CLINICS

BASIC RESEARCH

# Mibefradil reduces blood glucose concentration in *db/db* mice

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**OBJECTIVE:** Numerous recent studies suggest that abnormal intracellular calcium concentration ($[Ca^{2+}]_i$) is a common defect in diabetic animal models and patients. Abnormal calcium handling is an important mechanism in the defective pancreatic β-cell function in type 2 diabetes. T-type $Ca^{2+}$ channel antagonists lower blood glucose in type 2 diabetes, but the mechanism remains unknown.

**METHODS:** We examined the effect of the $Ca^{2+}$ channel antagonist mibefradil on blood glucose in male *db/db* mice and phenotypically normal heterozygous mice by intraperitoneal injection.

**RESULTS:** Mibefradil (15 mg/kg, i.p., b.i.d.) caused a profound reduction of fasting blood glucose from 430.92±20.46 mg/dl to 285.20±5.74 mg/dl in three days. The hypoglycemic effect of mibefradil was reproduced by NNC 55-0396, a compound structurally similar to mibefradil but more selective for T-type $Ca^{2+}$ channels, but not by the specific L-type $Ca^{2+}$ channel blocker nicardipine. Mibefradil did not show such hypoglycemic effects in heterozygous animals. In addition, triglycerides, basal insulin and food intake were significantly decreased by mibefradil treatment in the *db/db* mice but not in the controls. Western blot analysis, immunohistochemistry and immunofluorescence staining showed a significantly increased expression of T-type $Ca^{2+}$ channel α-subunits Cav3.1 and Cav3.2 in liver and brain tissues from *db/db* mice compared to those from heterozygous animals.

**CONCLUSIONS:** Collectively, these results suggest that T-type $Ca^{2+}$ channels are potential therapeutic targets for antidiabetic drugs.

**KEYWORDS:** Diabetes Mellitus; Hypoglycemic Effect; Insulin; Food Intake; T-type $Ca^{2+}$ Channel Antagonist.

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## INTRODUCTION

The hallmark of type 2 diabetes mellitus is chronic hyperglycemia under both fasting and postprandial conditions. Numerous recent studies in diabetic animal models and patients suggest that abnormal intracellular calcium concentration ($[Ca^{2+}]_i$) is a common defect in both insulin-dependent (type 1) and insulin-independent (type 2) diabetes (1). Abnormal calcium handling is an important mechanism in the defective pancreatic β-cell function in type 2 diabetes (2). Dysregulation of $[Ca^{2+}]_i$ may represent a common factor underlying metabolic, cardiovascular, ocular and neural complications of diabetes mellitus (3). Clinical administration of an L-type $Ca^{2+}$ antagonist has produced no detrimental or beneficial effects on glucose tolerance (4,5). Cerebrocrast, an L- and T-type calcium channel inhibitor, decreases blood glucose and food intake and increases glucose uptake by the brain (6,7). However, the specific effects of T-type $Ca^{2+}$ channel antagonists on blood glucose regulation remain unknown.

T-type $Ca^{2+}$ channels are different from other types of $Ca^{2+}$ channels with regard to their kinetics, pharmacological properties and activation/inactivation voltage range (8,9). T-type $Ca^{2+}$ currents have been described in human pancreatic islet cells (10,11). Studies on human islets have shown that a desensitization of glucose-induced insulin secretion is associated with $[Ca^{2+}]_i$ elevation (12). T-type $Ca^{2+}$ current density and the channels' mRNA levels increase markedly in rat pancreatic islets treated with high glucose (13).

Mibefradil blocks both L-type and T-type $Ca^{2+}$ channels (14). Clinically, mibefradil has significant therapeutic advantages in reducing blood pressure (15,16), preventing blood pressure-related arterial hypertrophy (17), lowering heart rate (18) and preventing and reducing hyperinsulinemia (19).



No potential conflict of interest was reported.

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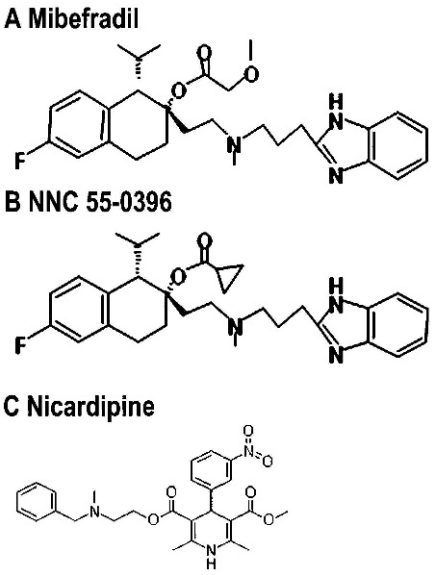



**Figure 1 -** Chemical structure of calcium channel blockers.

An analog of mibefradil, (1S,2S)-2-(2-(N-[(3-benzoimidazol-2-yl)propyl]-N-methylamino) ethyl)-6-fluoro 1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride [NNC 55-0396], is more selective for T-type $Ca^{2+}$ channels (20). The molecular structure of NNC55-0396 is more similar to that of mibefradil than to that of the L-type $Ca^{2+}$ channel blocker nicardipine (Figure 1). The clinical effect of NNC 55-0396 is unclear.

