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Acute exposure of beta-cells to troglitazone decreases insulin hypersecretion via activating AMPK

Ruyuan Deng [a,1], Aifang Nie [a,1], Fangfang Jian [a], Yun Liu [a], Hongju Tang [a], Juan Zhang [a], Yuqing Zhang [a], Li Shao [b], Fengying Li [a], Libin Zhou [a,*], Xiao Wang [a,*], Guang Ning [a]

[a] Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
[b] Department of Geratology, East Hospital, Shanghai Tongji University, Shanghai 200120, China

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ABSTRACT

Background: It has been recognized that insulin hypersecretion can lead to the development of insulin resistance and type 2 diabetes mellitus. There is substantial evidence demonstrating that thiazolidinediones are able to delay and prevent the progression of pancreatic β-cell dysfunction. However, the mechanism underlying the protective effect of thiazolidinediones on β-cell function remains elusive.
Methods: We synchronously detected the effects of troglitazone on insulin secretion and AMP-activated protein kinase (AMPK) activity under various conditions in isolated rat islets and MIN6 cells.
Results: Long-term exposure to high glucose stimulated insulin hypersecretion and inhibited AMPK activity in rat islets. Troglitazone-suppressed insulin hypersecretion was closely related to the activation of AMPK. This action was most prominent at the moderate concentration of glucose. Glucose-stimulated insulin secretion was decreased by long-term troglitazone treatment, but significantly increased after the drug withdrawal. Compound C, an AMPK inhibitor, reversed troglitazone-suppressed insulin secretion in MIN6 cells and rat islets. Knockdown of AMPKα2 showed a similar result. In MIN6 cells, troglitazone blocked high glucose-closed ATP-sensitive K^+ (K_{ATP}) channel and decreased membrane potential, along with increased voltage-dependent potassium channel currents. Troglitazone suppressed intracellular Ca^{2+} response to high glucose, which was abolished by treatment with compound C.
Conclusion: Our results suggest that troglitazone provides β-cell "a rest" through activating AMPK and inhibiting insulin hypersecretion, and thus restores its response to glucose.
General significance: These data support that AMPK activation may be an important mechanism for thiazolidinediones preserving β-cell function.

1. Introduction

Type 2 diabetes is characterized by insulin resistance and impaired insulin secretion. For many years, insulin resistance has been seen as the core defect responsible for the development of type 2 diabetes mellitus. Insulin hypersecretion is thought to result from insulin resistance [1]. However, in the past decade, it has been widely recognized that β-cell dysfunction is the key problem in type 2 diabetes [2]. There is a wealth of evidence to suggest that insulin hypersecretion can lead to the development of decreased insulin sensitivity and is regarded as the central pathogenetic factor in type 2 diabetes [1,3]. Thus, preserving pancreatic β-cell function is thought to be critical to preventing the progression of type 2 diabetes mellitus.

Many clinical studies have demonstrated that thiazolidinediones (TZDs) can delay or prevent type 2 diabetes in subjects with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) [4–7]. Although TZDs are considered primarily to target insulin resistance in peripheral tissues, evidence from several sources suggests that they also have profound effects on the β-cell [8,9]. In A Diabetes Outcome Progression Trial (ADOPT) study, TZDs are able to sustain glucose control in early type 2 diabetes better than metformin or sulfonylureas, suggesting that TZD must exert a long-term effect to preserve β-cell function [10]. In Troglitazone in Prevention of Diabetes (TRIPOD) study, the incidence of type 2 diabetes reduced by 56% in women with previous gestational diabetes mellitus who received troglitazone. The protective effect of the TZD was most prominent in women with a

Abbreviations: AMPK, AMP-activated protein kinase; TZD, thiazolidinediones; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; GSIS, glucose-stimulated insulin secretion; ACC, acetyl-CoA carboxylase; PPARγ, peroxisome proliferator-activated receptor γ; shRNA, short hairpin RNA; K_{ATP}, ATP-sensitive K^+ channel; Kv, voltage-dependent potassium channel

* Corresponding authors at: Shanghai Institute of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Road II, Shanghai 200025, China. Tel.: +86 21 64315587; fax: +86 21 64673639.
E-mail addresses: libin_zhou@yahoo.com (L. Zhou), wangxiao1976@hotmail.com (X. Wang).
[1] Contributed equally to this work.

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large reduction in insulin output from β-cells [5]. It is assumed that the insulin-sparing effect of TZDs is due to the reduction of peripheral insulin resistance. But many in vitro studies showed that TZDs exerted a direct suppressive effect on insulin secretion from β-cells [11–14]. As peroxisome proliferator-activated receptor-γ (PPARγ) is expressed in pancreatic β-cells, it is postulated that the direct effect of TZDs on beta-cell function depends on this nuclear receptor. However, Welters et al. found that this was not the case [15]. Increasing evidence revealed that TZDs activated AMP-activated protein kinase (AMPK) in various cell types, including β-cell. In β-cells, AMPK activity is rapidly decreased by elevations in glucose concentration over the physiological range [16,17]. Clamping AMPK activity at the elevated levels at low glucose concentrations by the expression of constitutively active AMPK subunits, the use of 5-aminoimidazole-4-car-boxamide riboside (AICAR), or metformin suppresses glucose-stimulated insulin secretion (GSIS) from islets as well as from β-cell lines [18–20]. However, whether TZDs regulate insulin secretion via activating AMPK has not been validated.

In the present study, we synchronously detected the effects of troglitazone on insulin secretion and AMPK activity under various conditions in isolated rat islets and MIN6 cells. The results showed that troglitazone-inhibited insulin hypersecretion was closely related to the activation of AMPK. Troglitazone increased ATP-sensitive K^+ (K_{ATP}) channel current and decreased intracellular Ca^{2+} concentration via activating AMPK, which contributes to the reduction of insulin secretion.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other culture reagents were obtained from Gibco Life Technologies (Grand Island, NY). The cell culture plates were purchased from Nalge Nunc International (Roskilde, Denmark). Troglitazone and 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-ylpyrrazolo[1,5-a] -pyrimidine (compound C) were purchased from Calbiochem (San Diego, CA). Collagenase type XI and tolbutamide were purchased from Sigma (St Louis, MO, USA). Materials for rat insulin ELISA were obtained from Mercodia (St Charles, MO, USA). Anti-AMPK, Anti-AMPKα2, anti-phospho-AMPK (Thr172), anti-acetyl-CoA carboxylase (ACC), anti-phospho-ACC (Ser79), and Anti-rabbit IgG conjugated with horseradish peroxidase were from Cell Signaling Technology (Beverly, MA, USA). Fura-2/AM was from Molecular Probes (Invitrogen, Oregon, USA).

2.2. Cell culture and treatment

MIN6 cells (passage 22–30) were cultured in DMEM with 25 mmol/l glucose, 10% FBS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and β-mercaptoethanol (5 μl/l) at 37 °C and 5% CO_2. Cells were seeded at 2×10^5 per well in 1 ml DMEM in a 24-well dish for secretory experiment and 1×10^6 per well in a 6-well dish for Western blotting. At 24 h before the acute experiment (24 h after seeding), the medium was replaced with DMEM containing 5.6 mmol/l glucose.

