < 0.001$) compared with KO females (7.49 ± 0.94 mM, n = 12), and there was no difference in WT groups (Fig. 1*C*). Whereas the fasting insulin levels between sexes were no significant difference (Fig. 1*D*). We calculated HOMA-IR (an index of insulin resistance) and HOMA-IS (an index of insulin sensitivity) from corresponding glucose and insulin levels (16). HOMA-IR suggested a tendency of insulin resistance in KO male mice compared with females, but no statistical significance between them (Fig. 1*E*), while HOMA-IS showed a decrease of insulin sensitivity in KO male mice compared with KO female mice (Fig. 1*F*).

Knockout of Hv1 results in glucose intolerance and defect of insulin secretion in both male and female mice

As described above, knockout of Hv1 leads to serious hyperglycemia in male mice than that in females. To examine whether the sex difference in glucose homeostasis is due to glucose intolerance and insulin resistance, we performed intraperitoneal (i.p.) glucose tolerance tests (IPGTT) and insulin tolerance tests (IPITT). KO male and female mice developed similar glucose intolerance (2 g/kg body weight) (Fig. 2*A*), which was more intolerant than WT groups. Corresponding serum insulin levels were much lower in both KO male and female mice throughout the IPGTT compared with WT group, providing an evidence for an insulin secretion

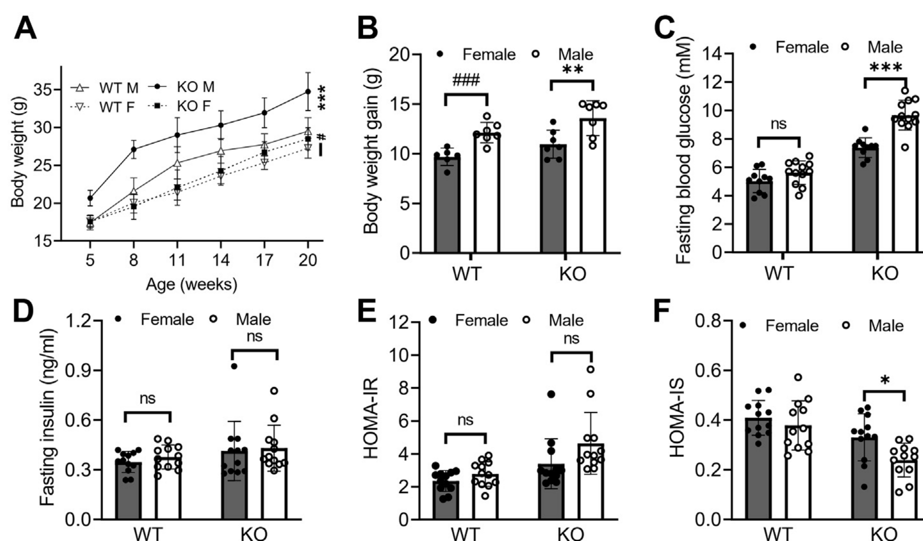

Figure 1. Knockout of Hv1 results in higher blood glucose levels in males than female mice. *A* and *B*, time course of body weight changes (*A*) and relative body weight gain (*B*) for male (○) and female (●) mice from wild-type (WT) and Hv1-knockout (KO) genotypes as indicated (WT males, WT M; WT females, WT F; KO males, KO M; KO females, KO F; n = 7/group). *C* and *D*, fasting blood glucose (*B*) and corresponding serum insulin (*C*) concentrations at 8-week-old mice after fasted for 6 h in each group (n = 12/group). *E* and *F*, HOMA parameters calculated from fasting blood glucose (*C*) and corresponding serum insulin concentrations (*D*). Data were shown as means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$ between the indicated groups. ns, no significant. Groups that share a vertical bar at the final time point did not significantly differ. HOMA-IR was calculated as follows: fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5. HOMA-IS is the reciprocal of HOMA-IR.