## MATERIALS AND METHODS

### Experimental animals

Experimental (male BKS Cg-Dock 7 $m^{+/+}$Lepr/J, *db/db*, 36–40 g) and control (male Dock7$m^{+/+}$$Lepr^{db}$, *db/+*, hereafter ''wild-type'', 23–26 g) eight-week-old mice purchased from Jackson Laboratory (Bar Harbor, ME) were randomly divided into two main groups. The mice were further randomly separated into two sub-groups, for a total of four groups (n = 6 mice per group): the control placebo group, control drug intervention group, *db/db* placebo group and *db/db* drug intervention group. Mice from both drug intervention groups were injected with mibefradil (i.p., 15 mg/kg per injection, b.i.d, for all experiments; Sigma-Aldrich, St. Louis, MS) in 100 μl normal saline with 0.05% DMSO. Normal saline with 0.05% DMSO was given to both placebo groups in an identical manner. During the drug intervention, fasting blood glucose (food deprivation for eight hours), blood pressure, body weight, food intake and insulin tolerance (by insulin tolerance test (ITT)) were continually measured. At the end of treatments, blood, liver and brain samples were collected.

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the Third Military Medical University. The organs of the animals were removed for histochemical analysis after euthanasia.

### Immunoassay for insulin, glycosylated hemoglobin A1c and total cholesterol measurements

Mouse cardiac blood (0.8 ml) was stored at 4℃ for three to four hours. After coagulation and clot retraction, samples were centrifuged at X1,000g for ten minutes, and serum was stored at −20℃ for later use. Serum basal insulin was measured using an insulin ELISA kit from Merodia (Uppsala, Sweden); HbA1c and total cholesterol were measured by using ELISA kits from R&D Systems (Minneapolis, MN).

### Western blot analysis

The tissue samples (50 mg) were homogenized in lysis buffer (Thermo Fisher Scientific Inc, Waltham, MA) containing a protease inhibitor cocktail (Roche, Basel, Sweden) and then centrifuged at X9,000g rpm for 15 minutes at 4℃. The supernatants were denatured and loaded for electrophoresis at a volume containing 90 μg total protein. After separation, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Blotted membranes were incubated overnight with primary antibodies against ß-tubulin (Santa Cruz Biotechnology Inc, Santa Cruz, CA, 1:200), α1G (Bioss, China, 1:200) and α1H (Santa Cruz Biotechnology Inc, 1:200). The membranes were then incubated with appropriate secondary antibodies at room temperature for 1.5 hours before detection with a chemiluminescence kit (Beyotime, BeyoECL Plus, China).

### Immunohistochemical analysis

The expression levels of Cav3.1 and Cav3.2 were evaluated using standard immunohistochemistry methods.