2.3. Islet isolation and treatment

Islets of Langerhans were isolated from male Sprague–Dawley rats by collagenase digestion and separated by density gradient centrifugation. Freshly isolated rat islets were transferred to 24-well plates (10 islets per well) or 6-well (150 islets per well) plates and cultured overnight in DMEM containing 3.3 or 11.1 mmol/l glucose, 10 mmol/l HEPES, 0.5% bovine serum albumin (BSA), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate at 37 °C and 5% CO_2. The treatment was the same as MIN6 cells.

2.4. Insulin secretion

Cultured cells or islets were washed once in KRB buffer (128.8 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, and 10 mmol/l HEPES, pH 7.4 with 0.1% BSA) containing 2.8 mmol/l glucose, and then they were preincubated for 30 min in 1 ml of the same medium at 37 °C. This buffer was then replaced with 1 ml of prewarmed KRB containing other additions as indicated for a further 60 min at 37 °C. An aliquot was then removed for analysis of insulin secretion. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content.

2.5. Lentivirus-mediated delivery of AMPKα2 short hairpin RNA (shRNA)

For knocking down mouse AMPKα2, specific three shRNAs (si-1:5′-CCAGCTTGCAGTGGCTTAT 3′; si-2: 5′-GGTAGTGAATGCATACCAT 3′; si-3: 5′-GCAAAGCGTGTGACATTAT3′) targeting AMPKα2 were designed. The lentiviral expression vector containing green fluorescent protein (GFP) was used to transduce siRNA into MIN6 cells according to the manufacturer's instructions (Shanghai GeneChem Co., Ltd.). The knock-down of AMPKα2 was examined by real-time PCR and Western blot. The specific primer for AMPKα2 was as follows: 5′-TGAAGCCAGAG AATGTGCTG-3′(forward), 5′-TGACCTCAGGTGCTGCATAA-3′ (reverse). The results of relative expression were normalized to β-actin mRNA levels in each sample.

2.6. Immunoblotting

150 islets or 1×10^6 cells in 6-well plates were washed twice with ice-cold PBS and placed immediately in lysis buffer containing 25 mmol/l HEPES (pH 7.4), 1% Nonidet P-40, 100 mmol/l NaCl, 2% glycerol, 5 mmol/l NaF, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, 1 mmol/l NaPPi, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Lysates were gently mixed for 10 min at 4 °C and then centrifuged at 13,000 *g* for 15 min at 4 °C. The protein concentration of the extracts was determined according to the method of Bradford, using BSA as the standard. Samples were separated by SDS-PAGE on 8% polyacrylamide gels and transferred to PVDF-Plus membranes (BIORAD). Primary antibodies were detected with donkey anti-rabbit IgG at 1:2000 for 1 h at room temperature. Blotted membrane was developed with ECL Advance (Cell Signaling Technology, Boston, MA) and imaged with a LAS-4000 Super CCD Remote Control Science Imaging System (Fuji, JAP).

2.7. Electrophysiological recordings

Whole-cell patch-clamp recordings in MIN6 cells were conducted using a patch clamp amplifier (EPC10; HEKA Electronik, Lambrecht, Germany). Stimulation and recordings were controlled by PULSE software. Capacitive transients and series resistance errors were minimized before recording. External medium for patch clamp contained 140 mmol/l NaCl, 5.6 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.6 mmol/l $CaCl_2$, 10 mmol/l HEPES, and 2.8 mmol/l glucose (pH 7.4 with NaOH). Patch electrodes were fabricated from borosilicate glass and had a resistance of 2.5–5 MΩ while filled with internal recording solution containing 70 mmol/l K_2SO_4, 10 mmol/l NaCl, 10 mmol/l KCl, 21 mmol/l $MgCl_2$, 10 mmol/l HEPES, and 40 mmol/l sucrose (pH 7.2 with KOH). Voltage-dependent potassium channel (Kv) currents were recorded by a series of 10 mV voltage steps to the potentials between −40 and + 60 mV from the holding potential of −70 mV. Membrane potential was recorded in I = 0 current-clamp mode, which maintains an average 0 pA holding current. All experiments were carried out at room temperature (22 °C − 24 °C).

2.8. Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

MIN6 cells were grown on coverslips and loaded with 5 μmol/l Fura-2/AM for 30 min at 37 °C in a solution containing 119 mmol/l NaCl, 4.75 mmol/l KCl, 5 mmol/l $NaHCO_3$, 1.2 mmol/l MgSO4, 1.18 mmol/l KH_2PO4, 2.54 mmol/l $CaCl_2$, 2.8 mmol/l glucose and 20 mmol/l HEPES (pH7.4). The coverslip was placed in a superfusion chamber under IX71 inverted microscope (Olympus, Tokyo, Japan). The $[Ca^{2+}]_i$ was measured using the Video-Imaging-System (Till Photonics, Munich, Germany). Cells were illuminated by alternative excitation light of 340 nm and 380 nm wavelength, which was produced by a monochromator (Till Photonics, Munich, Germany). The images were captured with emission light of 510 nm wavelength using an image-intensifying CCD camera (SensiCam, PCO, Kelheim, Germany) and processed using an image processing system (TillVision, Till Photonics, Munich, Germany). The ratio images were captured at intervals of every 10 s. Calcium concentrations were indicated as ratio of F340/F380. Cells were continuously superfused with the solution as described above throughout the experiment. All drugs were applied through superfusion.

2.9. Statistics

Data were presented as mean ± SEM. Comparisons were performed using ANOVA for multiple groups or the Student's *t* test for two groups. Significance was established at $P < 0.05$.

3. Results

3.1. Long-term exposure to high glucose stimulates insulin hypersecretion and inhibits AMPK activity

It has been demonstrated that glucose acutely inhibits AMPK activity and stimulates insulin release in β-cells [16]. To determine whether high glucose-potentiated insulin secretion is attributed to the persistent reduction of AMPK activity, rat islets were incubated with 3.3, 8.3, 11.1, and 16.7 mmol/l glucose for 20 h, and then stimulated with 3.3, 8.3, 16.7 mmol/l glucose and 35 mmol/l KCl for 1 h. As shown in Fig. 1A, with the increased concentrations of glucose during the pretreatment period, the insulin secretory response of islets to glucose at various concentrations significantly enhanced in the last hour of incubation. Compared with the 3.3 mmol/l glucose pretreatment group, the other three groups all showed significant increase in insulin secretion, even at the basal status (3.3 mmol/l glucose). These islets preincubated with moderate (8.3 or 11.1 mmol/l) and high (16.7 mmol/l) concentrations of glucose also exhibited a higher response to 35 mmol/l KCl. Meanwhile, we detected the AMPK activity of these islets preincubated with various concentrations of glucose for 20 h. Fig. 1B showed that elevations in glucose from 3.3 to 16.7 mmol/l markedly decreased the levels of phosphorylated AMPK and its substrate ACC, suggesting that the sustained reduction of AMPK activity may contribute to insulin hypersecretion.

