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# 1-deoxynojirimycin inhibits glucose absorption and accelerates glucose metabolism in streptozotocin-induced diabetic mice

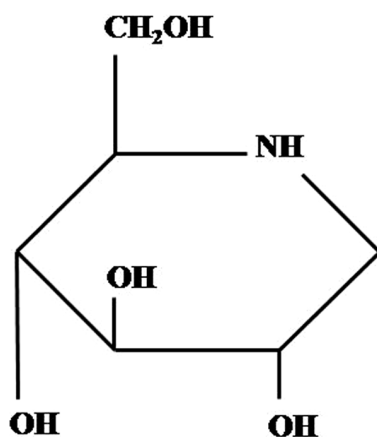
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We investigated the role of 1-deoxynojirimycin (DNJ) on glucose absorption and metabolism in normal and diabetic mice. Oral and intravenous glucose tolerance tests and labeled <sup>13</sup>C<sub>6</sub>-glucose uptake assays suggested that DNJ inhibited intestinal glucose absorption in intestine. We also showed that DNJ down-regulated intestinal SGLT1, Na<sup>+</sup>/K<sup>+</sup>-ATP and GLUT2 mRNA and protein expression. Pretreatment with DNJ (50 mg/kg) increased the activity, mRNA and protein levels of hepatic glycolysis enzymes (GK, PFK, PK, PDE1) and decreased the expression of gluconeogenesis enzymes (PEPCK, G-6-Pase). Assays of protein expression in hepatic cells and *in vitro* tests with purified enzymes indicated that the increased activity of glucose glycolysis enzymes was resulted from the relative increase in protein expression, rather than from direct enzyme activation. These results suggest that DNJ inhibits intestinal glucose absorption and accelerates hepatic glucose metabolism by directly regulating the expression of proteins involved in glucose transport systems, glycolysis and gluconeogenesis enzymes.

Diabetes mellitus is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism that result from deficient insulin secretion and/or insulin resistance<sup>1</sup>. Under normal circumstances carbohydrates in the diet are hydrolyzed into monosaccharides, which are then absorbed through the intestine by a transepithelial transport system. There is ample evidence to show that the increases capacity of the small intestine to absorb glucose in type 2 diabetes is a result of changes occurring at the brush border membrane (BBM) and basolateral membranes (BLM)<sup>2</sup>. These changes are mainly due to enhanced activity, mRNA and protein levels of sodium glucose transport protein (SGLT1), Na<sup>+</sup>/K<sup>+</sup>-ATPase and glucose transporter 2 (GLUT2)<sup>3-5</sup>. Supra-physiological levels of glucose absorption cause an imbalance in the carbohydrate metabolism and overload the endocrine system, which attempts to correct it in return. This results in progressive deterioration in endocrine control<sup>6</sup>. Continuing deterioration of endocrine control further exacerbates the metabolic disturbances by altering the activities of key enzymes such as glucokinase (GK), phosphofructokinase (PFK), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase). These changes impair peripheral glucose utilization and augment hepatic glucose production<sup>7,8</sup>. Therefore, inhibiting glucose absorption at BBM or BLM and/or modulating the activity of the hepatic enzymes involved in carbohydrate metabolism provide a plausible strategy for lowering blood glucose levels in subjects with type-2 diabetes mellitus (T2DM).

Several studies have demonstrated that phytochemicals from natural resources provide new opportunities for treating diabetes<sup>9</sup>. Mulberry leaf extracts have been used in China and other Asian countries to treat diabetes on the basis of reports of anti-diabetic effects in experimental animals<sup>10-12</sup>. In previous studies we demonstrated that 1-deoxynojirimycin (DNJ, Fig. 1) derived from Mulberry leaves is a potent inhibitor of intestinal  $\alpha$ -glucosidases, which acts as an antihyperglycemic agent by slowing the rate of carbohydrate degradation to monosaccharides. This delays glucose absorption and significantly reduces post prandial blood glucose levels<sup>13,14</sup>. We also showed that DNJ was readily absorbed and retained in the small intestine and liver of diabetic mice for more than 7 h<sup>13</sup>, which attracts our considerable interest. The present study was designed to investigate whether the antidiabetic effects of DNJ were mediated by attenuating glucose absorption in small intestine and modulating key enzymes involved in glucose metabolism in liver.



**Figure 1** | Structure of 1-deoxyojirimycin.

## Results

**DNJ inhibits glucose absorption by suppressing intestinal glucose transport.** We previously demonstrated that DNJ resulted in reversible, noncompetitive inhibition of  $\alpha$ -glycosidases and slowed the conversion of carbohydrate to monosaccharide<sup>13,14</sup>. Does DNJ possess the potential of inhibiting the glucose absorption? To address this question, oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) was performed. In OGTT, maximal blood glucose levels 30 min after glucose administration were  $16.56 \pm 1.07$  mmol/L in normal mice and  $35.64 \pm 1.47$  mmol/L in diabetic mice. Pretreatment with DNJ (50 mg/kg) improved glucose tolerance in both normal and diabetic mice (Fig. 2A, B) and significantly reduced the calculated relative area under the glucose concentration curve (AUC) (Fig. 2C, D). Maximal blood glucose levels after DNJ pretreatment were  $11.88 \pm 1.83$  mmol/L in normal mice and  $27.61 \pm 1.91$  mmol/L in diabetic mice. In IVGTT, maximal blood glucose levels were seen 15 min after injection of glucose solution into the tail vein. However, there was no statistically significant difference in glucose levels neither between NaCl and DNJ pre-treated normal mice (Fig. 2E) nor between NaCl and DNJ pretreated diabetic mice (Fig. 2F). These findings suggest that the decrease in maximal blood glucose levels seen in the OGTT was the result of inhibition of intestinal glucose absorption.

To verify the result, we used labeled  $^{13}\text{C}_6$ -glucose to trace glucose absorption in the small intestine. As seen in Fig 2G, blood  $^{13}\text{C}_6$ -glucose levels were significantly lower in DNJ treated diabetic mice ( $3.37 \pm 0.33$  mg/mL) than in control diabetic mice ( $4.22 \pm 0.30$  mg/mL;  $P < 0.01$ ). Values in DNJ and control normal mice were  $2.18 \pm 0.19$  and  $2.96 \pm 0.28$  mg/mL, respectively. In the small intestine glucose levels were significantly higher in DNJ treated diabetic mice ( $46.68 \pm 7.41$   $\mu\text{g/g}$ ) than in diabetic controls ( $26.29 \pm 5.01$   $\mu\text{g/g}$ ,  $P < 0.01$ ). Corresponding values in control mice without diabetes were  $49.66 \pm 3.47$  vs.  $34.28 \pm 5.05$   $\mu\text{g/g}$  (Fig. 2H). Collectively, these results suggested that DNJ could inhibit glucose absorption in the small intestine.

To explore whether the inhibitory mechanism of DNJ on glucose absorption were related to changes in the transepithelial transport system, levels of SGLT1,  $\text{Na}^+/\text{K}^+$ -ATP, GLUT2 mRNA and corresponding proteins in jejunum were assayed using real time (RT)-PCR and Western blot analysis. The results showed that mRNA (Figs 3A) and the corresponding protein expression (Figs 3B, C) of SGLT1 in BBM, and mRNA and protein expression of  $\text{Na}^+/\text{K}^+$ -ATP, GLUT2 in the BLM were all significantly up-regulated in diabetic mice. These results are in accordance with previous reports<sup>2,3,15</sup>. Following treatment with DNJ (50 mg/kg) levels of the three proteins were significantly lower than in normal and diabetic control mice, indicating

that DNJ attenuated glucose absorption by inhibiting the expression of glucose transport proteins.