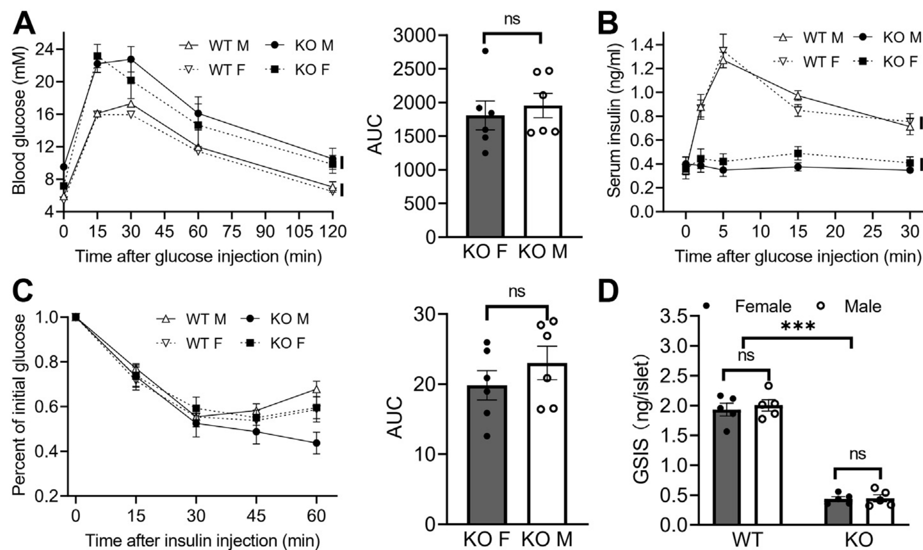

Figure 2. Glucose intolerance and defect of insulin secretion in both Hv1-deficient male and female mice. *A*, glucose tolerance tests (GTTs) were performed at 8-week-old after fasted for 6 h in each group as indicated (n = 6/group). Blood glucose was plotted at the indicated time intervals after i.p. injection of 2 g glucose/kg body weight. The area under the curve (AUC) is shown in the *right panel*. *B*, serum insulin content was plotted at the indicated time intervals under the GTT in each group as indicated. *C*, insulin tolerance tests (ITT, 0.75 U/kg, i.p.) at 8-week-old mice in each group as indicated (n = 5–6/group). The area under the curve (AUC) is shown in the *right panel*. *D*, GSIS (glucose-stimulated insulin secretion) of isolated islets from 8-week-old males and females (n = 5/group). Results were expressed as means ± SEM. ns, no significant, KO M *versus* KO F. ***$p < 0.001$ WT *versus* KO group. Groups that share a vertical bar at the final time point did not significantly differ.

defect in response to glucose (Fig. 2*B*). Meanwhile, the similar curves of IPITT indicated that KO male and female mice have same insulin tolerance (Fig. 2*C*). On the other hand, the insulin secretion from isolated islets in both KO males and females was much lower compared with normal male and female mice, indicating that the low serum insulin levels in both KO male and female mice is due to defect of insulin secretion in pancreatic β-cells (Fig. 2*D*). These data suggested that sexually dimorphic response to blood glucose in KO mice is not explained by sex differences in whole-body insulin action.

Knockout of Hv1 results in elevated gluconeogenesis and decreased glycolysis in male livers

Hepatic glucose production through gluconeogenesis and glycogenolysis plays an important role in maintaining glucose homeostasis (1). To examine whether the loss of insulin secretion in KO male mice results in hepatic glucose dysregulation, next, we focused on liver that plays a crucial role for glucose metabolism by regulating glycogenolysis, glycolysis, and gluconeogenesis. As shown in Figure 3, *A* and *B*, the mRNA levels of key glycolytic enzymes Gck and Pklr were downregulated by 31% and 46% *versus* KO female mice, respectively. Furthermore, the mRNA level of key gluconeogenic enzyme G6pc in livers from KO male mice was significantly higher than that in females (KO female: 5.09 ± 0.86-fold, KO male: 10.59 ± 1.59-fold, n = 3, $p < 0.01$), which was consistent with the expression of the protein level (KO female: 0.76 ± 0.03, KO male: 1.01 ± 0.08, n = 3, $p < 0.05$) (Fig. 3, *C* and *E*). Thus, the preserved blood glucose in KO female mice might be due to the low gluconeogenesis and high glycolysis. Meanwhile, the mRNA level of Pck1 in livers from KO mice (female: 2.76 ± 0.15-fold, male: 2.52 ± 0.35-fold, n = 3) was significantly higher than that in WT (female: 1.01 ± 0.11-fold, male: 0.61 ± 0.10-fold, n = 3) group, but there is no difference in the expression of the protein level (Fig. 3, *D* and *F*), which may be explained by the diversity of enzyme regulation. Additionally, the mRNA levels of Gck and Pklr were both upregulated in KO female group compared with KO male group. By contrast, the mRNA levels of G6pc and Pck1 in livers were both upregulated in KO group compared with WT group, especially in KO males. In addition, there were no changes in the mRNA level of key glycogenolysis enzyme Pygl (Fig. 3*G*) and the hepatic glycogen content (Fig. 3*H*) in livers between the WT and KO groups, suggesting that there are no effects on the breakdown and synthesis of liver glycogen in Hv1-knockout mice. These data suggested that the direct reason of hyperglycemia in KO mice may be the disorders of hepatic glucose production induced by the decreased insulin secretion.

Hepatic deficiency of insulin signaling in Hv1-knockout male mice

Many studies demonstrated that FoxO1 significantly contributes to hyperglycemia by promoting gene transcription of the gluconeogenic enzyme G6pc and Pck1 (17–19) and that the IRS1/2-PI3K-Akt-FoxO1 branch of insulin signaling is largely responsible for hepatic insulin-regulated glucose homeostasis (20). To further elucidate the mechanism of the preserved blood glucose in females, we firstly analyzed the mRNA expression patterns of genes involved in insulin action in livers. The mRNA level of IRS1 was significantly downregulated by 57% in KO males compared with KO females (Fig. 4*A*). The mRNA level of IRS2 was upregulated by