3.2. Troglitazone decreases glucose-potentiated insulin hypersecretion along with AMPK activation

It was previously reported that the knockout or inhibition of AMPK increased insulin secretion at the low concentration of glucose, and the overexpression or activation of AMPK reduced GSIS [17]. In the present study, we simultaneously detected the effects of troglitazone on insulin secretion and AMPK activity at various concentrations of glucose. After rat islets were pretreated with 3.3 mmol/l glucose for 20 h, these islets were then stimulated with 3.3, 8.3, and 16.7 mmol/l glucose for 1 h in the presence or absence of troglitazone. The results showed that troglitazone suppressed insulin secretion and increased AMPK activity at the concentrations of 8.3 and 16.7 mmol/l glucose, not at 3.3 mmol/l glucose (Figs. 2A and B). However, when rat islets

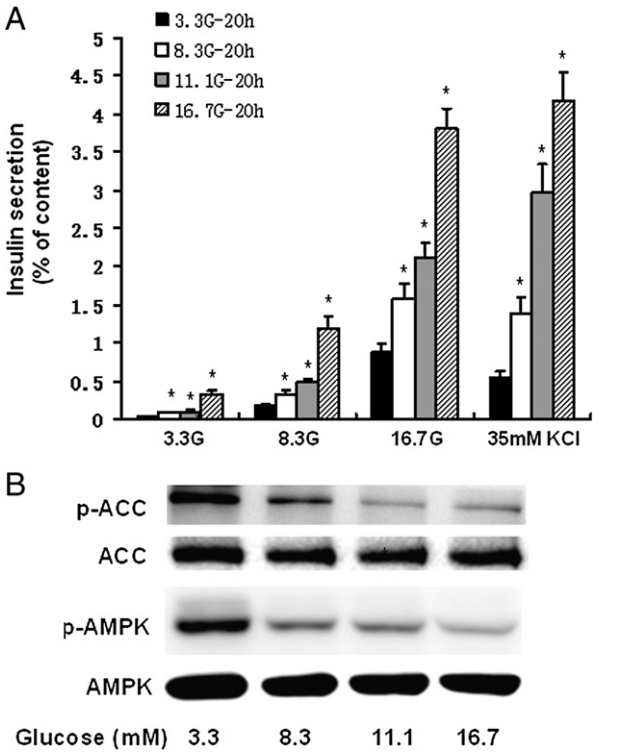

Fig. 1. Long-term exposure to high glucose inhibits AMPK activity and stimulates insulin hypersecretion in rat islets. Rat islets were isolated and preincubated in DMEM containing 3.3, 8.3, 11.1, and 16.7 mmol/l glucose for 20 h. (A) These islets were further cultured in KRB buffer with 3.3, 8.3, 16.7 mmol/L and 35 mmol/l KCl for 1 h and assayed for insulin secretion. (B) Total cell lysates in these islets pretreated with various concentrations of glucose for 20 h were analyzed for the phosphorylations of ACC and AMPK by Western blot. Data are expressed as mean ± SEM for three separate experiments. $^*P < 0.05$ vs. 3.3 mmol/l glucose pretreatment group. A representative blot from three independent experiments is shown. All three experiments showed similar results.

were pretreated with 11.1 mmol/l glucose for 20 h, only at 8.3 mmol/l glucose was insulin secretion reduced by troglitazone ($P < 0.05$), accompanied by the increased levels of AMPK and ACC phosphorylation. Troglitazone had no significant effects on insulin secretion and AMPK activity at both 3.3 and 16.7 mmol/l glucose (Figs. 2C and D). To further observe whether troglitazone-suppressed insulin secretion can restore after withdrawal of the drug, rat islets were pretreated with 10 μmol/l troglitazone for 20 h, and then stimulated with 16.7 mmol/l glucose for 1 h in the presence or absence of troglitazone. As expected, GSIS was decreased by 38% in the last hour of incubation with troglitazone, but increased by 43% compared with control group after drug withdrawal ($P < 0.05$, Fig. 2E). In consistent with the result of insulin secretion, troglitazone-stimulated AMPK and ACC phosphorylations returned to basal levels at 1 h after this drug withdrawal (Fig. 2F), suggesting that troglitazone-suppressed insulin secretion is closely related to the activation of AMPK.

3.3. Inhibition of AMPK activity reverses troglitazone-suppressed insulin secretion

To further confirm whether troglitazone inhibits insulin secretion via activating AMPK, we detected the effect of compound C, an AMPK inhibitor on troglitazone-suppressed GSIS in isolated rat islets and MIN6 cells. As expected, 10 μmol/l compound C completely reversed troglitazone-suppressed GSIS in rat islets ($P < 0.05$) while antagonized troglitazone-stimulated AMPK and ACC phosphorylations. At 16.7 mmol/l glucose, compound C alone was without effects on insulin secretion and AMPK activity (Figs. 3A and B). In MIN6 cells, compound C showed similar results in insulin secretion and AMPK activity at 25 mmol/l glucose (Figs. 3C and D). We further