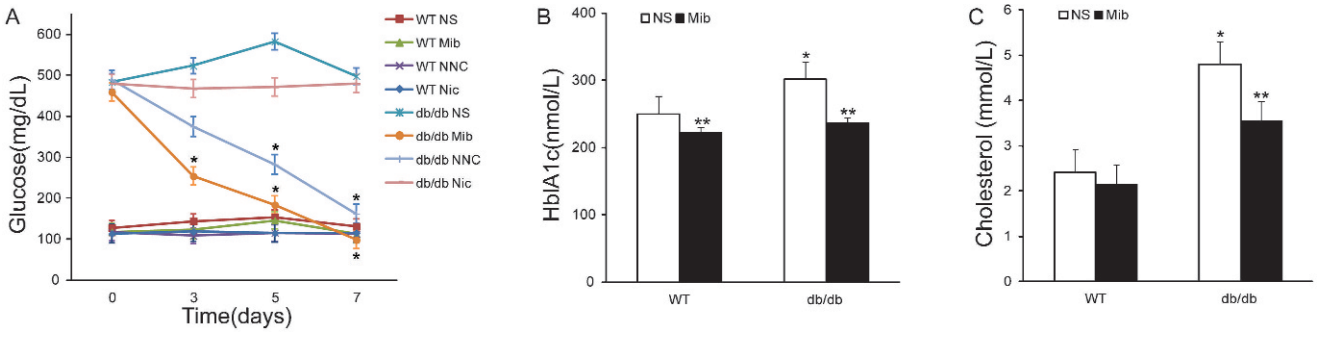



**Figure 2 -** The hypoglycemic and hypolipidemic effects of $Ca^{2+}$ channel antagonists in *db/db* mice. **A)** Time-dependent changes in blood glucose concentration during drug treatment. **B)** Serum HbA1c in mice with and without mibefradil injection. **C)** Serum total cholesterol in mice with and without mibefradil injection. Abbreviations: WT, nondiabetic wild-type mice; *db/db*, *db/db* mice; NS, normal saline; Mib, mibefradil; NNC, NNC 55-0396; Nic, nicardipine. * $p<0.05$, n = 6, compared with WT NS group; ** $p<0.05$, n = 6, compared with NS group of the same type of mice at the same time. Statistical analyses were performed with Student's t-test (A) or ANOVA (B and C) according to the specific application.

Fresh tissue samples were washed with PBS, followed by paraformaldehyde fixation (4%) and paraffin embedding. The samples were sliced into 4 μm serial sections for xylene dewaxing and alcohol dehydration. After blocking nonspecific antigens with goat serum, the samples were incubated overnight at 4℃ with diluted primary antibodies (50 μl). Then, biotin-labeled secondary antibodies were added for incubation at room temperature for one hour in the dark. The samples were then stained with DAPI for five minutes in the dark, dried and sealed with anti-fluorescence quenching mounting medium and kept at 4℃ in the dark. Samples without primary antibodies were employed as negative controls.

### Statistical analysis

All data are expressed as the mean ± SEM. Statistical analyses were performed with Student's t-test or ANOVA according to the specific application. *P*-values ≤0.05 were considered significant. Data were organized with Adobe Illustrator CS3 and analyzed with SPSS 17.0 (Statistical Product and Service Solutions, IBM, Chicago, USA). Statistical significance for Western blot experiments was determined by analyzing gray-level values using Quantity One software.

## RESULTS

The effects of $Ca^{2+}$ channel antagonists on blood glucose were examined in *db/db* mice. As shown in Figure 2A, mibefradil (15 mg/kg, b.i.d) intraperitoneal injection significantly decreased blood glucose concentration from 430.92±20.46 mg/dl to 285.20±5.74 mg/dl (n = 6, $p<0.05$) by day 3. Fasting blood glucose decreased to a level similar to that of wild-type controls at day 5. The injection of normal saline (with 0.05% DMSO vehicle) had no effect on blood glucose in *db/db* mice. The application of NNC 55-0396, an antagonist that is more specific to the T-type $Ca^{2+}$ channel (21) at the same dose (15 mg/kg, b.i.d), also effectively reduced blood glucose. In contrast, the L-type $Ca^{2+}$ channel blocker nicardipine (12.5 mg/kg, b.i.d) had no significant effect on blood glucose when administered at a concentration commonly used for *in vivo* study (22). These results indicate that $Ca^{2+}$ channel antagonists have a profound blood glucose-lowering effect in *db/db* mice, and this effect is likely attributed to the inhibition of T-type $Ca^{2+}$ channels rather than any effects on L-type $Ca^{2+}$ channels. The application of mibefradil, NNC 55-0396 or nicardipine for one week had no significant effect on fasting blood glucose in wild-type mice (Figure 2A), indicating that the effects of T-type $Ca^{2+}$ channel blockers are specific to the *db/db* mice.

The effects of T-type $Ca^{2+}$ channel blockers on blood glucose were also evaluated by measuring hemoglobin glycosylation in *db/db* and wild-type mice. Similar to the above results, mibefradil effectively reduced HbA1c in *db/db* mice from 301.5±9.50 to 236.9±9.83 ($p<0.05$, n = 6; Figure 2B). Interestingly, mibefradil slightly reduced HbA1c