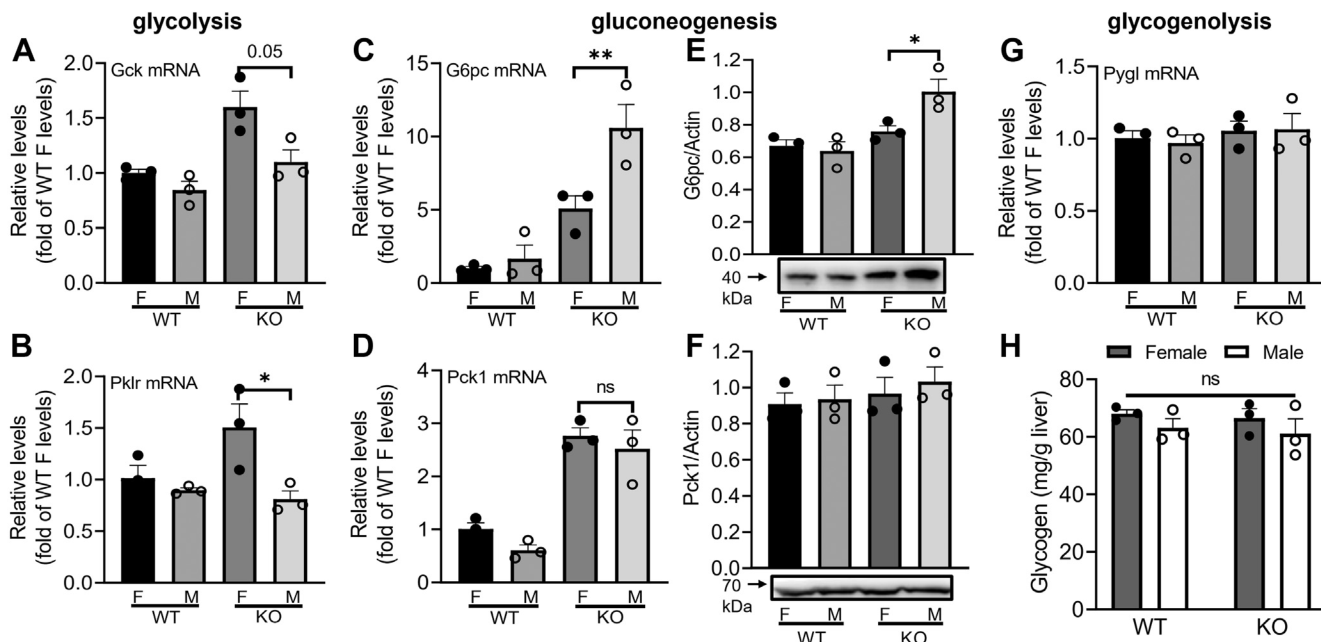

Figure 3. Knockout of Hv1 results in elevated gluconeogenesis and decreased glycolysis in male liver. *A–D*, relative mRNA levels of glycolytic (Gck and Pklr) (*A* and *B*) and gluconeogenic genes (G6pc and Pck1) (*C* and *D*) in livers of each group, normalized to WT female mice (n = 3/group). *E* and *F*, western blots and corresponding quantification of G6pc and Pck1 proteins in liver lysates of each group (n = 3/group). *G*, relative mRNA levels of glycogenolytic gene (Pygl) in livers of each group, normalized to WT female mice (n = 3/group). *H*, hepatic glycogen content was measured in liver lysates of each group (n = 3/group). Data were collected from 8-week-old male and female mice of WT and KO genotypes as indicated and shown as means ± SEM. *$p < 0.05$; **$p < 0.01$ KO females *versus* KO males. ns, no significant.