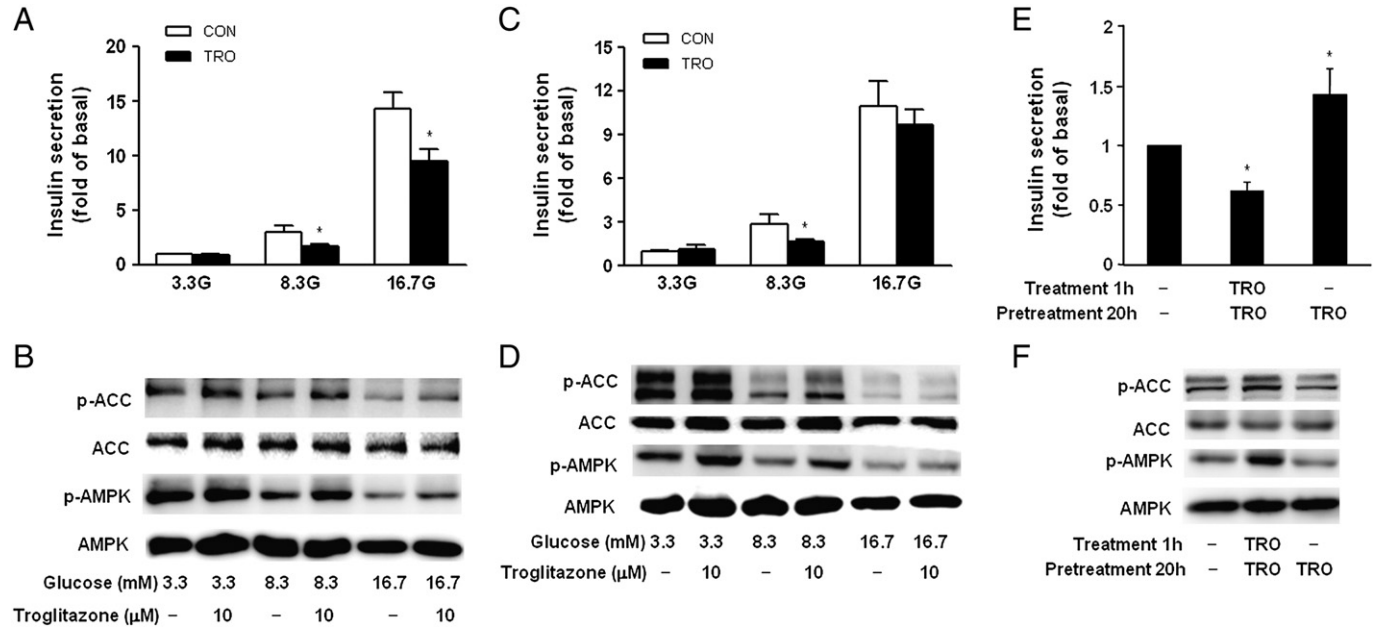

Fig. 2. Effects of troglitazone on glucose-stimulated insulin secretion and AMPK activity. Rat islets were pretreated in DMEM containing 3.3 or 11.1 mmol/l glucose for 20 h. (A and B) The islets pretreated with 3.3 mmol/l glucose were further cultured in KRB with the indicated concentrations of glucose in the presence or absence of 10 μmol/l troglitazone (TRO) for 1 h. The culture medium was taken for insulin secretion assay and the islets were lyzed for measuring the phosphorylations of ACC and AMPK by Western blot. (C and D) The islets pretreated with 11.1 mmol/l glucose were further treated as the indicated conditions for detecting insulin secretion and the phosphorylation of AMPK and ACC. (E and F) Isolated rat islets were pretreated with 10 μmol/l troglitazone for 20 h and were stimulated with 16.7 mmol/l glucose for 1 h in the presence or absence of troglitazone. Insulin secretion and the phosphorylations of AMPK and ACC were detected. Data are expressed as mean ± SEM for three separate experiments. *$P < 0.05$ vs. control (CON) group. A representative blot from three independent experiments is shown. All three experiments showed similar results.

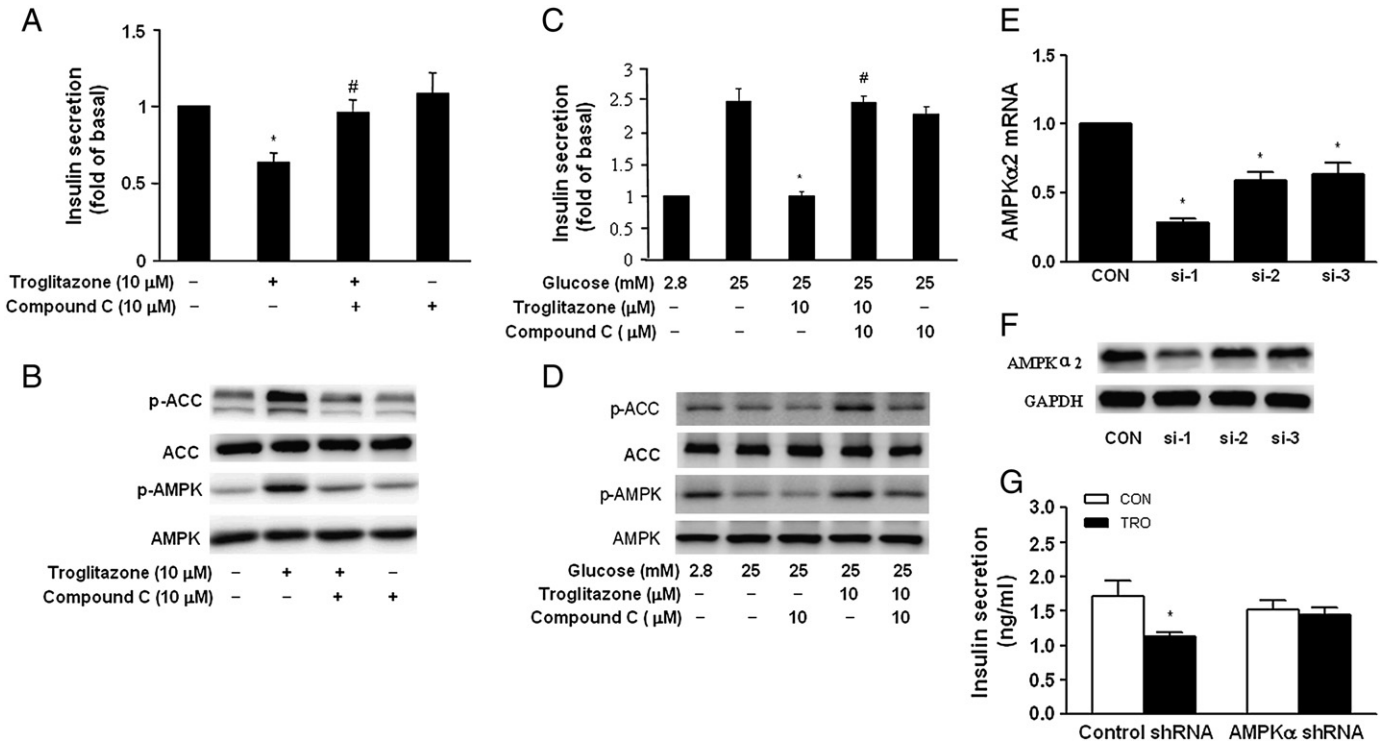

Fig. 3. Compound C reverses troglitazone-inhibited insulin secretion and -stimulated AMPK activity in isolated rat islets and MIN6 cells. Rat islets were preincubated with 10 μmol/l compound C for 30 min, and then treated with 10 μmol/l troglitazone at 16.7 mmol/l glucose for 1 h. Insulin secretion (A) and the phosphorylations of AMPK and ACC (B) were detected. MIN6 cells were preincubated with 10 μmol/l compound C for 30 min, and then treated with 10 μmol/l troglitazone at the indicated concentrations of glucose for 1 h. Insulin secretion (C) and the phosphorylations of AMPK and ACC (D) were detected. Three shRNAs for AMPKα2 were transfected into MIN6 cells by lentiviral vector containing GFP. 72 h later, AMPKα2 mRNA and protein expressions were detected by real-time PCR (E) and Western blot (F). (G) MIN6 cells infected with lentiviral vector containing control shRNA and si-1 were cultured in KRB with 2.8 mmol/l glucose for 1 h and further incubated with 10 μmol/l troglitazone in the presence of 25 mmol/l glucose for another 1 h. Insulin secretion was assayed. Data are expressed as mean ± SEM for three separate experiments. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. troglitazone alone. A representative blot from three independent experiments is shown. All three experiments showed similar results.

transfected AMPKα2 shRNA into MIN6 cells with lentiviral vectors. 72 h later, AMPKα2 mRNA expression in si-1 group was knocked down by 72% (Fig. 3E). Western blot exhibited a similar decrease in AMPKα2 protein expression (Fig. 3F). We used this shRNA for the analysis of insulin secretion. In shRNA control group, troglitazone markedly decreased insulin secretion ($P < 0.05$), but not in AMPKα knockdown group (Fig. 3G).