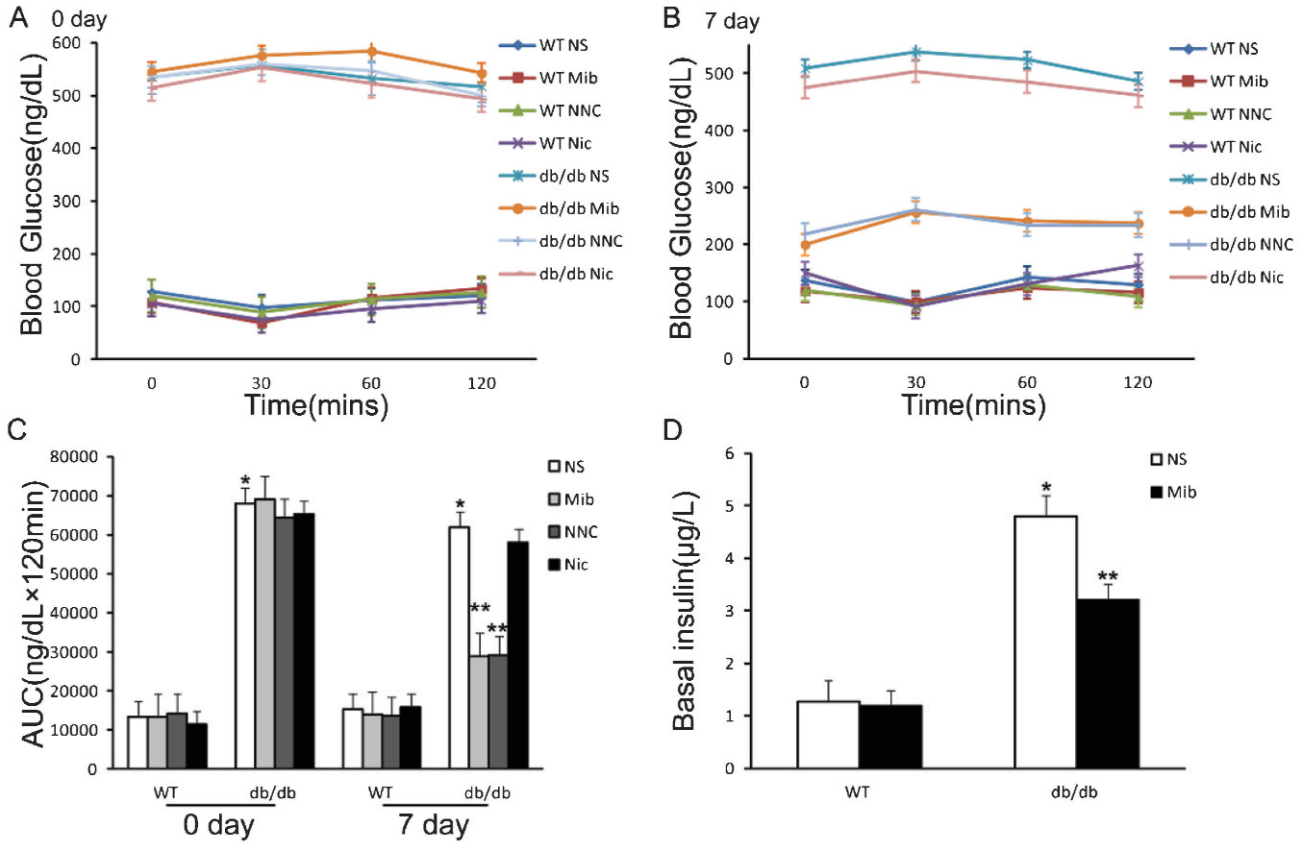



**Figure 3 -** Effects of mibefradil on insulin sensitivity and basal insulin release in diabetic and nondiabetic mice. **A and B)** Glucose disposal measured by insulin tolerance test (ITT) before (A) and after (B) mibefradil treatment for seven days. **C)** Difference in the area under the curve before and after drug injection. **D)** Effect of mibefradil on basal insulin in diabetic and nondiabetic mice. * $p<0.05$, n = 6, compared with WT NS group; ** $p<0.05$, n = 6, compared with NS group of the same type of mice at the same time. Statistical analyses were performed with Student's t-test (D) or ANOVA (C), according to the specific application.

in wild-type animals (from $250.0 \pm 8.28$ to $221.7 \pm 4.50$ nmol/L, $p<0.05$, n = 6), which indicates a physiological role for T-type $Ca^{2+}$ channels in glucose regulation in normal mice. The effect of mibefradil on blood triglycerides was evaluated in *db/db* mice. Figure 2C shows that after one week of mibefradil treatment, plasma cholesterol was significantly reduced ($p<0.05$, n = 6) in a manner similar to the reduction of blood glucose. In contrast, there was no significant change in cholesterol level between the wild-type mice with and without the treatment with mibefradil. Cholesterol was significantly different between *db/db* and wild-type control groups ($p<0.05$, n = 6).