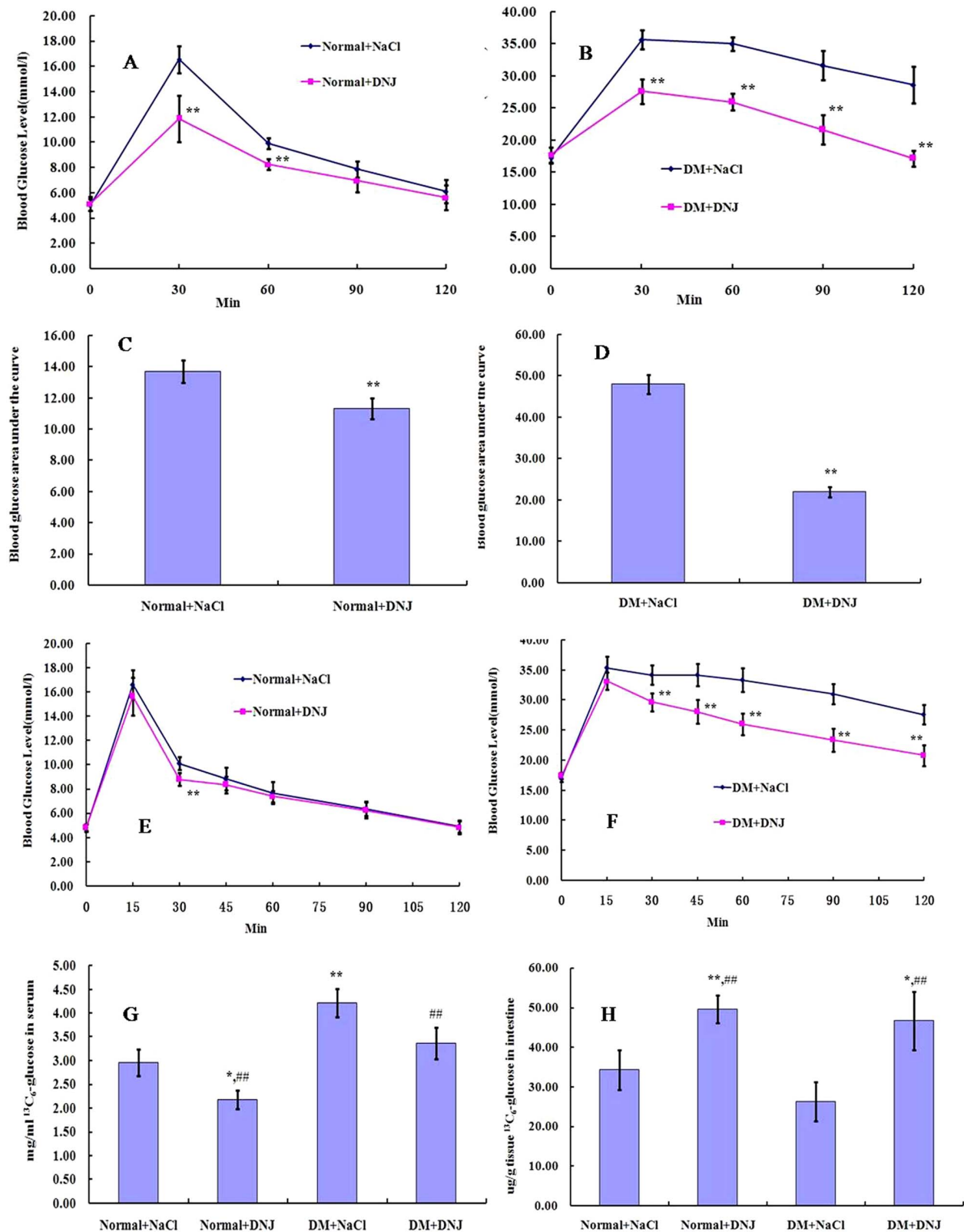
**DNJ accelerates glucose utilization by regulating hepatic glucose metabolism enzymes.** Blood glucose concentrations decreased sharply in diabetic mice pretreated with DNJ (Fig. 2B, F). In this group, maximal values were  $27.61 \pm 1.91$  mmol/L at 30 min after OGTT and  $35.17 \pm 1.45$  mmol/L at 15 min after IVGTT. Two hours after the GTTs these values decreased to  $17.18 \pm 1.21$  and  $20.78 \pm 1.69$  mmol/L, respectively. Control diabetic mice had only small decreases in glucose concentrations after OGTT or IVGTT. The liver is an important organ that plays a pivotal role in glycolysis and gluconeogenesis<sup>16</sup>. After a glucose load, we observed a significant increase of hepatic glucose production (HDP) in both normal and diabetic control animals. This was markedly reduced in DNJ pre-treated groups (Fig. 4A). The results were in accordance with the observed changes in blood glucose, suggesting that DNJ accelerates hepatic glucose metabolism and/or inhibits gluconeogenesis (Fig. 4A).

We next determined the dynamic activities of GK, PFK and PK to investigate whether the effects of DNJ involved modulation of hepatic enzymes responsible for glucose metabolism. The activities of GK, PFK and PK were significantly diminished in streptozotocin (STZ)-induced diabetic mice after glucose administration. By contrast, in normal and diabetic mice pretreated with DNJ, the activities of these enzymes significantly increased 120 min after glucose administration (Fig. 4B, C and D). The levels of hepatic GK, PFK and PK mRNA and its accompanying protein were also significantly increased compared to normal control and diabetic control mice (Fig. 5A, D and E).

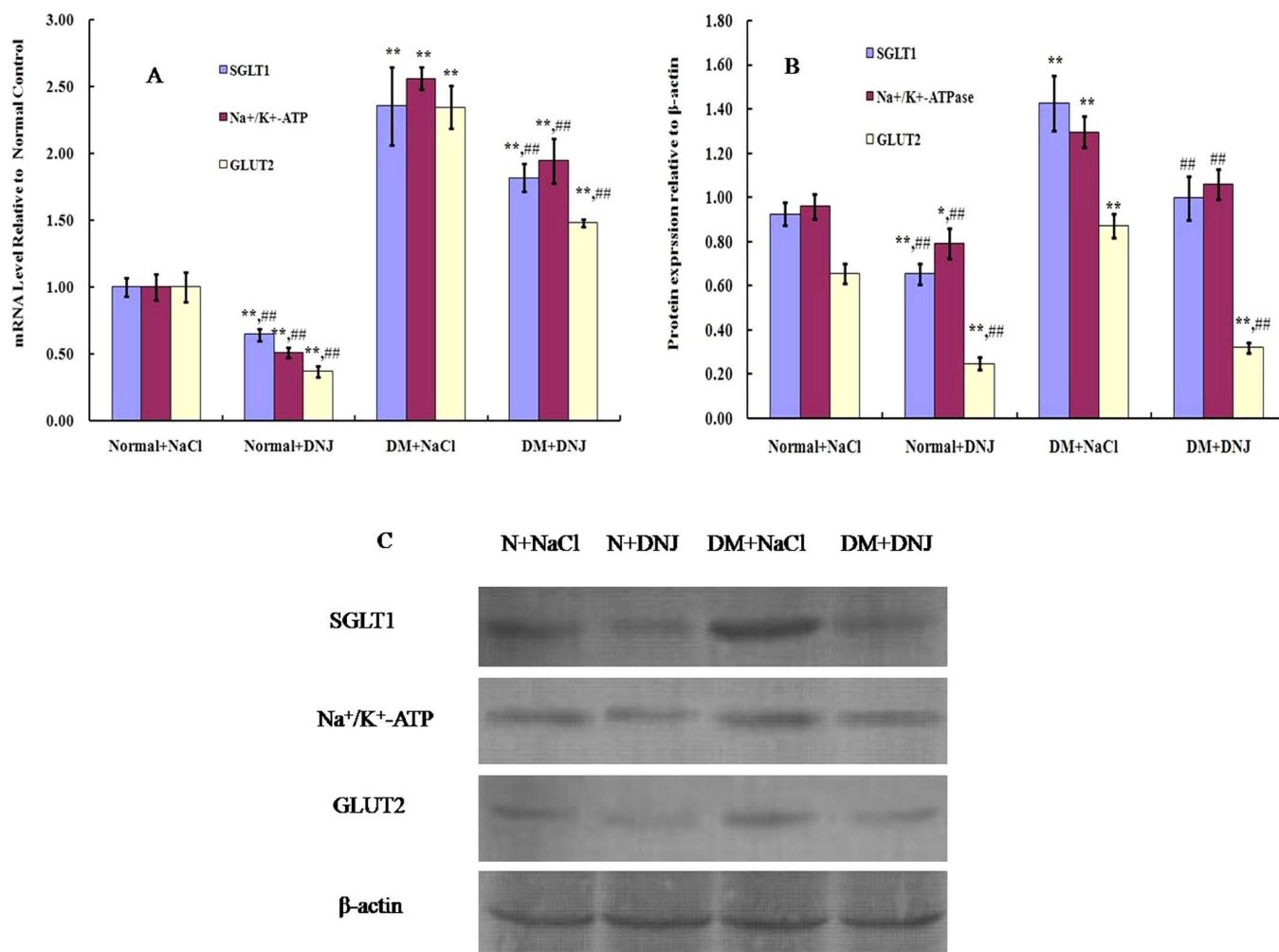
Pyruvate decarboxylase E1 (PDE1) is an important component of the pyruvate dehydrogenase complex (PDC), which is inversely modulated by PDC kinases (PDK)<sup>17</sup>. In our experiments DNJ markedly increased PDE1 mRNA levels in normal and diabetic mice (Fig. 5B). It also attenuated PDK2 mRNA and its corresponding protein expression (Fig. 5B, D and E). These findings indicate that DNJ might catalyze dephosphorylation reactions and result in activation of PDE1. To test this hypothesis, we analyzed pyruvate levels after glucose administration and found that hepatic pyruvate concentrations decreased more rapidly in DNJ treated mice than control mice suggesting that DNJ accelerated an oxidative decarboxylation reaction (Fig. 4E). However, NADH/NAD<sup>+</sup> ratios were not significantly different between DNJ and control groups (Fig. 4F) suggesting that PDE1 dephosphorylation was not a result of an intracellular energy change. Taken together, these results suggest that pyruvate metabolic responses were accelerated by increased PDE1 expression whereas attenuated PDK expression resulted in PDE1 activation via a dephosphorylation pathway.