3.4. Troglitazone inhibits tolbutamide-stimulated insulin secretion

GSIS is involved in K_{ATP} channel-dependent and independent pathway. It has been demonstrated that K_{ATP} channel trafficking is regulated by cellular energy status via AMPK. An extracellular high potassium concentration depolarizes the cell membrane without any need for the K_{ATP} channel current, activates voltage-sensitive calcium channels, and stimulates insulin secretion [21]. In our study, troglitazone had no significant effect on 35 mmol/l KCl-stimulated insulin secretion (Figs. 4A and B). Tolbutamide promotes insulin secretion by binding to the regulatory sulfonylurea receptor-1 (SUR1) and inhibiting the K_{ATP} channel current [22]. Figs. 4A and B revealed that troglitazone suppressed tolbutamide-stimulated insulin secretion in rat islets and MIN6 cells ($P < 0.05$), suggesting that troglitazone affected the activity of K_{ATP} channel. In the presence of diazoxide (250 μmol/l) and 35 mmol/l KCl, K_{ATP} channel-mediated pathways would be expected to be completely inactivated. High glucose stimulates insulin secretion via K_{ATP} channel-independent pathway. As shown in Figs. 4C and D, troglitazone did not inhibit GSIS in the presence of diazoxide and high potassium.

3.5. Troglitazone increases K_{ATP} and Kv channel

As mentioned previously, K_{ATP} channel is key to regulating GSIS [21]. Therefore, we investigated whether the suppressive effect of troglitazone on insulin secretion was mediated by the regulation of the channel activity. For this purpose, whole-cell patch-clamp recordings in MIN6 cells were performed. The increase of the glucose concentration in the extracellular solution from 2.8 to 25 mmol/l led to a significant inhibition of the K_{ATP} current density (Fig. 5A). However, in the presence of 10 μmol/l troglitazone, high glucose-mediated K_{ATP} channel closure was prevented (Fig. 5B).

To test if the stimulated-effect of troglitazone on K_{ATP} currents might be reflected in an altered excitability of the cells, we measured action potential of MIN6 cells. The increase in the glucose concentration in the extracellular solution from 2.8 to 25 mmol/l led to a significant depolarization of membrane potential (Fig. 5C). When 10 μmol/l troglitazone was added, the high glucose-stimulated action potential was inhibited (Figs. 5D and E).

Kv currents have been detected in β-cells and are demonstrated to mediate membrane potential repolarization, and limit Ca^{2+} influx and insulin secretion [23-25]. In this study, 25 mmol/l glucose decreased Kv current as compared with 2.8 mmol/l glucose (Fig. 6A). 10 μmol/l troglitazone increased Kv current at 25 mmol/l glucose. Fig. 6B showed the current–voltage curves of Kv from −40 mV to +60 mV with a 10 mV increment. In the presence of 10 μmol/l troglitazone, the amplitude of Kv was significantly increased at potential + 10 mV through + 60 mV ($P < 0.01$).

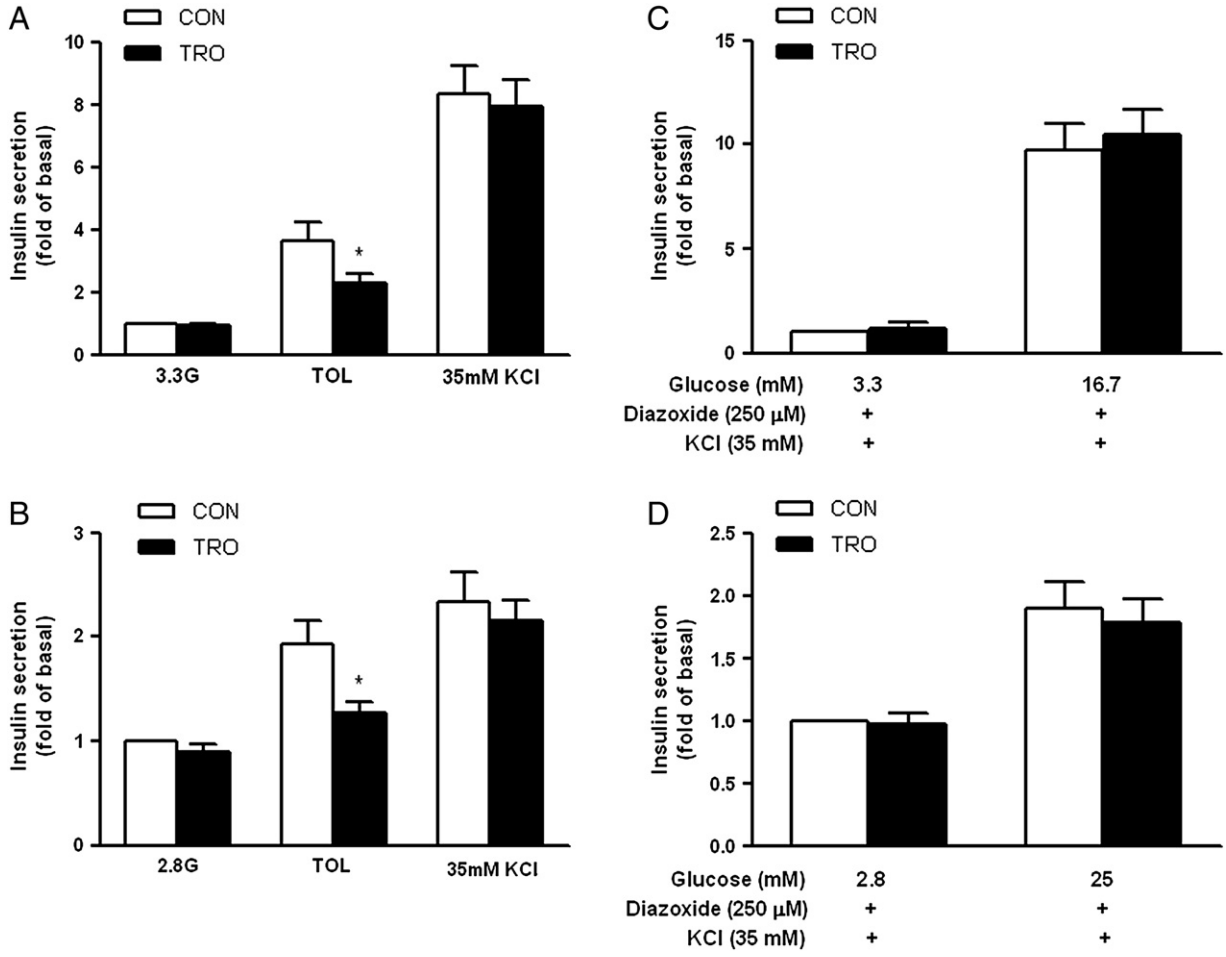

Fig. 4. Effects of troglitazone on high potassium and tolbutamide-stimulated insulin secretion. (A) Isolated rat islets and (B) MIN6 cells were incubated with 3.3 (3.3G) or 2.8 (2.8G) mmol/l glucose, 2.5 mmol/l tolbumide (TOL), and 35 mmol/l KCl in the presence or absence of 10 μmol/l troglitazone (TRO) for 1 h. Insulin secretion was assayed. (C) Rat islets and (D) MIN6 cells were incubated with or without 10 μmol/l troglitazone for 1 h at the indicated concentrations of glucose in the presence of 250 μmol/l diazoxide and 35 mmol/l KCl. Insulin secretion was assayed. Data are expressed as mean ± SEM for three separate experiments. *$P < 0.05$ vs. control (CON) group.