To delineate the mechanism underlying the effects of T-type $Ca^{2+}$ channel antagonists on blood glucose in *db/db* mice, an ITT was performed after six hours of fasting. Mice were injected intraperitoneally with porcine insulin (Xinbai Pharmaceutical, Nanking, China) at 0.75 unit/kg of body weight. Blood glucose was measured from tail bleeds taken at the indicated times. Figures 3A and 3B show the results of ITTs before and after three days of mibefradil injection in wild-type and *db/db* mice. The ITT results show no significant difference in the glucose tolerance in response to insulin stimulation between the mibefradil-treated and saline-treated groups (Figures 3A, 3B, 3C). This similar response may be attributed to the existing high level of insulin in the *db/db* mice, which may have attenuated the effects of further insulin application. In contrast, basal insulin (after food deprivation for eight hours) in *db/db* mice was significantly reduced by seven days of treatment with mibefradil, from $4.80 \pm 0.35$ to $3.21 \pm 0.12$ ng/ml ($p<0.05$, n = 6) (Figure 3D). This finding indicates that T-type $Ca^{2+}$ channel antagonists suppress insulin release from pancreatic β-cells of *db/db* mice. Therefore, T-type $Ca^{2+}$ channels may play a role in the pathogenesis of hyperinsulinemia in this type 2 diabetic model.

The maintenance of the basal glucose level is regulated by the release of glucose from the liver and the uptake of glucose into muscle, adipose and brain tissue. The profound blood glucose-lowering effect of T-type $Ca^{2+}$ channel

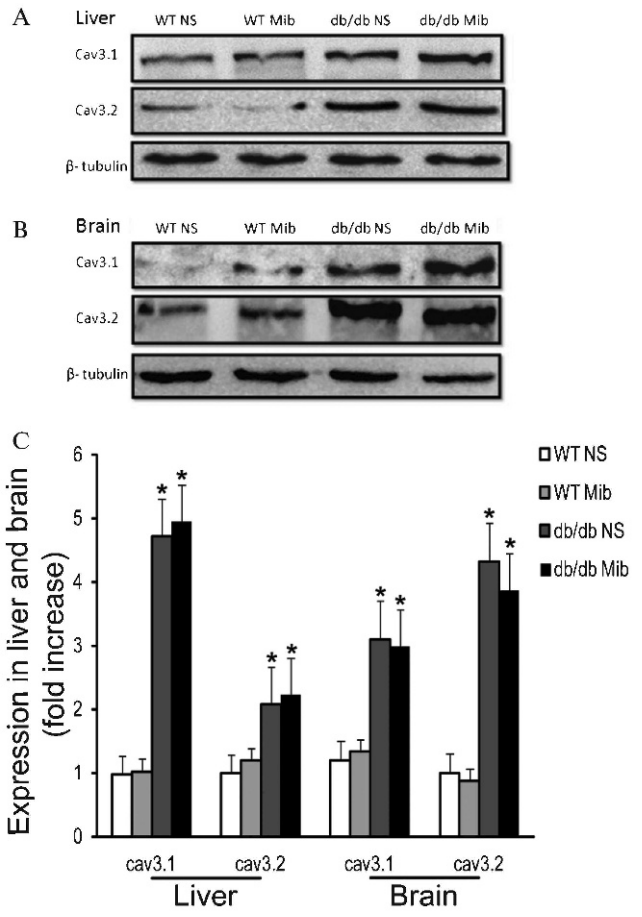



**Figure 4 -** Western blot analysis of T-type $Ca^{2+}$ channel expression in liver and brain tissues of diabetic and nondiabetic mice. Increased expression of α1G (Cav3.1) and α1H (Cav3.2) subunits of T-type $Ca^{2+}$ channels (n = 3). The molecular weights of Cav3.1 and Cav3.2 are ~262 kDa. * $p<0.05$, n = 6, compared with WT NS group. Statistical analyses were performed with ANOVA.