We next analyzed gene expression of the gluconeogenic enzymes PEPCK and G-6-Pase in the liver of normal and diabetic mice. The mRNA level of both enzymes was significantly up-regulated in STZ-treated mice (Fig. 5C). In DNJ pretreated mice, there was a prominent reduction in PEPCK expression evidenced by mRNA and protein levels (Fig. 5D, E). Hepatic G-6-Pase expression was also significantly reduced in DNJ pretreated mice (Fig. 5C), with resulting suppression of dephosphorylation of glucose-6-phosphate to free glucose. These findings were consistent with previous results showing that DNJ decreases hepatic glucose production in diabetic mice (Fig. 4A). Analysis of hepatic  $\text{Na}^+/\text{K}^+$ -ATP levels indicated that DNJ decreased the activity (Fig. 4G), mRNA (Fig. 5C) and protein expression (Fig. 5D, E) of this complex. These results are consistent with the observed changes in gluconeogenic genes (PEPCK, G-6-Pase). However, the relationship between  $\text{Na}^+/\text{K}^+$ -ATP and gluconeogenesis requires further research.

**DNJ directly regulates expression of hepatic enzymes involved in glucose metabolism.** It has been previously shown that insulin



**Figure 2 | Oral glucose tolerance test (OGTT), intravenous glucose tolerance test (IVGTT) and labeled  $^{13}\text{C}_6$ -glucose uptake assay.** After an overnight fast (16 h), normal and STZ-induced diabetic mice were intragastrically administered with equal volumes of 0.9% saline or DNJ (50 mg/kg b.w/d). 15 min later, a 30% glucose solution (3 g/kg) was orally administered for OGTT and 30% glucose solution (1 g/kg) was injected at the base of the tail vein for IVGTT. Blood samples were collected from the tail tip vein 0, (15), 30, (45), 60, 90, 120 min after glucose challenge. Results represent OGTT in normal (A) and diabetic mice (B), and the corresponding calculated relative area under the curve (AUC) for glucose concentration (C, D). IVGTT results are shown in normal (E) and diabetic mice (F).  $^{13}\text{C}_6$ -glucose concentrations in serum and intestine are shown in G and H, respectively. Results are expressed as means  $\pm$  SD (A–F,  $n = 10$ ; G and H,  $n = 5$  per group). \* $P < 0.05$  and \*\* $P < 0.01$  vs control groups.



**Figure 3 | SGLT1, Na<sup>+</sup>/k<sup>+</sup>-ATP and GLUT2 expression analysis in small intestine.** Panel A shows RT-PCR analysis of SGLT1, Na<sup>+</sup>/k<sup>+</sup>-ATP and GLUT2 mRNA expression in normal (N) and diabetic mice (DM) with or without DNJ pretreatment. Panel B and C shows western blot analysis of SGLT1, Na<sup>+</sup>/k<sup>+</sup>-ATP and GLUT2 protein expression in the following groups: Normal + NaCl + Glucose (N + NaCl), Normal + DNJ + Glucose (N + DNJ), DM + NaCl + Glucose (DM + NaCl), DM + DNJ + Glucose (DM + DNJ). Western blot results were quantified using image J software (National Institutes of Health, USA). Density values were normalized to β-actin. Data are mean ± SD for three experiments per group. \*P < 0.05, \*\*P < 0.01 vs Normal; #P < 0.05, ##P < 0.01 vs DM.

activates glycogen synthesis and suppresses gluconeogenesis and glycogenolysis by affecting post-translational modification of the key enzymes and their gene expression<sup>18</sup>. The opposing hormone glucagon, induces gluconeogenesis enzymes and activates glycogenolysis resulting in hepatic glucose output<sup>8</sup>. Our results show that under normal physiological conditions, glucose ingestion induces insulin secretion and reduces glucagon and epinephrine levels (Fig. 6A, B, and C). Moreover, significantly increased serum insulin and decreased levels of glucagon and epinephrine were detected in the DNJ pre-treated normal group (Fig. 6A, B, C). For diabetic mice, elevated serum insulin levels were observed for 2 h after glucose administration in both DNJ treated and untreated groups, but insulin concentrations remained constantly lower in DNJ treated mice than that in untreated diabetic mice (Fig. 6A). In addition, glucagon and epinephrine levels both decreased in to a lesser degree after glucose absorption in DNJ treated diabetic mice (Fig. 6B, C). These finding indicate that DNJ did not significantly improve insulin response in diabetic mice and imply that the sharp decrease in blood glucose levels in DNJ pretreated diabetic mice might be the consequence of rapid glucose disposal, resulting from increased activity of the glucose metabolism enzymes in liver (Fig. 4A, B, C).