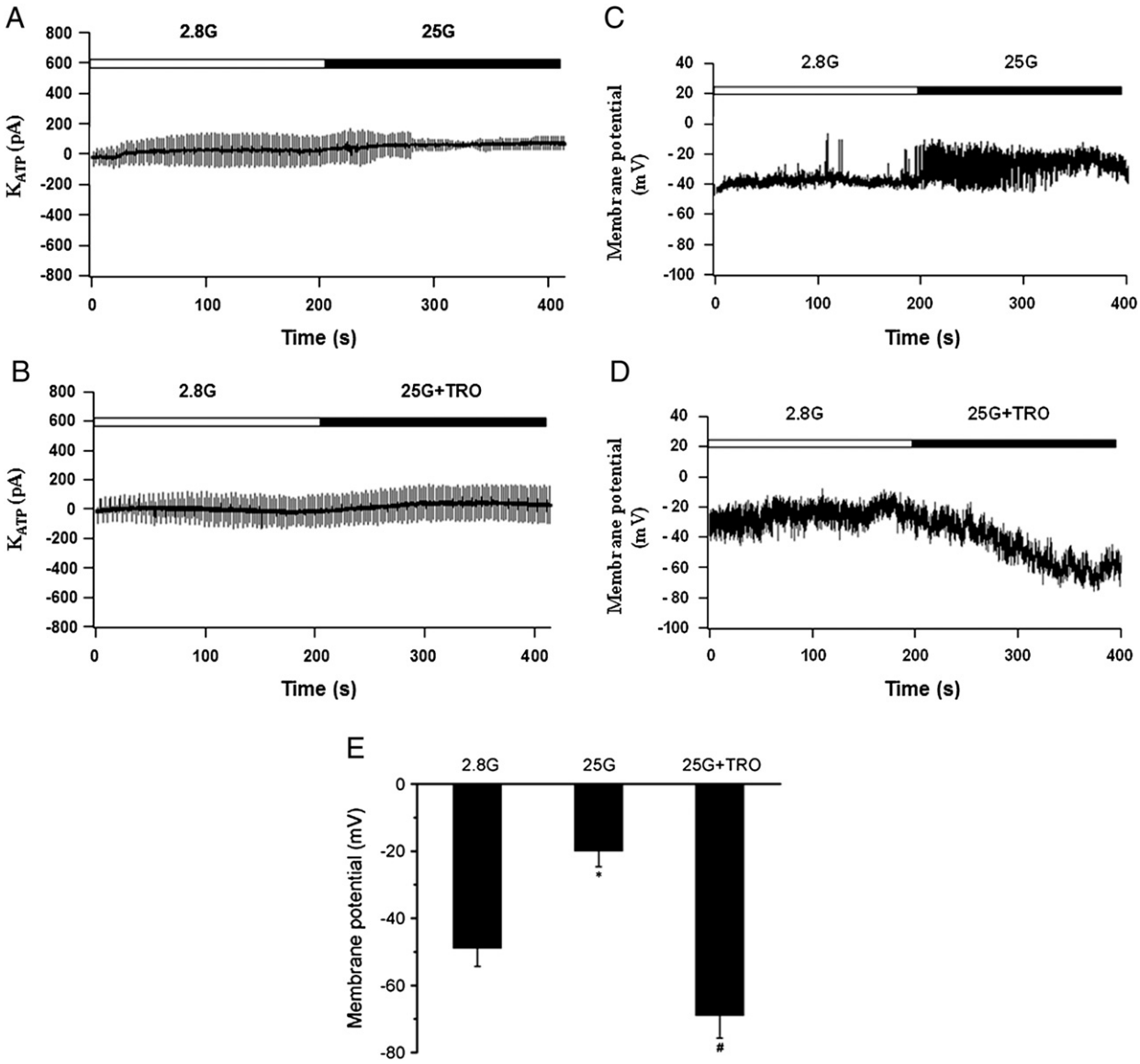

Fig. 5. Effects of troglitazone on K_{ATP} current and membrane potential in MIN6 cells. After establishing a perforated whole-cell clamp in HKRB containing 2.8 mmol/l glucose(2.8G), 25 mmol/l glucose (25G) closed K_{ATP} channel and 10 μmol/l troglitazone (TRO) opened the channel (A and B). 25 mmol/l glucose-elicited membrane potential was reduced by 10 μmol/l troglitazone (C and D). The histograms in (E) is the mean values for membrane potential in MIN6 cells exposed to 2.8 mmol/l glucose, 25 mmol/l glucose, and 25G plus 10 μmol/l troglitazone. Values are derived from 10 individual β-cell recordings per group and expressed as mean ± SEM. *$P < 0.05$ vs. 2.8G group; #$P < 0.05$ vs. 25G.

3.6. Troglitazone decreases intracellular Ca^{2+} response to glucose

Since a rise in $[Ca^{2+}]i$ is a crucial step in insulin secretion, we next studied whether the suppressive effect of troglitazone on GSIS was associated with changes in $[Ca^{2+}]i$ measured in MIN6 cells after preloading the cells with the fluorescent Ca^{2+} probe Fura-2. As shown in Figs. 7A–D, 25 mmol/l glucose stimulated the increase of $[Ca^{2+}]i$. Troglitazone suppressed $[Ca^{2+}]i$ response to high glucose and the effect was abolished by treatment with compound C.

4. Discussion

Progressive β-cell dysfunction and ultimate β-cell failure are fundamental pathogenic features of type 2 diabetes. Many clinical studies show that TZDs have the potential to preserve β cell function for delaying, preventing, or stabilizing type 2 diabetes [26-29]. However, the mechanism underlying TZDs stabilizing β cell function remains elusive. In clinical studies it has been shown that insulin levels decrease during TZDs treatment [5,30], which seems to be contradictory to its protective effect on β-cell function. At present, it has been accepted that hyperinsulinemia can cause insulin resistance and that lowering insulin secretion in hyperinsulinemic individuals may be beneficial. In the current study, troglitazone exerted a direct action on β cell to reduce insulin secretion in vitro as pioglitazone [11]. Recently Lamontagne et al. reported the surprising ability of pioglitazone to acutely inhibit GSIS in vivo prior to any significant change in insulin sensitivity, suggesting that the acute TZD effect to lower insulinemia is not due to changes in insulin sensitivity [31]. We further detected whether the suppressive effect of troglitazone on insulin secretion was reversible. It was shown that GSIS in the islets preincubated with troglitazone increased and was even higher than that in control islets after drug withdrawal (Fig. 2E). The result suggests that TZDs may provide β-cell rest by reducing insulin release, protect against the negative effects of overstimulation, and preserve its response to glucose. Therefore, the protective effect of TZDs on β-cell function may be, at least in part, attributed to their suppressive effect on insulin secretion.