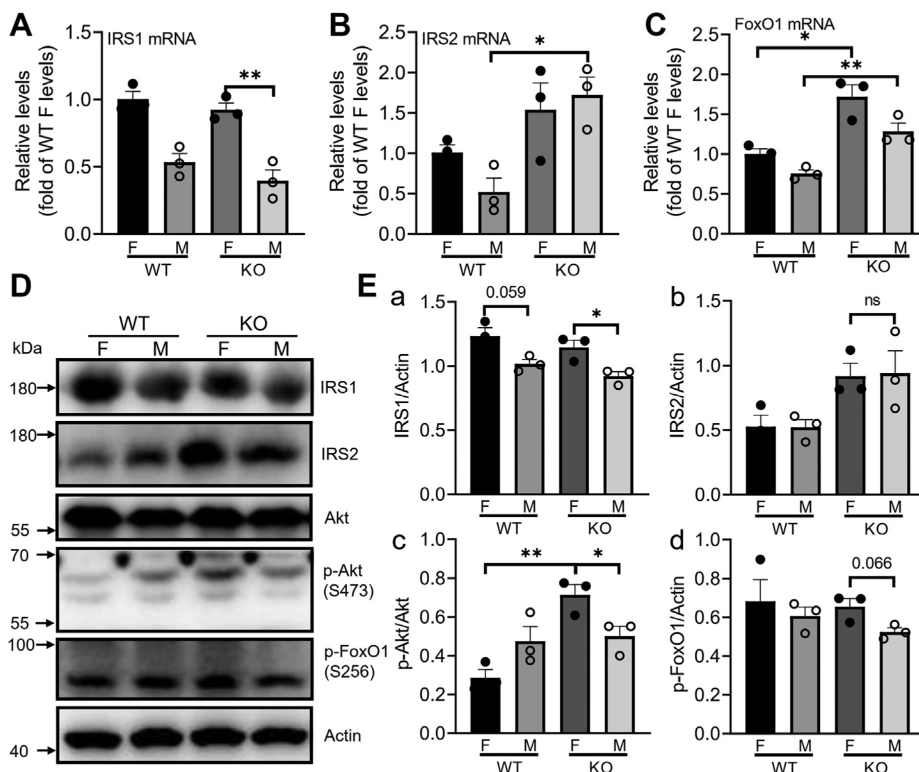

Figure 4. Hepatic deficiency of insulin signaling in Hv1-knockout male mice. *A–C*, relative mRNA levels of insulin signaling genes (IRS1, IRS2 and FoxO1) in livers of each group, normalized to WT female mice (n = 3/group). *D*, representative immunoblots of IRS1, IRS2, Akt, p-Akt^{S473}, and p-FoxO1^{S256} proteins in liver lysates of each group as indicated (n = 3/group). *E*, western blot corresponding quantification of IRS1 (*a*), IRS2 (*b*), p-Akt^{S473}/Akt (*c*), and p-FoxO1^{S256} (*d*) proteins in liver lysates of each group. Data were collected from 8-week-old male and female mice of WT and KO genotypes as indicated. Data were shown as means ± SEM. *$p < 0.05$, **$p < 0.01$ between the indicated groups. ns, no significant.

1.53- and 3.3-fold ($p < 0.05$) in KO group compared with that in WT group, whereas there is no change between KO males and females (Fig. 4*B*). Next, we detected the mRNA level of FoxO1, which is a predominant regulator for hepatic glucose production and can be suppressed by insulin action (7–9). The mRNA level of FoxO1 was upregulated by 1.7-fold ($p < 0.05$) in KO group compared with that in WT group (Fig. 4*C*). Based on the above findings, knockout of Hv1 appears to somewhat inhibiting insulin signaling in the male livers.

To further verify these results, we next determined whether the protein expression levels in insulin signaling pathway are different between KO male and female mice. Western blot analysis and corresponding quantification of IRS1, IRS2, Akt, p-Akt^{S473}, and p-FoxO1^{S256} proteins in livers lysates of each group were shown in Figure 4, *D* and *E*. Consistently, the protein level of IRS1 was significantly downregulated in KO males compared with KO females (female: 1.15 ± 0.06, male: 0.92 ± 0.04, n = 3, $p < 0.05$) (Fig. 4*E*, *a*). Moreover, there was no difference between these two groups (Fig. 4*E*, *b*). Combined with the mRNA and protein expression levels, IRS1 manifests a sexual dimorphism in liver rather than IRS2 in KO mice. Activated IRS1 activates its downstream Akt by phosphorylation (20). The ratio of p-Akt^{S473}/Akt was significantly downregulated in KO males compared with females (female: 0.71 ± 0.05, male: 0.50 ± 0.05, n = 3, $p < 0.05$) (Fig. 4*E*, *c*), which suggested that knockout of Hv1 results in the loss of insulin action in male livers. In addition, the protein level of p-FoxO1^{S256} showed a downward trend in KO males compared with females but without a statistical difference (Fig. 4*E*, *d*), suggesting that there may be another mechanism to inactivate FoxO1. Taken together, impaired insulin signaling in the liver is associated with the severe hyperglycemia in KO males, thus contributing to the sex dimorphism in glucose homeostasis in Hv1-deficient mice.

Knockout of Hv1 results in testosterone deficiency in males

Both clinical and experimental studies have shown that sex hormones after puberty have largely contributed to gender differences in diabetes susceptibility (10). All sex hormones exist in both males and females, although the levels are different between the two sexes. The most important biologically relevant forms of estrogens and androgens are estradiol (E2) and testosterone, respectively (21). To further elucidate whether the sex steroid hormones result in the sex dimorphism in glucose homeostasis in Hv1-deficient mice, we measured the serum E2 and testosterone levels in 8-week-old KO female and male mice and WT mice as a control group. There was no difference of serum E2 level in female and male mice between WT and KO group (Fig. 5, *A* and *B*). Interestingly, the level of testosterone showed a significantly lower in KO males compared with the WT males (Fig. 5*C*). KO male mice exhibited a 0.26-fold lower serum testosterone level compared with WT males (Fig. 5*C*), while there were no difference of serum testosterone level in females between WT and KO group (Fig. 5*D*). Since the serum testosterone content of female mice was below the detection range of the ELISA kit, the OD450 was directly used. Thus, hypotestosteronemia in Hv1-knockout male mice may be responsible for the dysregulation of glucose homeostasis.