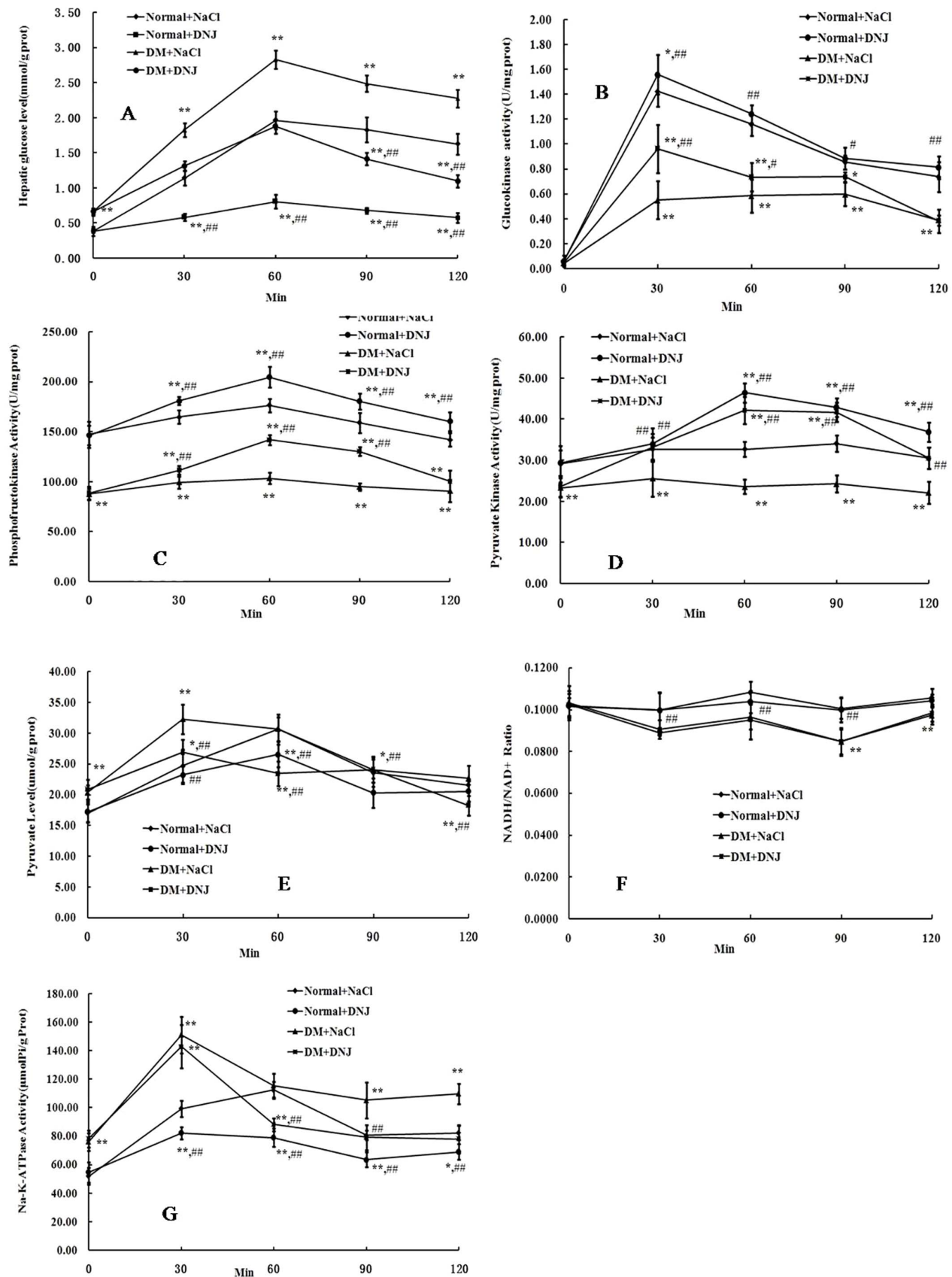
To verify this hypothesis and exclude the interference of the hormones (insulin, glucagon and epinephrine) on hepatic glucose metabolism enzymes, we evaluated GK, PFK, PK, PDK and PEPCK protein expression in mice hepatic cells (NCTC1469) directly incubated with DNJ for 2 h. As shown in Fig. 7, GK, PFK and PK protein expression increased, and PDK, PEPCK expression decreased in cells exposed to DNJ, indicating that expression of glucose metabolism enzymes was directly regulated by DNJ.

To further explore these findings, we used an *in vitro* assay to quantify the effect of DNJ on enzyme activities of GK, PFK and PK (Fig. 8). In the DNJ dose range 40 to 320 μg/mL, there was a progressive increase in PK activity which became maximal at 320 μg/mL and decreased with further increases in DNJ dose. However, GK and PFK activities were directly inhibited by the DNJ. Considering the highest concentration of DNJ determined in mice liver is just 0.96 μg/g<sup>13</sup>, we concluded that the increased activity of glucose metabolism enzymes was not related to the direct effect of DNJ, but were probably related to the relative increased in protein expression.

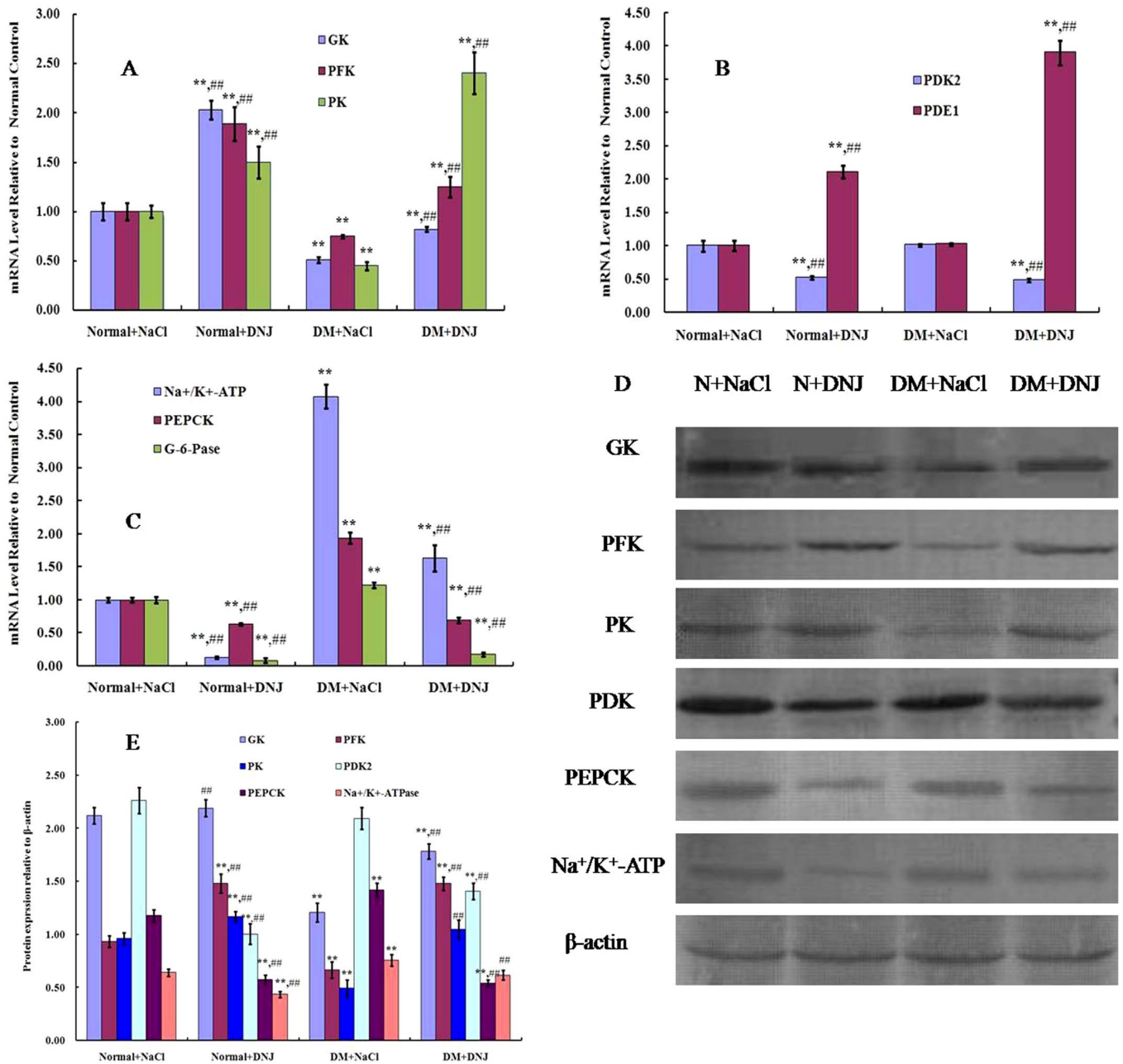
## Discussion

Glycosidases were the first therapeutic targets for the treatment of diabetes. DNJ obtained from Mulberry leaves has been reported to be





**Figure 4** | Effect of DNJ on dynamic changes in different biochemical values in liver of normal and diabetic mice. Hepatic glucose level (A), GK activity (B), PFK activity (C), PK activity (D), Pyruvate concentration (E), NADH/NAD<sup>+</sup> ratio (F), and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (G) were assayed 0, 30, 60, 90, 120 min after glucose administration. Results are expressed as means ± SD for 10 animals per group. \*P < 0.05 and \*\*P < 0.01 vs control mice; #P < 0.05, ##P < 0.01 vs diabetic mice.