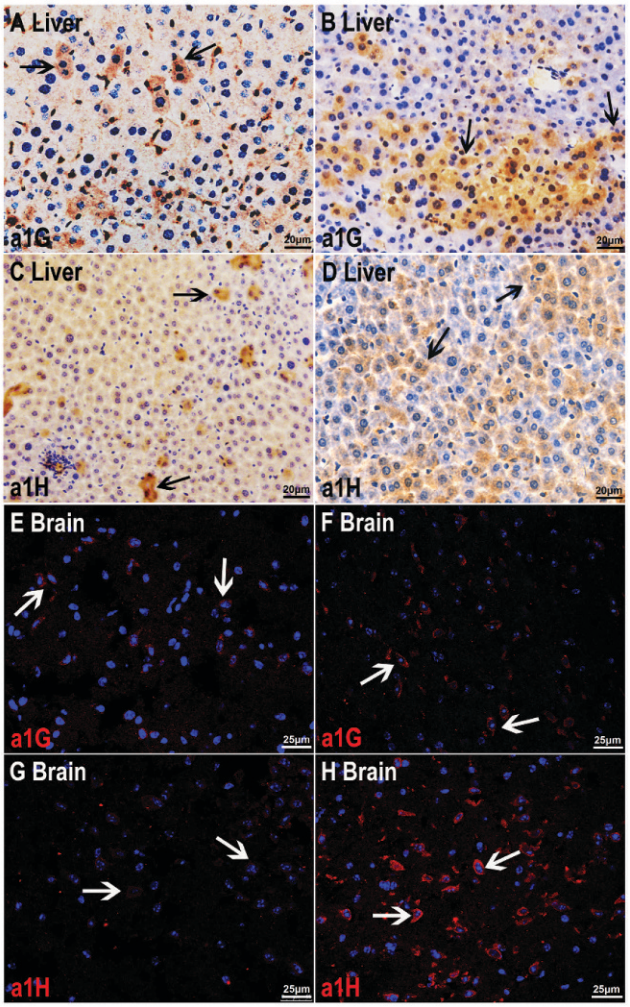



**Figure 5 -** Immunohistochemical and immunofluorescence staining showing an increased expression of α1G and α1H subunits of T-type $Ca^{2+}$ channels in liver and brain slide preparations. **A and C)** Immunohistochemical staining for T-type $Ca^{2+}$ channel α1G (A) and α1H (C) subunits in liver slide preparations from non-diabetic mice (scale bar = 20 μm). **B and D)** Immunohistochemical staining of *db/db* animal liver preparations (scale bar = 20 μm). **E and G)** Immunofluorescence staining showing the expression of α1G (E) and α1H (G) subunits of T-type $Ca^{2+}$ channels in brain slide preparations from nondiabetic mice (scale bar = 25 μm). **F and H)** Immunofluorescence staining of brain slide preparations from *db/db* mice (scale bar = 25 μm).

antagonists under high insulin concentrations suggests that the target organs of mibefradil may include the liver and the brain, which is the upper-level control center for hepatic glucose production. We examined the protein levels of Cav3.1 (α1G) and Cav3.2 (α1H) α1 subunits of T-type $Ca^{2+}$ channels in the liver and brain of *db/db* mice and control wild-type mice. As shown in Figure 4, both Cav3.1 and Cav3.2 in the liver and brain were significantly higher in *db/db* mice. These data may explain the difference in the blood glucose-lowering effects of mibefradil and NNC 55-0396 observed in *db/db* and wild-type animals. These findings also suggest that the targets of mibefradil and NNC 55-0396 are in the liver and the brain in addition to pancreatic β-cells. Immunohistochemistry confirmed the results of Western blot analysis. Figure 5 shows that the expression of α1G and α1H was higher in *db/db* liver (Figure 5B and 5D) and brain (Figure 5F and 5H) preparations compared to those in the wild-type animals.

Because *db/db* mice are characterized by continual feeding as a result of a mutation in the leptin receptor, we