TZDs improve insulin resistance in type 2 diabetes through activation of PPARγ in adipose tissue [32]. There is a debate about

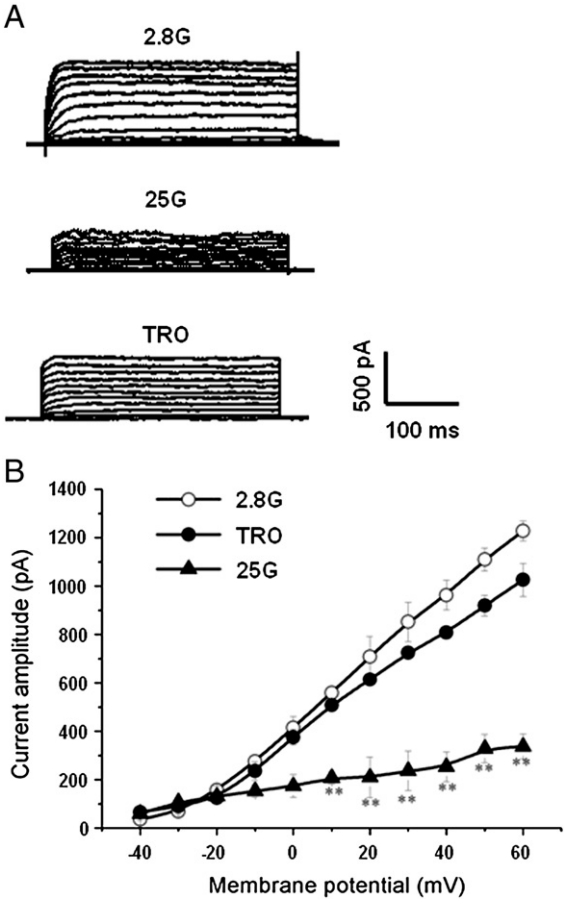

Fig. 6. Troglitazone increases Kv channel conductance in MIN6 cells. Voltage-dependent outward K current were recorded from MIN6 cells by whole-cell voltage clamp.(A)The K^+ current at 2.8 mmol/l glucose (2.8G), 25 mmol/l glucose (25G), and 25G plus 10 μmol/l troglitazone (TRO). (B) Current–voltage relationship curve of the peak current value at 2.8 mmol/l glucose, 25 mmol/l glucose, and 25G plus 10 μmol/l troglitazone. Values are expressed as mean ± SEM. $^{**}P < 0.01$ vs. TRO group.

whether TZDs regulate β-cell function via PPARγ activation. In our study, GSIS significantly decreased after troglitazone treatment for 1 h. In a study reported previously, troglitazone exhibited a regulatory effect on insulin secretion within 10 min [33]. Therefore, it is unlikely that this effect is mediated via PPARγ and gene expression changes within such a short time. It has been postulated that activation of AMPK by TZDs, metformin, or AICAR contributes to decreased GSIS in β-cell lines and rodent islets [11,18,19]. However, this has been not validated because of the lack of consistent documentation of AMPK activity with these agents using the same conditions in the insulin secretion experiments. Though overexpression of constitutively active AMPK, mimicking conditions of chronic pharmacological activation of AMPK [20], reveals a role for AMPK in regulating β-cell function, it does not permit evaluation of acute activation of AMPK on GSIS. In the current study, we detected both AMPK activity and insulin secretion under the same conditions and found that troglitazone-inhibited insulin secretion was inversely related to the activation of AMPK.

Continuous overstimulation of the β-cell by glucose could eventually lead to depletion of insulin stores, worsening of hyperglycemia, and finally deterioration of β-cell function [34]. In this study, the islets preincubated with 8.3, 11.1, and 16.7 glucose showed significantly increased responses to various concentrations of glucose and high potassium. AMPK acts as a cellular integration node for various nutrient and hormone signals, and changes in AMPK activity regulates multiple metabolic pathways of glucose metabolism [35]. In the rodent insulinoma INS1, MIN6, and HIT cell lines, AMPK activity was observed to be inversely related to insulin secretion across a wide range of glucose concentrations [16,36]. These studies all demonstrated the acutely suppressive effect of glucose on AMPK activity. It has been noted recently that inhibition of AMPK activity within the β-cell is necessary for the stimulation of insulin secretion by glucose in β-cells with selective inactivation of both AMPK catalytic subunits or with a deletion of LKB1, an upstream activator of AMPK [37,38]. In accordance with acute results of increased glucose concentrations reported previously [36], the long-term treatment with moderate and high concentrations of glucose also decreased AMPK activity in our study. Therefore, the inhibition of AMPK activity seems to be necessary for insulin hypersecretion and its activation may protect β-cell against chronic overstimulation and loss of islet function.

Though the preponderance of evidence indicated that AMPK was a key negative regulator of insulin secretion, there were some studies showing that AMPK activation by pharmaceutical agents promoted insulin secretion [39,40]. The conflicting results may attribute to the different experimental conditions including the use of primary β-cells or cell lines, of whole pancreas or single cells, of different concentrations of glucose, and the acute or long-term application of AMPK activators. In the present study, we performed all experiments at 10 μmol/l troglitazone, a concentration commonly used in other in vitro studies. Troglitazone exerted different effects on insulin secretion and AMPK activity at various concentrations of glucose for different time intervals. In the islets pretreated with both 3.3 and 11.1 mmol/l glucose, troglitazone was without effect on insulin secretion and AMPK activity under the condition of 3.3 mmol/l glucose stimulation for 1 h. At basal status, low glucose has stimulated AMPK phosphorylation to such a great extent that troglitazone cannot further enhance its activity. At the islets pretreated with 3.3 mmol/l glucose, troglitazone inhibited high glucose-stimulated insulin secretion and increased AMPK activity, but not at the islets pretreated with 11.1 mmol/l glucose. However, troglitazone inhibited moderate glucose-stimulated insulin secretion and increased AMPK and ACC phosphorylations in the islets pretreated with both 3.3 and 11.1 mmol/l glucose. The result is similar to that of Lamontagne et al. [11]. In their study, the suppressive effect of pioglitazone on insulin secretion was obvious at the intermediate glucose. Maybe the effect of low glucose or high glucose itself on AMPK activity is beyond troglitazone action so that troglitazone cannot affect insulin secretion. As reported previously, AICAR increased GSIS after drug withdrawal [41]. Our study showed that GSIS decreased in the presence of troglitazone in the last one hour and significantly increased after the drug withdrawal. At that time, troglitazone-stimulated AMPK and ACC phosphorylations returned to basal level. Recently Garcia-Haro et al. reported that a protein phosphatase holoenzyme composed of PP1 and the regulatory subunit R6 participated in glucose-induced dephosphorylation and inactivation of AMPK [42]. Thus, troglitazone-stimulated AMPK phosporylation may be dephosphorylated by high glucose via this pathway within 1 h, which restores the response of the islets to glucose for insulin secretion. In rat islets and MIN6 cells, compound C, an AMPK inhibitor, reversed troglitazone-stimulated AMPK activation and restored the suppressive insulin secretion, and knockdown of AMPKα2 also showed a similar result, which further verifies the hypothesis that troglitazone inhibits insulin secretion via activating AMPK.