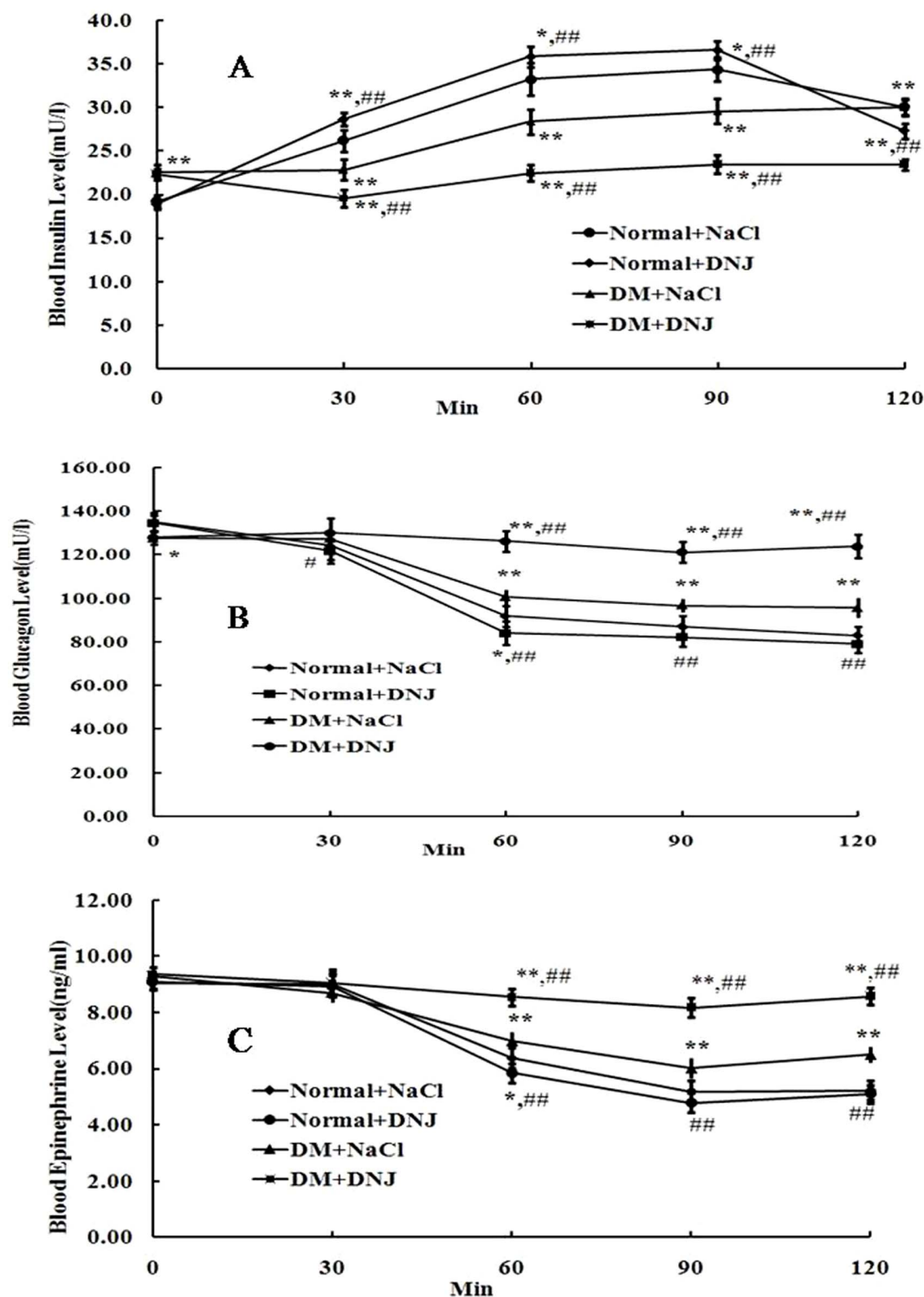


**Figure 5 | Hepatic expression of GK, PFK, PK, PDK2, PDE1, PEPCK, G-6-Pase and Na<sup>+</sup>/K<sup>+</sup>-ATPase.** Panels A, B and C show RT-PCR analysis of mRNA expression in normal mice and in diabetic mice (DM) with or without DNJ pretreated. Panel D and E shows western blot analysis of protein expression in the following groups: Normal + NaCl + Glucose (N + NaCl), Normal + DNJ + Glucose (N + DNJ), DM + NaCl + Glucose (DM + NaCl), DM + DNJ + Glucose (DM + DNJ). Western blot results were quantified using image J software (National Institutes of Health, USA). Density values were normalized to  $\beta$ -actin. Data are mean  $\pm$  SD of three mice in each group. \*P < 0.05, \*\*P < 0.01 vs Normal; #P < 0.05, ##P < 0.01 vs DM.

a potent inhibitor of intestinal sucrase and isomaltase which slows carbohydrate conversion to monosaccharides, thus delaying absorption and significantly reducing the glycemic peak response to a meal<sup>12,13</sup>. In earlier studies from our laboratory, we showed that DNJ is not only a potent inhibitor of intestinal  $\alpha$ -glycosidases, but that, it also modulated hepatic glucose metabolism and gluconeogenesis by up- or down-regulating mRNA expression of rate-limiting enzymes (GK, PEPCK and G-6-Pase) in alloxan-induced diabetic mice<sup>13</sup>. The current study for the first time demonstrates that DNJ prevents glucose absorption by inhibiting the expression of proteins involved in the transepithelial glucose transport system. At the same time it accelerates hepatic glucose utilization by directly

regulating enzyme protein levels correlated with glycolysis and gluconeogenesis.

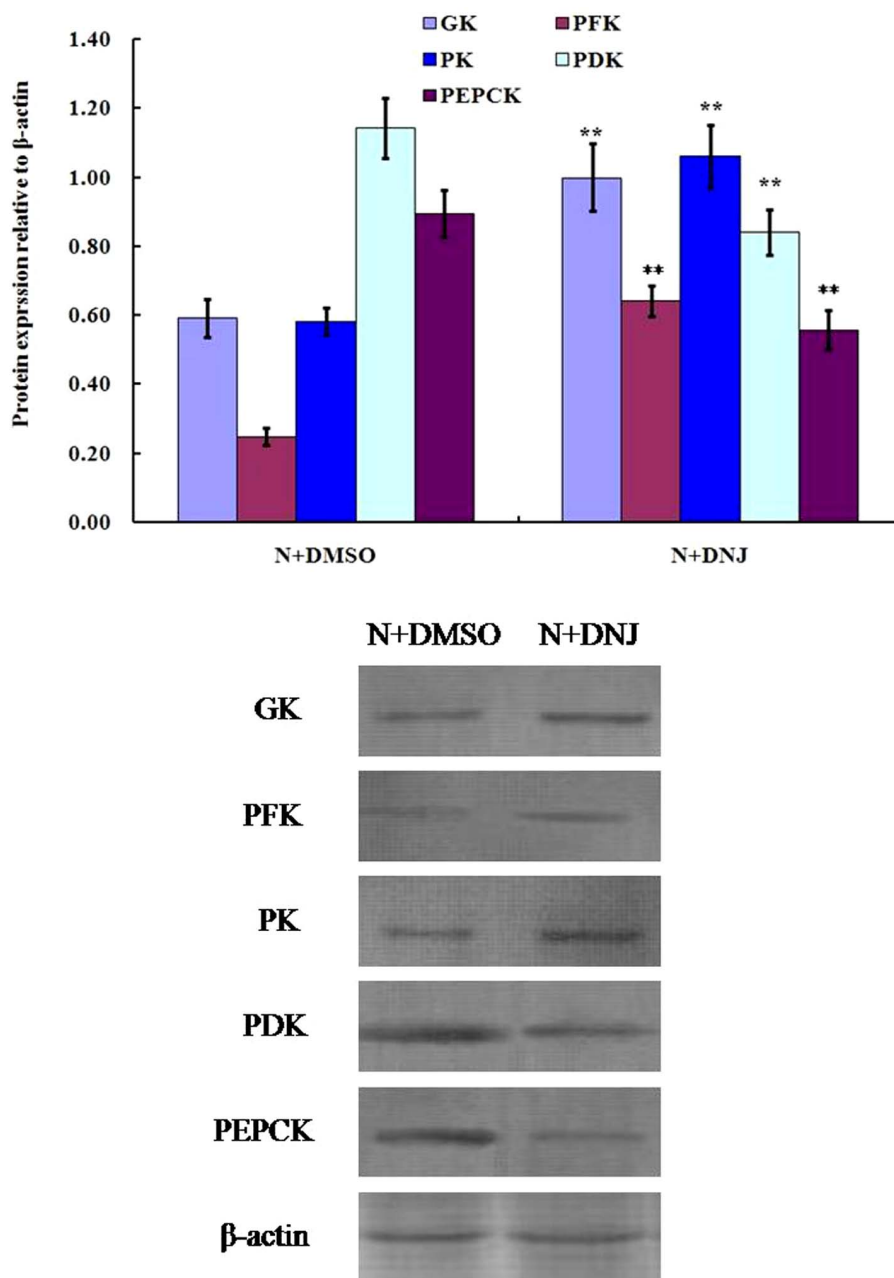
Carbohydrates, which are one of the three major nutrients in mammal diet, are hydrolyzed by digestive enzymes in the gastrointestinal tract. The resulting monosaccharides are absorbed from the small intestine via influx hexose transporters<sup>19</sup>. Two types of hexose transporters have been identified in human and murine small intestine: sodium-dependent Na<sup>+</sup>/glucose co-transporters (SGLT) and sodium-independent glucose transporters (GLUT)<sup>20–22</sup>. Glucose and galactose have been shown to be transported across the BBM domain of the enterocyte by SGLT<sup>23</sup>, while fructose absorption is mediated by GLUT<sup>5</sup>. The transport of all three sugars