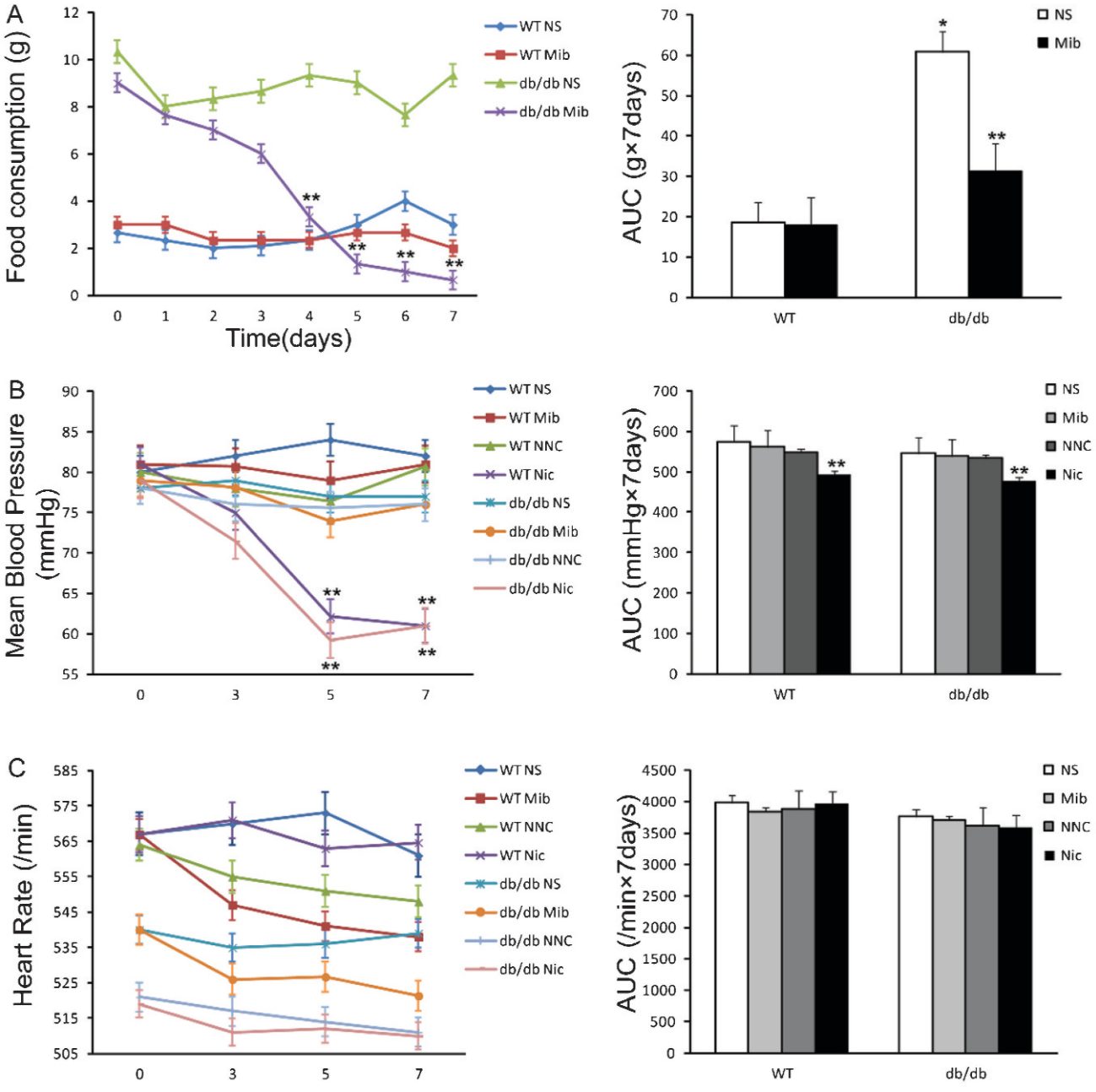
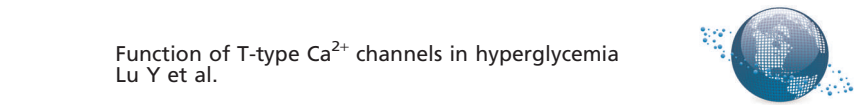



**Figure 6 -** Effects of mibefradil and/or nicardipine on the food consumption, mean blood pressure and heart rate of *db/db* and nondiabetic mice. **A)** Effect of mibefradil injection on the food intake of diabetic and nondiabetic mice. **B)** Effects of mibefradil and nicardipine on the mean blood pressure of diabetic and nondiabetic mice. **C)** Effects of mibefradil and nicardipine on the heart rate of diabetic and nondiabetic mice. * $p<0.05$, n = 6, compared with WT NS group; ** $p<0.05$, n = 6, compared with NS group of the same type of mice at the same time. Statistical analyses were performed with Student's t-test (A) or ANOVA (B and C) according to the specific application.





also set up experiments to evaluate the effect of mibefradil and NNC 55-0396 on the feeding behavior of these animals. Mibefradil significantly reduced the food consumption of *db/db* mice but had no significant effect on the control animals (Figure 6A). This result also suggests mibefradil has a central nervous system (CNS) target that may affect the feeding behavior of the diabetic animals (15,16).

Mibefradil also has antihypertensive effects; thus, we examined the effects of mibefradil and NNC 55-0396 on the cardiovascular functions of *db/db* mice. The mean blood pressure (MBP) and heart rate after drug injection in the *db/db* and wild-type mice showed no significant difference in MBP (Figure 6B) or heart rate (Figure 6C) among mibefradil-, NNC 55-0396- and vehicle-treated groups. The physiological functions of T-type $Ca^{2+}$ channels in cardiovascular regulation in *db/db* mice remain unclear.

## DISCUSSION

The *db/db* mouse is a well-established diabetic rodent model for studying the mechanisms of hyperglycemia, hyperinsulinemia, hyperlipidemia and related metabolic abnormalities. The present study is the first attempt to utilize this model for characterizing the role of T-type $Ca^{2+}$ channels in hyperglycemia *in vivo*. The hallmark of diabetes mellitus is chronically high fasting blood glucose, resulting from impaired insulin sensitivity in the peripheral tissues and defective insulin production by pancreatic β-cells. Using the *db/db* model, we showed that T-type $Ca^{2+}$ channels might be novel therapeutic targets for hyperglycemia and that antagonists of T-type $Ca^{2+}$ channels might lower basal glucose by reducing liver glucose output and decreasing basal insulin release or synthesis by pancreatic β-cells (Figure 7).