K_{ATP} channels are metabolic sensors that couple cellular energy status to electrical activity and play key roles in energy-dependent insulin secretion in pancreatic β-cells [21]. Stimulation of insulin secretion by glucose involves the enhanced synthesis of ATP by mitochondria and closure of K_{ATP}. Subsequent depolarization of the plasma membrane then opens voltage-sensitive (L-type) Ca^{2+} channels causing insulin-containing vesicles to fuse at the plasma membrane [21]. It has been demonstrated that AMPK activation increased K_{ATP} conductance [43]. AMPK decreases the cytosolic Ca^{2+} concentration, either by blocking Ca^{2+} channels in the plasma membrane or by activating Ca^{2+} pump extrusion to the endoplasmic reticulum [17]. Moreover, activated AMPK

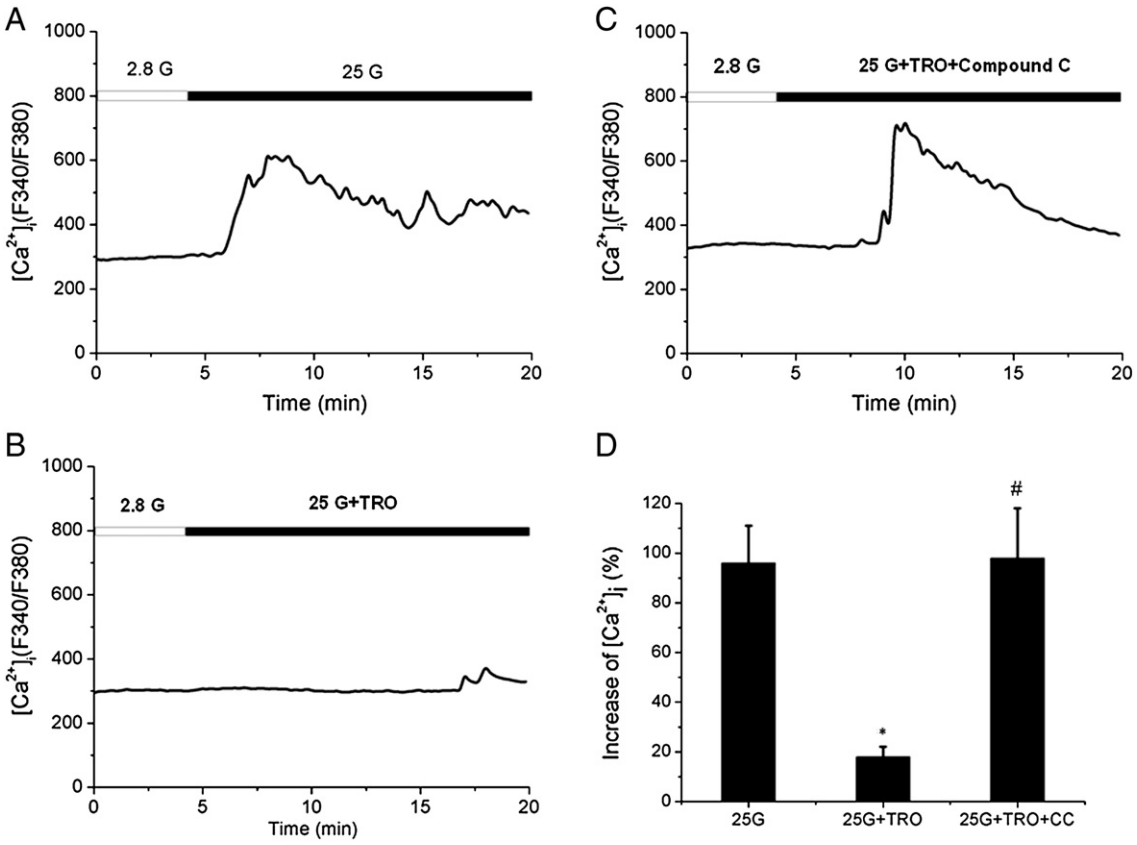

Fig. 7. Troglitazone decreases the intracellular Ca^{2+} level. (A and B)MIN6 cells were incubated at 2.8 mmol/l glucose (2.8G), and stimulated with 25 mmol/l glucose (25G) in the presence or absence of 10 μmol/l troglitazone (TRO). (C) MIN6 cells were incubated with 25G plus 10 μmol/l troglitazone and 10 μmol/l compound C (CC). The mean values of intracellular Ca^{2+} levels in three groups were shown in (D). Values are derived from 20 to 30 individual β-cell recordings per group and expressed as mean ± SEM. $^{*}P < 0.05$ vs. 25G group; $\#P < 0.05$ vs. 25G + TRO group.

inhibits glucose-stimulated insulin-containing vesicle movements and decreases the number of these vesicles docked or fused at the plasma membrane [19,44]. However, there was evidence demonstrating that AMPK activated by AICAR inhibited K_{ATP} current and increased membrane potential [39]. In our study, troglitazone antagonized high glucose-stimulated K_{ATP} current closure, which contributed to the decrease in membrane potential. In pancreatic β-cells, activation of Kv channels repolarizes cells and attenuates glucose-stimulated action potentials, limiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels to suppress insulin secretion [45]. Blockade of Kv channels can promote glucose-dependent insulin secretion [46]. The present study showed that exposure to 10 μmol/l troglitazone increased outward K currents, which may cause a rapid repolarization and shortening of bursting action potentials, leading to the attenuation of glucose-induced $[Ca^{2+}]i$ increases. Because of decreased membrane potential and increased Kv currents, troglitazone decreased the content of $[Ca^{2+}]i$ as expected. In the presence of compound C, troglitazone-inhibited $[Ca^{2+}]i$ increase was reversed, further suggesting that troglitazone suppresses GSIS via activating AMPK and decreasing $[Ca^{2+}]i$ level.

5. Conclusions

Troglitazone inhibited GSIS via activating AMPK, increasing K_{ATP} and Kv currents, decreasing $[Ca^{2+}]i$ level. This direct effect on the pancreatic islets was the most profound at moderate glucose level. Troglitazone provides β cell "a rest" through inhibiting insulin hypersecretion and restores its response to glucose, which may partially explain the protective effect of TZDs on beta-cell function in prediabetic or type 2 diabetic patients.

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