**Figure 6** | Effect of DNJ on the dynamic change of insulin (A), glucagon (B) and epinephrine (C) in serum of normal and diabetic mice assayed at 0, 30, 60, 90, 120 min after glucose administration. Results are expressed as means  $\pm$  SD for 10 animals per group. \* $P < 0.05$  and \*\* $P < 0.01$  vs control mice, # $P < 0.05$ , ## $P < 0.01$  vs diabetic mice.

across the basolateral membrane domain of the enterocyte is mediated by a single transport protein, GLUT2<sup>3,21</sup>. Previous studies have reported that increased glucose absorption is due to increased activity and expression of SGLT1, and GLUT2<sup>3-5</sup>. The uptake of glucose across the BBM is also mediated by a Na<sup>+</sup>/K<sup>+</sup>-ATPase mechanism, which is responsible for establishing and maintaining the Na<sup>+</sup> gradient required for the activity of the Na<sup>+</sup>/glucose cotransporter (SGLT1)<sup>3</sup>. Recent studies have shown that modifications of systemic

glycemia in OGTT reflect the activity of the intestinal glucose transporter SGLT1<sup>24</sup>. We, therefore, examined the effect of DNJ on normal and diabetic mice subjected to an OGTT. DNJ significantly reduced the overall OGTT response after acute glucose administration in normal and diabetic mice, but there was no statistically significant difference in peak blood glucose levels between control and DNJ pre-treated groups subjected to an IVGTT. These results indicate that DNJ reduces intestinal transport *in vivo* and support



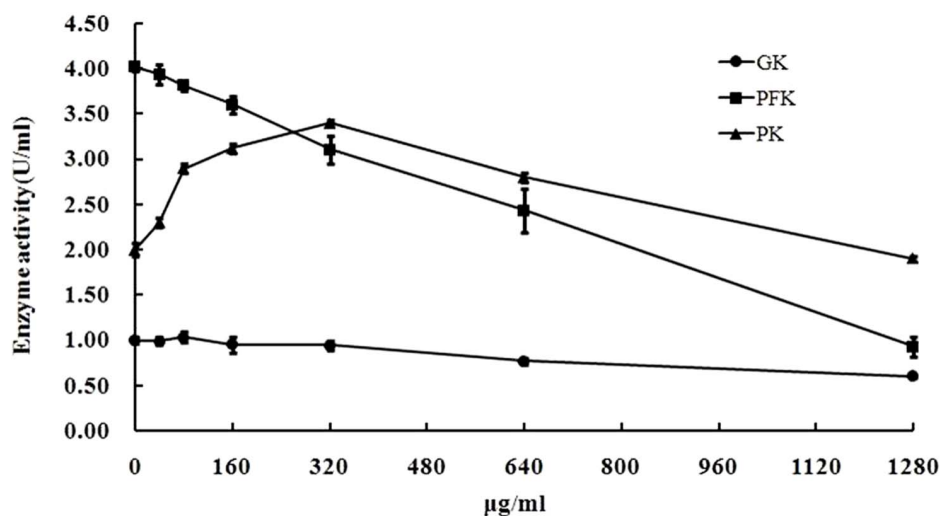
**Figure 7** | GK, PFK, PK, PDK2 and PEPCK protein expression in NCTC1469 mice hepatic cells. The results show western blot analysis of the protein expression in Normal cells + DMSO (N + DMSO), Normal cells + 40  $\mu$ g/mL DNJ (N + DNJ). Western blot results were quantified using image J software (National Institutes of Health, USA). In all experiments density values were normalized to  $\beta$ -actin. Data are means  $\pm$  SD for three experiments per group. \* $P < 0.05$ , \*\* $P < 0.01$  vs control.

previous studies<sup>13</sup> showing that DNJ improved OGTT in alloxan-induced diabetic mice. To further confirm this conclusion, we used labeled <sup>13</sup>C<sub>6</sub>-glucose to trace glucose absorption in the intestine. Blood <sup>13</sup>C<sub>6</sub>-glucose levels were lower in DNJ treated mice than controls, but opposite findings were seen in the small intestine (Fig. 2G, H). These results support the hypothesis that DNJ inhibits glucose absorption. To explore the inhibitory mechanism of DNJ on glucose absorption, levels of SGLT1, Na<sup>+</sup>/K<sup>+</sup>-ATP, GLUT2 mRNA and corresponding proteins were assayed. These experiments showed that mRNA and corresponding proteins for SGLT1, Na<sup>+</sup>/K<sup>+</sup>-ATPase and GLUT2 were up-regulated in the jejunum of STZ-induced diabetic mice. This finding supports evidence provided by other workers<sup>3-5,25</sup>. Treatments with DNJ (50 mg/kg) for three days evidently attenuated the expression of the three proteins in both normal and diabetic mice.

It is thought that the expression of SGLT1 and Na<sup>+</sup>/K<sup>+</sup>-ATPase may be controlled by cellular events acting at the post-transcriptional level<sup>3</sup>. Considering the pivotal role that Na<sup>+</sup>/K<sup>+</sup>-ATPase plays in glucose transport across the BBM, it is not surprising that the alterations in SGLT1 expression were paralleled by corresponding changes in Na<sup>+</sup>/K<sup>+</sup>-ATPase gene expression. These findings further support the contention that DNJ possess the potential to inhibit glucose absorption.

Elevated endogenous glucose production is a common abnormality associated with diabetes<sup>26</sup>. The liver is mainly responsible for maintaining normal concentrations of blood glucose by regulating glycolysis and gluconeogenesis<sup>16</sup>. GK is a cytoplasmic enzyme that phosphorylates glucose to glucose-6-phosphate (Glu-6-P), which is the first, and rate-limiting step in the oxidation of glucose<sup>27</sup>. PFK is a





**Figure 8** | Effect of DNJ on the enzyme activities of GK, PFK and PK *in vitro*. DNJ at different concentrations was incubated with the GK (PFK, PK) enzymes for 15 min before assay. Results are expressed as means  $\pm$  SD of three repeated experiments in each group.

rate limiting enzyme involved in glycolysis and represents a major control point in the metabolism of glucose<sup>8</sup>. PK is a ubiquitously expressed glycolytic enzyme that catalyzes the conversion of phosphoenol pyruvate to pyruvate. Under physiological conditions this irreversible reaction is considered to play a critical role in the regulation of metabolic flux in the second part of glycolysis<sup>28</sup>. A number of researchers have reported that GK, PFK and PK are the most sensitive indicators of the glycolytic pathway in the diabetic state<sup>29,30</sup>. Enhanced hepatic glucose production (HGP) has been shown to be involved in the pathogenesis of diabetes mellitus<sup>31,32</sup>. This was first shown in alloxan diabetic dogs<sup>33</sup> and obese insulin-resistant Zucker (fa/fa) rats<sup>34</sup>. Insulin resistance in the liver may be associated with the transient elevation in HGP after ingesting glucose<sup>32</sup>. In the current study, blood glucose concentrations following both OGTT and IVGTT decreased sharply within 120 min in diabetic mice pretreated DNJ. We also observed marked decreases in HGP in DNJ pretreated normal and diabetic mice. Based on these findings, we hypothesized that DNJ may activate glucose metabolism enzymes (GK, PFK, PK) and accelerate hepatic glucose utilization after glucose administration. Our results showed that the activity of hepatic GK was close to zero in both normal and diabetic mice after an overnight fast; and that hepatic PFK and PK activity in diabetic mice was significantly lower than in normal mice. We also showed that glucose administration activated GK, PFK and PK, and that DNJ pretreatment further enhanced its activities in liver. Levels of hepatic GK, PFK, PK mRNA and its accompanying protein were also significantly increased in DNJ pretreated mice compared to control mice (Fig. 5 A, D).