Basal insulin release from pancreatic β-cells may be controlled by a different mechanism from glucose-stimulated insulin release. Hyperinsulinemia is commonly observed in pre-diabetic and type 2 diabetic patients. Chronic exposure to elevated insulin may result in the development of insulin resistance (23) and, in our opinion, may also lead to the attenuation of first-phase insulin release observed in type 2 diabetic patients. Therefore, the fact that T-type $Ca^{2+}$ channel antagonists can reduce the basal insulin is certainly an encouraging result because it may not have a significant impact on glucose-stimulated insulin release, which is mostly mediated by the activation of L-type $Ca^{2+}$ channels.

We have shown that T-type $Ca^{2+}$ channel antagonists significantly reduced basal insulin release in wild-type animals compared to untreated *db/db* diabetic animals, indicating that the basal release of insulin from pancreatic β-cells might involve a T-type $Ca^{2+}$ channel-mediated mechanism. However, residual basal insulin is still elevated after T-type $Ca^{2+}$ channel antagonist treatment, suggesting that T-type $Ca^{2+}$ channel antagonists may also suppress glucose output from the liver during fasting.

As shown in Figure 3, the results of ITT indicated that treatment with T-type $Ca^{2+}$ channel antagonists failed to improve glucose disposal in *db/db* mice compared to untreated animals. We are currently unable to draw a definitive conclusion about the effects of these drugs on improving insulin sensitivity in muscle, adipose or brain tissue. It is possible that the *db/db* mouse is not a suitable model for studying the role of T-type $Ca^{2+}$ channels in insulin sensitivity because the hyperglycemia of the *db/db* mice cannot be reversed by an injection of insulin (The Jackson Laboratory, http://jaxmice.jax.org/list/ra66.html).

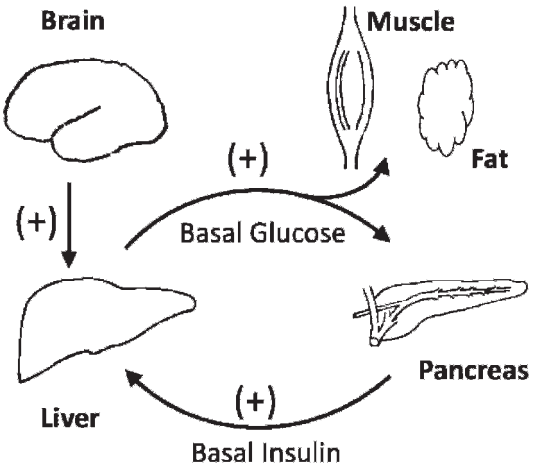



**Figure 7 -** A diagram showing the possible targeting sites of mibefradil on the system regulating basal glucose. (+), secretion of hormones or nerve signals increased by the activity of T-type $Ca^{2+}$ channels.

The overexpression of T-type $Ca^{2+}$ channel α1G and α1H subunits in *db/db* mice compared with the wild-type animals suggests that either a genetic predisposition favors the T-type $Ca^{2+}$ channels or a secondary pathological regulation of these proteins occurs in these mice. Nevertheless, the fact that T-type $Ca^{2+}$ channel antagonists have an effect on glucose regulation in wild-type animals (Figure 2B) suggests that T-type $Ca^{2+}$ channels may also have physiological functions in non-diabetic animals at low expression levels.

Further investigation is required to confirm the effects of T-type $Ca^{2+}$ channel antagonists on metabolic regulation in the CNS. The results of the present study show an increased expression of T-type $Ca^{2+}$ channel α1G and α1H subunits in the brain of *db/db* mice, suggesting that these channels may play critical roles in the mechanisms of hyperglycemia and hyperlipidemia in these animals. We also show that T-type $Ca^{2+}$ channel antagonists caused a decrease in appetite in the later phases of drug treatment, as well as a decrease in body weight (data not shown), in *db/db* mice, indicating that T-type $Ca^{2+}$ channels indeed play an important role in metabolic regulation in *db/db* mice; nevertheless, further investigation of this subject is necessary.

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## AUTHOR CONTRIBUTIONS

Lu Y, Li M and Xu Z participated in the research design. Lu Y, Long M and Xu Z conducted the experiments. Lu Y, Long M, Li M, Zhou S, Hu F

and Xu Z contributed to the new reagents or analytic tools. Lu Y, Zhou S, Hu F, Li M and Xu Z performed the data analysis. Li M, Lu Y and Xu Z wrote the manuscript.

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