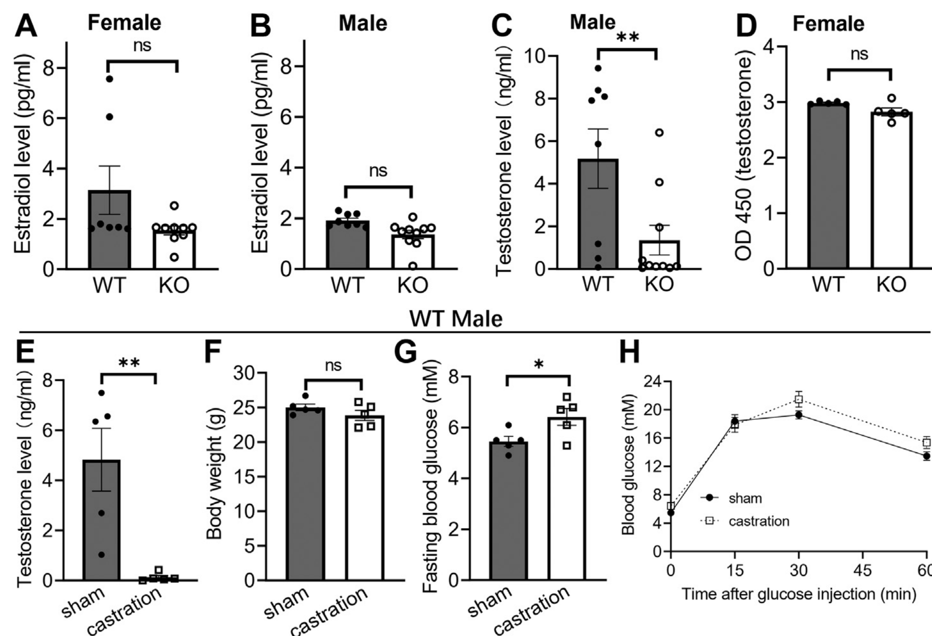

Figure 5. Knockout of Hv1 results in testosterone deficiency in males and castration-induced testosterone deficiency increases fasting glucose in WT mice. *A* and *B*, serum estradiol levels in 8-week-old WT and KO female (*A*) mice and male (*B*) mice (n = 7–10/group). *C* and *D*, serum testosterone levels in 8-week-old WT and KO male (*C*) mice and female (*D*) mice (n = 7–10/group). Since the serum testosterone content of female mice was below the detection range of the ELISA kit, the OD450 was directly used. *E–H*, serum testosterone level (*E*), body weight (*F*), fasting blood glucose (*G*), and GTT (*H*) in shame-operated and castration-operated WT male mice (n = 5/group). Data were shown as means ± SEM. *$p < 0.05$, **$p < 0.01$ between the indicated groups. ns, no significant.

Castration-induced testosterone deficiency increases fasting glucose in WT males

To verify whether a lower level of testosterone in KO male mice is a reason of hyperglycemia, we performed the castration of WT male mice to examine the phenotypes displayed in KO mice. Six weeks after surgery, the serum testosterone levels of the castration-operated group were significantly lower compared with those in the sham-operated group (sham: 4.826 ± 1.42 ng/ml, castration: 0.1267 ± 0.07 ng/ml, $p < 0.01$) (Fig. 5*E*). But the mean body weight did not display a statistical significance between these two groups (Fig. 5*F*), which may be due to the short time period. Interestingly, the fasting blood glucose levels were significantly higher after bilateral testicles were removed (sham: 5.46 ± 0.19 mM, castration: 6.42 ± 0.33 mM, $p < 0.05$) (Fig. 5*G*). To examine whether differences in the use of glucose contribute to the increase of blood glucose, we performed the GTT. As shown in Figure 5*H*, castration resulted in rising trend of glucose intolerance, but without a statistical significance. Previous studies also reported that the castration-induced testosterone deficiency results in an increase in fasting blood glucose levels, and reintroducing testosterone partly rescues normal glucose metabolism (22, 23). These results demonstrated that the effect of physiological testosterone deficiency on metabolic disorders partly provides a basis for explaining the phenotype of KO mice.

Testosterone supplementation reduces blood glucose levels via IRS1/Akt pathway in liver

To estimate the effect of testosterone on the glucose homeostasis, we investigated whether testosterone administration ameliorated the hyperglycemia in KO male mice. Surprisingly, the supplementation of exogenous testosterone significantly decreased blood glucose in both WT and KO males compared with the placebo replacement groups (WT: placebo: 6.26 ± 0.21 mM, testosterone: 5.27 ± 0.20 mM, n = 8, $p < 0.01$; KO: placebo: 9.04 ± 0.33 mM, testosterone: 6.71 ± 0.42 mM, n = 8, $p < 0.001$) (Fig. 6*B*), accompanied by the elevated testosterone levels after testosterone administration in KO mice (placebo: 1.78 ± 0.68 ng/ml, testosterone: 6.87 ± 0.86 ng/ml, n = 8, $p < 0.01$) (Fig. 6*A*). These data suggested that exogenous testosterone treatment could ameliorate glucose homeostasis in testosterone-deficiency-associated hyperglycemia, which is consisted with the previous reports that testosterone deficiency has a high prevalence in men with T2DM (24, 25).

Consistent with androgen action on glucose control, after low-dose and short-term testosterone treatment, the expression of IRS1 in cultured adipocytes and skeletal muscle cells is upregulated (26). To verify whether the decrease in blood glucose caused by testosterone is related with insulin signaling pathway in liver, we measured the protein levels of IRS1, Akt,