Pyruvate decarboxylase E1 (PDE1) is an important enzyme in the pyruvate dehydrogenase complex (PDC) that catalyzes the rate-limiting step of pyruvate to acetyl CoA in glycolysis<sup>35</sup>. This process is inversely mediated by PDC kinases (PDK)<sup>17</sup>. Inhibition of PDC activity impairs mitochondrial oxidation of pyruvate and promotes its cytoplasmic reduction to lactate. Diminished PDC activity also decreases the availability of acetyl CoA for the tricarboxylic acid (TCA) cycle<sup>17</sup>. Our results show that STZ-induced diabetes did not significantly alter the regulation of PDE1 gene expression in liver; whereas DNJ markedly increased the PDE1 mRNA levels in normal and diabetic mice. However, reduced PDK mRNA and protein expression were observed in DNJ pretreated mice, which could catalyze PDE1 dephosphorylation and result in increased PDE1 activity. To confirm whether increased PDE1 accelerated the translation of pyruvate to acetyl CoA, we analyzed the dynamic change in pyruvate levels in treated mice. The hepatic pyruvate concentration decreased

more rapidly in DNJ treated mice than in control animals suggesting that oxidative decarboxylation reactions were accelerated prior to entering the Krebs cycle. When intracellular NADH/NAD<sup>+</sup> ratio are elevated, serine residues on PDE1 are phosphorylated by special phosphate kinases, which result in PDE1 becoming less active. Therefore, reducing the NADH/NAD<sup>+</sup> ratio strengthens PDE1 dephosphorylation, and speeds up pyruvate decarboxylation reactions. In our study there was no significant difference in the NADH/NAD<sup>+</sup> ratio between DNJ and control groups, indicating that PDE1 dephosphorylation reaction was not the consequence of intracellular energy changes. These findings suggest that acceleration of pyruvate metabolic responses was mediated by the increased PDE1 expression and the inhibitory effect of DNJ on PDK expression resulted in PDE1 being activated by the dephosphorylation pathway.

PEPCK and G-6-Pase, expressed mainly in the liver, play a critical role in providing glucose to other organs during diabetes, prolonged fasting or starvation<sup>36</sup>. PEPCK is involved in the synthesis of glucose-6-phosphate from non-carbohydrate precursors; G-6-Pase catalyzes the dephosphorylation of glucose-6-phosphate to free glucose as the terminal step in gluconeogenesis and glycogenolysis. Expression of the PEPCK and G-6-Pase genes is enhanced in the liver in most models of diabetes, and is thought to contribute to the increased hepatic glucose output associated with this disease<sup>13,37</sup>. In our study DNJ significantly attenuated the hepatic expression of mRNA in both genes, resulting in a reduction in protein expression of PEPCK. These results suggest that decreased modulation of PEPCK/G-6-Pase gene expression by DNJ may play an important role in regulating STZ-induced glucose output. In addition, we also showed that DNJ decreased hepatic levels of Na<sup>+</sup>/K<sup>+</sup>-ATP as well as its activity and corresponding mRNA and protein expression. These results are consistent with changes in gluconeogenic genes (PEPCK, G-6-Pase). However, additional studies are needed to more fully elucidate the relationship between decreased hepatic expression of Na<sup>+</sup>/K<sup>+</sup>-ATP and glucose metabolism.

Under normal physiological conditions insulin activates glycolysis enzymes, and represses gluconeogenesis enzyme activity in order to achieve post prandial homeostatic regulation of blood glucose. Both glucagon and epinephrine play opposite roles in glucose metabolism. STZ has been used in animal models to induce both insulin-dependent (IDDM, type 1) and non-insulin-dependent diabetes mellitus (NIDDM, type 2)<sup>39</sup>. It has been reported that STZ produces mild to severe types of diabetes according to the dosage administered<sup>38</sup>. Low doses of STZ (100 mg/kg) have been shown to induce slowly-progressive diabetes mellitus, in which non-fasting serum glucose



levels increase gradually without a decline of the serum insulin level<sup>40</sup>. Conversely, elevated serum insulin levels were detected within 1 week after low dose STZ administration<sup>40</sup>. Based on these findings it was postulated that the low dose STZ-induced diabetic mouse model is similar to NIDDM and the increase of serum insulin in the first week is due to over secretion of insulin from  $\beta$ -cells that escape from the attack of STZ<sup>40</sup>. Interestingly, we found that the serum insulin level in low dose STZ-induced diabetic mice increased on Day 10, which is in accordance with the previous report<sup>40</sup>.

Insulin deficiency and/or resistance is a significant feature of diabetes mellitus, and results in dysregulation of carbohydrate metabolism and decreased activity of GK, PFK and PK<sup>8</sup>, resulting in impaired peripheral glucose utilization and augmented hepatic glucose production. Our results show that DNJ enhances the activity of the glycolysis enzymes (GK, PFK and PK) and suppresses the expression of the gluconeogenesis enzymes (PEPCK and G-6-Pase) in both normal and diabetic mice. It is interesting to note that diabetic mice responded differently to DNJ than normal mice. Although the serum levels of insulin, glucagon and epinephrine in DNJ treated mice changed in the same direction as in the NaCl treated group (Fig. 6A, B, C), DNJ pretreatment significantly affected the secretion levels of serum hormones (Fig. 6A). In normal mice the level of serum insulin increased significantly after DNJ treated in comparison to untreated group, while in DNJ treated diabetic mice the serum insulin level response was less marked than in the untreated diabetic mice. These findings suggest that hormones levels were affected by DNJ treatment and that DNJ does not significantly improve the insulin response of diabetic mice. The mechanism of action of DNJ in regulating hormone secretion in STZ-induced diabetic mice and normal mice remains unknown. However current results imply that the decline of blood glucose by DNJ treatment in diabetic mice may be due to rapid peripheral glucose disposal, caused by increased activity of glucose metabolism enzymes. The relationship between DNJ and the hormone secretion requires further investigation.

To exclude hormonal interference on hepatic glucose metabolism enzymes, we determined GK, PFK, PK, PDK and PEPCK protein expression in mouse NCTC1469 hepatic cells incubated with DNJ for 2 h. Protein expression of GK, PFK and PK were increased, and PDK, PEPCK were impaired after exposure to DNJ suggesting that DNJ may directly regulate the expression of glucose metabolism enzymes. We also showed that GK and PFK activities were directly inhibited by *in vitro* exposure to DNJ, whereas the activity of PK was gradually increased after exposure to DNJ in the concentration range 40 to 320  $\mu\text{g}/\text{mL}$ . However, as the highest concentration of DNJ determined in mice liver is 0.96  $\mu\text{g}/\text{g}$ <sup>13</sup>, we concluded that increases in the activity of glucose metabolism enzymes in normal and diabetic mice were not associated with a direct effect of DNJ, but may have resulted from increased hepatic protein expression.

In summary, the current study demonstrates that DNJ inhibited glucose absorption in the small intestine by attenuating the expression of proteins involved in the transepithelial glucose transport system, and maintained stable blood glucose levels by directly regulating the expression of enzyme proteins involved in hepatic glycolysis and gluconeogenesis. DNJ may, therefore, provide a future therapeutic option for diabetic disease.

## Methods

**DNJ preparation.** DNJ was extracted from Mulberry (*Morus Multicaulis* Perr.) leaves and purified by LC-MS systems according to the method of Li et al.<sup>13</sup>. The extract had a purity >95%.