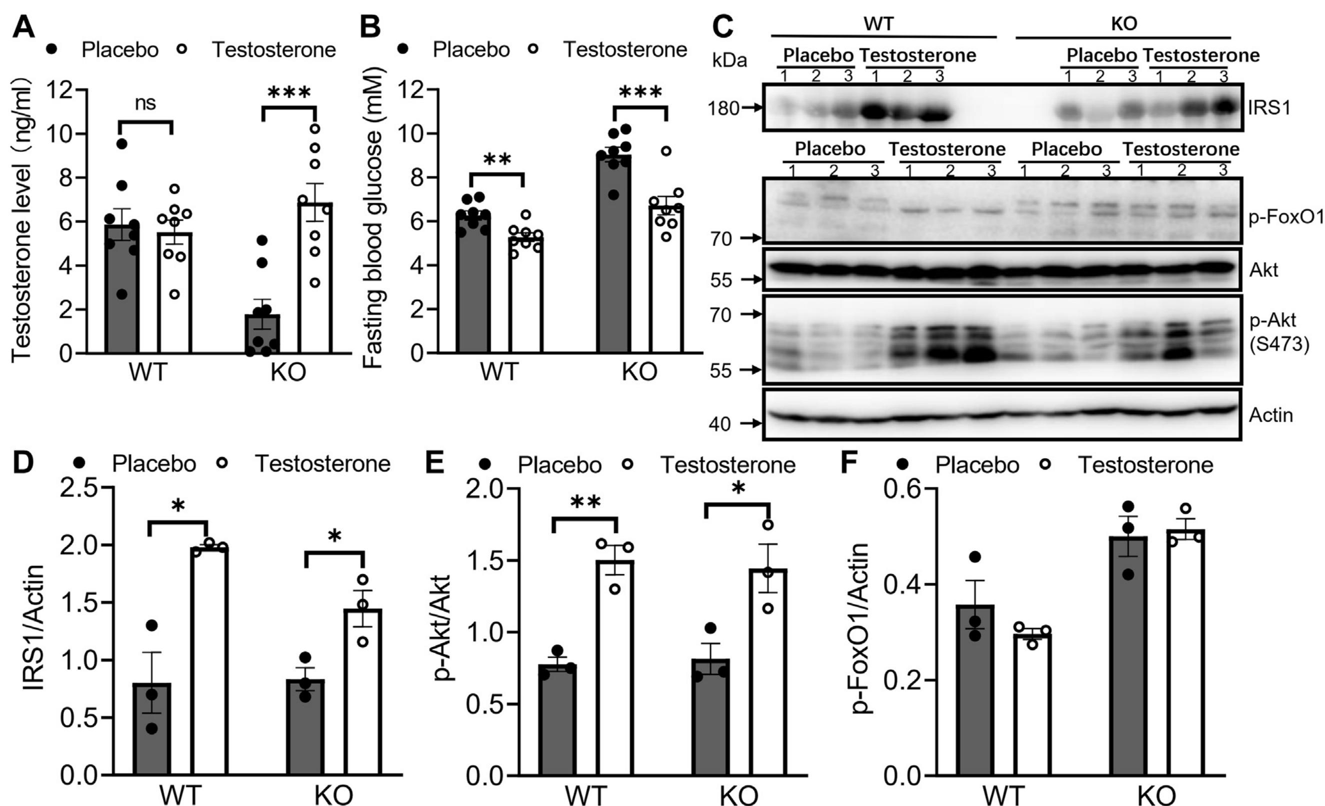

Figure 6. Testosterone supplementation reduces blood glucose via IRS1/AKT pathway in the liver. *A*, serum testosterone levels in WT and KO male mice after treated with testosterone supplementation (testosterone) and placebo replacement (placebo). Exogenous testosterone was administered once every 2 days for 2 weeks with 100 μg/g body weight from 5 weeks of age (n = 8/group). *B*, fasting blood glucose concentrations after fasted for 6 h in placebo and testosterone group (n = 8/group). *C*, representative immunoblots of IRS1, Akt, p-Akt^{S473}, and p-FoxO1^{S256} proteins in liver lysates of each group as indicated (n = 3/group). *D–F*, western blot corresponding quantification of IRS1 (*D*), p-Akt^{S473}/Akt (*E*), and p-FoxO1^{S256} (*F*) proteins in liver lysates of each group (n = 3/group). Data were shown as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between the indicated groups. ns, no significant.

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p-Akt^{S473}, and p-FoxO1^{S256} in the liver. As shown in Figure 6*C*, the hepatic IRS1 protein level and the ratio of p-Akt^{S473}/Akt in both WT and KO mice were significantly upregulated after treatment with testosterone, providing the evidence of improved insulin action. The protein level of IRS1 increased 2.47-fold and 1.74-fold ($p < 0.05$) in WT and KO mice, respectively (Fig. 6*D*). Accordingly, the ratio of p-Akt^{S473}/Akt increased 1.94-fold ($p < 0.01$) and 1.77-fold ($p < 0.05$) in WT and KO mice, respectively (Fig. 6*E*). However, there was no significant difference in the p-FoxO1^{S256} expression levels before and after treatment with testosterone. Based on the above findings, we confirmed that the short-term testosterone treatment partly reduces blood glucose levels *via* IRS1/Akt pathway in the liver. Taken together, these results demonstrated that the sexual dimorphism in glucose homeostasis in Hv1-deficient mice is associated with the hypotestosteronemia in Hv1-knockout male mice, which could regulate glucose homeostasis in the liver by insulin pathway.

Discussion

Our previous works demonstrated that the voltage-gated proton channel Hv1 is highly expressed in pancreatic β-cells and positively regulates insulin secretion (13, 14). Knockout of Hv1 inhibits insulin secretion in pancreatic β-cells, leads to hyperglycemia and glucose intolerance in male mice (14). In present work, we showed a sex-specific difference in glucose homeostasis in KO male and female mice, which resulted from the deficiency of serum testosterone in males that affects glucose production in livers by IRS1-AKT-p-AKT pathway.

In recent years, addressing gender and sex differences has become a priority issue in several medical fields including metabolic diseases (27). Sex dimorphism relies on basic biological differences, which affect the physiological or pathophysiological processes of males and females differently. So, recent guidelines underline the need to consider such sex differences from preclinical to clinical studies, avoiding traditional male predominance when using these approaches (4). It is now clear that sex has an important influence on the pathogenesis of metabolic diseases, such as type 2 diabetes. The first dimorphism is associated with the prevalence of diabetes, in which males predominate in humans and most animal models, while women are usually not affected by dietary metabolic disorders (3). Consisting with the previous studies, in our present work, we found that knockout of Hv1 results in the deficiency of serum testosterone in male mice, accompanied by hyperglycemia, compared with WT males. It is worth noting that KO male mice are more prone to obesity induced by a high-fat diet in our previous report (15). In that obesity model, we also performed the experiment using KO females; however, there is no effect on body weight and fat accumulation in KO female mice after challenged with overnutrition (data not shown). Now, we speculate that the deficiency of testosterone in KO males may be another mechanism for the obesity, while females are generally protected from diet-induced metabolic disorders.

In present work, we speculate that the lower physiological levels of testosterone produced in KO male mice testicles contributed to the impaired glucose metabolism. If this is the case, female mice, which have a much lower level of testosterone, should show a higher body weight and fasting blood glucose level compared with male mice. However, the fact is that the normal female mice generally show lower body weight than male mice (28–30). There are sex differences in the body weight variation and distribution, energy conversion to body weight, and body composition changes (28), which may be contributed by estrogen level (31), sex-specific leptin resistance (32, 33), and differences in gross locomotor activity (34). In addition, there is no significant difference in fasting blood glucose between normal male and female mice (28, 30) even if female mice have low level of testosterone (35). In both males and females, estradiol is derived from the aromatization of testosterone (21). Moreover, the animal models and human studies showed that estrogen action is important in both males and females in the regulation of glucose homeostasis, improving glucose tolerance and insulin sensitivity (21). Based on our present results, the androgen/estrogen ratio undoubtedly has an impact on metabolic regulation. Therefore, we suspect that the disproportion caused by knockout of Hv1 is more severe in males and results in more severe metabolic disorders than females.

Liver plays a major role in the regulation of glucose homeostasis and insulin sensitivity. In diabetes, hyperglycemia is partly attributed to dysregulated hepatic glucose production, particularly enhancing gluconeogenesis (4). Many studies demonstrated that FoxO1 significantly contributes to hyperglycemia by promoting gene transcription of the gluconeogenic enzyme G6pc and Pck1 (17–19) and that the IRS1/2-PI3K-Akt-FoxO1 branch of insulin signaling is largely responsible for hepatic insulin-regulated glucose homeostasis (20). Consistent with the previous reports, the livers from Hv1-deficient male mice have significantly higher mRNA levels of key gluconeogenic enzymes Pck1, G6pc, and the key transcription factor FoxO1 than that of WT male mice, which account for the hyperglycemia in Hv1-deficient male mice. Exceptionally, the IRS2 showed a high mRNA expression level in Hv1-deficient male mice compared with WT. This might explain why knockout of Hv1 does not lead to insulin resistance in male mice (14).

A series of studies have reported the existence of sexual dependence in the regulation of metabolic homeostasis, especially in the liver tissues (30, 36, 37). Females are more insulin sensitive than males (10). As expected, the expression level of hepatic G6pc is downregulated, whereas the key glycolytic enzymes including Gck and Pklr were upregulated in Hv1-deleted female mice *versus* males, demonstrating a lower gluconeogenesis and higher glycolysis in Hv1-deficient females than males. Meanwhile, the higher expression level of IRS1 in Hv1-deleted female mice may also account for the lower blood glucose levels in females than male mice. Previous studies have shown that the estrogen receptor alpha signaling in hepatocytes mediates the protective effects against steatosis and insulin resistance after challenged with high-fat diet in female

mice (38). Therefore, the protection of female mice against hyperglycemia induced by Hv1 deficiency may be achieved through lower gluconeogenesis and higher glycolysis in the liver mediated by estrogen.

Accumulating evidence suggests that testosterone deficiency is an independent cardiovascular risk factor, and many recent reviews have focused on the link between hypogonadism, type 2 diabetes mellitus, and cardiovascular disease (39, 40). Testosterone levels themselves are considered to be lowered by chronic disease, making the designation of a causal relationship difficult to delineate (41). Similarly, testosterone administration in orchidectomized adult male rats increased insulin receptor mRNA and protein expression in liver tissue and was associated with a normalization of the castration-induced impairment of glucose oxidation (42). The present results further support the sexual dimorphism in glucose homeostasis in livers resulted from the physiological sex hormone. In our case, testosterone leads to a decrease in blood glucose in Hv1-deficient male mice, indicating the important role of testosterone in maintaining glucose homeostasis. Overall, our findings provide an evidence for more effective sex-specific therapies for obesity, diabetes, and metabolic syndrome.