**Experimental design.** Experiments were performed with male ICR mice (25  $\pm$  2 g) as previously described<sup>40</sup>. The animals received a standard diet with free access to tap water. The experiments were approved by the Regulations of Experimental Animal Administration issued by State Committee of Science and Technology of the People's Republic of China on November 14th, 1988. After 1 week of acclimatization, weight-matched mice were subjected to a 16-h fast. Diabetes was induced by single intraperitoneal injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA)

(65 mg/kg b.w.) dissolved in 0.1 M cold citrate buffer (pH 4.5). Ten days after STZ administration, mice with a 12 h fasting blood glucose concentration between 16 and 20 mmol/L were selected for the experiment. Normal and diabetic mice were divided into four groups. Group I comprised normal mice who received 0.9% saline orally. Group II comprised normal mice who received DNJ (50 mg/kg b.w.). Group III comprised STZ-induced diabetic mice who received 0.9% saline orally and Group IV comprised STZ-induced diabetic mice who received DNJ (50 mg/kg b.w. in 0.9% saline) administered intragastrically twice daily (at 08:00 and 20:0 h) for 3 days. Previous studies indicate that treatment of diabetic mice with DNJ for 3 days does not significantly decrease fasting blood glucose<sup>13</sup>, and fasting concentrations of blood glucose regulating hormones (insulin, glucagon or epinephrine) remain relatively stable. Therefore, there is no significant difference between the DNJ and control groups in term of the basal levels of blood sugar and hormones. Thus, oral glucose tolerance tests (OGTT) and intravenous glucose tolerance tests (IVGTT) were carried out after an overnight fast. Normal and STZ-induced diabetic mice were intragastrically administered equal volumes of 0.9% saline or DNJ (50 mg/kg b.w./d). Fifteen minutes later, a 30% glucose solution (3 g/kg) was orally administered for the OGTT and or injected at the base of the tail vein for the IVGTT. Glucose concentrations were measured in peripheral blood taken from the tail tip vein at various time points. Animals (n = 10) were killed at 0, 30, 60, 90 and 120 min after OGTT, and the serum and liver tissue were collected immediately for biochemical estimations. In animals sacrificed at 120 min, BBM (for SGLT1) and BLM (for Na<sup>+</sup>/K<sup>+</sup>-ATPase and GLUT2) were isolated from the jejunum as described by Boyer et al.<sup>2</sup>, and stored in liquid nitrogen for subsequent isolation of RNA and proteins.

DNJ (50 mg/kg b.w.) and labeled<sup>13</sup>C<sub>6</sub>-glucose (1 g/kg b.w.; Sigma, St. Louis, MO, USA) was administered intragastrically at 15 min intervals to fasting mice for the assessment of intestinal glucose absorption and transport. Blood and small intestine (duodenum, jejunum and ileum) samples were collected after 30 min and processed for GC/MS analysis as previously described<sup>11</sup>.

**Biochemical estimations.** Serum insulin, glucagon and epinephrine levels were determined by ELISA according to the manufacturer's instructions (R&D Systems, USA). GK<sup>27</sup>, PFK<sup>32</sup>, PK<sup>33</sup>, Na<sup>+</sup>/K<sup>+</sup>-ATPase<sup>44</sup> activities were assayed as described previously. Hepatic glucose and pyruvate content were measured using assay kits (Su Zhou Keming Bioengineer Company, China) according to the manufacturer's instructions. NADH and NAD<sup>+</sup> levels in liver were determined using HPLC analyses (column: Sun Fire<sup>TM</sup>-C18 (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Waters, USA), with the mobile phase containing 0.1 M NaH<sub>2</sub>PO<sub>4</sub> in water (A) and acetonitrile (B) at a ratio of 90 : 10. The flow rate was 0.8 mL/min and detection was undertaken at 2487 and 254 nm.

**Quantitative real-time reverse transcription-PCR analysis.** Total RNA was extracted using TRIZOL reagent according to the supplier's instruction. Reverse-transcription was performed using a Revert Aid<sup>TM</sup> First-Strand cDNA Synthesis Kit for RT-PCR, and performed as previously described<sup>13</sup>. The primer sequences are shown in Table 1.

**Table 1 | Primers used in quantitative real-time reverse transcription-PCR**

Primer	Sequence 5'-3'	PCR product size (bp)
GK-F	AGGGAACAACATCGTGGGAC	132
GK-R	TCACATTGGCGGTCTTCATA	
PFK-F	CGTCTTTGAGGACCCCTTCA	132
PFK-R	TCTGTGGTGTAGTGTTCGTGACA	
PK-F	AAGAAGGGAGCCACTCTGAA	84
PK-R	CTTGTAGTCCAGCCACAGGAT	
PDK2-F	TAGAGGGCTACGGGACAGAT	92
PDK 2-R	CCAGGCGGCTTATTGTA	
PDE1-F	AGCCAGCACGGACTACTACAA	107
PDE1-R	AATAGGCAGCCGAAACTTT	
PEPCK-F	CCCTTGCTATGAAGCCCTCA	150
PEPCK-R	GCCGAAGTTGTAGCCGAAGA	
G-6-Pase-F	CCAGAATGGGTCCACCTTGA	148
G-6-Pase-R	GAAGCGGAATGGGAGCAACT	
Na <sup>+</sup> /K <sup>+</sup> -ATPase-F	CCTGGGAGGCTTCTTCACTT	146
Na <sup>+</sup> /K <sup>+</sup> -ATPase-R	CTGCTCGTAGGTCACCTGCT	
SGLT1-F	TTGCCTATGGAAGTGGGAGC	107
SGLT1-R	TGATGACGCTGATGACGAAGA	
GLUT2-F	GCTGTCTCTGTGCTGCTTGT	136
GLUT2-R	CGTAACTCATCCAGGCGAAT	
$\beta$ -actin-F	CAGCCITCCTTCTTGGGTAT	105
$\beta$ -actin-R	CTGTGTTGGCATAGAGGCTCT	

KeyF: Forward primer, R: Reverse primer, bp: base pairs (length of nucleic acid sequence).



**Western blot analysis.** Samples were suspended in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, phenylmethylsulfonyl fluoride 100 µg/mL, aprotinin 2 µg/mL, pepstatin 1 µg/mL, and leupeptin 10 µg/mL, and placed on ice for 30 min. After centrifugation (15,000 g) for 15 min at 4°C, the suspension was solubilized with SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol and 50 mM Tris; pH 6.8) and total protein was measured using a bicinchoninic acid assay (Su Zhou Keming Bioengineer Company, China) according to the manufacturer's recommendations. Samples containing 50 µg of protein were separated by SDS-PAGE using 10% gels. The proteins were transferred to nitrocellulose membranes using 400 mA current (3 h at 4°C). The membranes were blocked with 5% skimmed milk for 1 h, followed by 2.5% gelatin for 1 h. The primary antibody anti-(GK-ab37796, PFK-ab119796, PK-ab38240, PDK2-ab92959, PEPCK-ab70358, Na<sup>+</sup>/K<sup>+</sup>-ATPase-ab110730, SGLT1-ab652 and GLUT2-ab54460) was obtained from Abcam (UK). The blots were developed using an enhanced chemiluminescent (ECL) kit (Amersham, UK).

**Cell culture.** The mice hepatic cells (NCTC1469) obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences were cultured according to the manufacturer's instructions. The cells were plated at a density of  $5 \times 10^5$  cells/well on 6-well plates and subjected to DMSO (A) or 40 µg/mL DNJ (B). Following incubation for 2 h, viable cells were collected for GK, PFK, PK, PDK2 and PEKCK expression analysis by Western blot.

**Effect on the activities of GK, PFK and PK by DNJ in vitro.** Standard GK, PFK and PK enzymes were purchased from Sigma-aldrich (St. Louis, MO, USA). Enzyme activities were measured according to previous methods<sup>27,42,43</sup>. DNJ at final concentrations of 0, 40, 80, 160, 320, 640, 1280 µg/mL was incubated with the GK (PFK, PK) enzymes for 15 min before each assay.

**Statistical analysis.** Statistics analysis was undertaken using SPSS for Windows version 12.0 (Chicago, USA). Results were presented means and standard deviations ( $\pm$ S.D.). Analysis of variance (ANOVA) was used to evaluate differences between multiple groups. In cases where significant differences were observed, Duncan's multiple range test (DMRT) was used for pairwise comparisons. Values of  $P < 0.05$  were considered to be statistically significant.

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## Author contributions

The experiments were designed by Y.G. Li and D.F. Ji and were performed by Y.G. Li, S. Zhong, T.B. Lin, Z.Q. Lv, and G.Y. Hu. The data were analyzed by Y.G. Li, D.F. Ji and S. Zhong. Y.G. Li wrote the paper. Dr. X. Wang contributed to the discussion section and edited the manuscript. All authors discussed the results and commented on the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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