Experimental procedures

Animals

$Hv1^{-/-}$ (KO) mice provided by Dr Y. Okamura (School of Medicine, Osaka University) were backcrossed eight times in the C57BL/6J background, as previously described (14). Animals were kept in a pathogen-free facility under a 12-h light/dark cycle with access to water and a standard mouse diet (Lillico Biotechnology). All animal husbandry and experiments were approved by and performed in accordance with guidelines from the Animal Research Committee of Nankai University.

Metabolic Analyses

Blood glucose levels were measured using a glucometer (One Touch, Johnson & Johnson) from the tail vein after fasting for 6 h, and the blood samples were collected simultaneously for determination of serum insulin by an ELISA kit (Mercodia). Glucose tolerance tests (GTT) and Insulin tolerance tests (ITT) were performed in mice fasted for 6 h from 9:00 to 15:00. All mice used in above experiments were at 8-week-old, and the procedures were implemented as previously reported (14). To measure the blood testosterone and estradiol (E2) levels, tail vein blood was collected in 8-week-old mice with free to eat at 10:00 AM. Blood samples were centrifuged to measure the concentrations of testosterone (IBL America) and E2 (Calbiotech) using ELISA kit.

Liver glycogen content analysis

The glycogen content assay was performed as previously described (16). Briefly, 8-week-old mice with free to eat were euthanized, and then about 50 mg of liver samples was quickly weighed and boiled with 0.4 ml of 2 mol/L HCl for 2 h. After that, 0.4 ml of 2 mol/L NaOH and 22 μl of 1 mol/L Tris-HCl (pH 7.4) were added to neutralize the acid. The glucosyl units from above glycogen breakdown were measured using Amplex Red Glucose Assay Kit (Invitrogen).

Insulin secretion in static incubation

Pancreata were perfused *in situ* with collagenase solution, and experiments of static incubation were performed as previously described (14).

Western blotting

Mice at 8-week-old with free to eat were euthanized at 10:00 AM, and then the tissues of liver were removed and frozen immediately on dry ice and stored at −80 °C. Frozen tissue was homogenized in RIPA (LABLEAD) buffer containing 1 mM PMSF. An equal amount of protein was resolved in SDS-PAGE and transferred to nitrocellulose membrane for Western blot. Antibodies for IRS1 and p-FoxO1 (phosphorylated FoxO1 at Ser256) were purchased from Cell Signaling Technology, Akt and p-Akt (phosphorylated Akt at Ser473) from Proteintech, Pck1 from Abcam, G6pc from Novus Biologicals, and β-Actin from Sigma. Protein densitometry was performed and analyzed using ImageJ as previously described (14).

Quantitative real-time PCR

Frozen tissues of liver were used to determine the mRNA levels. The procedures of quantitative RT-PCR were performed as described previously (15). Gene-specific mouse primers were used as indicated in Table 1.

Testosterone supplement

Testosterone propionate (MACKLIN, China) was dissolved in sesame oil at a concentration of 20 μg/μl. Testosterone was

Table 1
Primer pair sequences

Gene	Primer pairs
Gck	
Forward	GGCTTCACCTTCTCCTTCCC
Reverse	TGTTGTTCCCTTCTGCTCCG
FoxO1	
Forward	GTGGATGGTGAAGAGCGTGC
Reverse	AAGGGACAGATTGTGGCGAA
Pck1	
Forward	TGACATTGCCTGGATGAAGT
Reverse	GTCTTAATGGCGTTCGGATT
Pygl	
Forward	TGGCAGAAGTGGTGAACAATGAC
Reverse	CCGTGGAGATCTGCTCCGATA
G6pc	
Forward	TCGGAGACTGGTTCAACCTC
Reverse	TCACAGGTGACAGGGAACTG
Pklr	
Forward	GAGTCGGAGGTGGAAATTGT
Reverse	GTCCACCCACACTGTCTTTG
Irs1	
Forward	GCCAGAGGATCGTCAATAGC
Reverse	AGACGTGAGGTCCTGGTTGT
Irs2	
Forward	GGTCCAGGCACTGGAGCTTTG
Reverse	GGGGCTGGTAGCGCTTCACT
GAPDH	
Forward	CTCATGACCACAGTCCATGCCATCACTG
Reverse	CATGAGGTCCACCACCCTGTTGCTGTA

injected subcutaneously into mice at 100 μg/g body weight/2 days for 2 weeks from 5 weeks of age, and blood testosterone levels were measured at 7 weeks of age.

Surgical castration

Five-week-old WT male mice were castrated according to the previous report (22). Under barbiturate anesthesia, an abdominal incision was made into the abdomen. The testes, epididymides, and vas deferentia were pulled out of the body while avoiding injury to the intestines. For each testis, the vas deferens was ligated at two sites and cut between ligations. The testes were then resected and the wound sutured.

Statistical analysis

All statistics were performed using GraphPad Prism 8 software. Comparison of the mean between groups was performed by *t* test. p values <0.05 were considered significant.

Data availability

All data are contained within the manuscript.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: G6pc, glucose-6-phosphatase; Gck, glucokinase; IPGTT, intraperitoneal (i.p.) glucose tolerance test; IPITT, intraperitoneal (i.p.)insulin tolerance test; IRS1, insulin receptor substrate 1; KO, knockout; Pck1, phosphoenolpyruvate carboxykinase-1; PI3K, phosphoinositide 3-kinase; Pygl, glycogen phosphorylase.

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