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Glucose fluctuations promote vascular BK channels dysfunction via PKCα/NF-κB/MuRF1 signaling

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ABSTRACT

Glucose fluctuations may contribute to large conductance calcium activated potassium (BK) channel dysfunction. However, the underlying mechanisms remain elusive. The aim of this study was to investigate the molecular mechanisms involved in BK channel dysfunction as a result of glucose fluctuations. A rat diabetic model was established through the injection of streptozotocin. Glucose fluctuations in diabetic rats were induced via consumption and starvation. Rat coronary arteries were isolated and coronary vascular tensions were measured after three weeks. Rat coronary artery smooth muscle cells were isolated and whole-cell BK channel currents were recorded using a patch clamp technique. Human coronary artery smooth muscle cells in vitro were used to explore the underlying mechanisms. After incubation with iberiotoxin (IBTX), the Δ tensions (% Max) of rat coronary arteries in the controlled diabetes mellitus (C-DM), the uncontrolled DM (U-DM) and the DM with glucose fluctuation (GF-DM) groups were found to be 84.46 ± 5.75, 61.89 ± 10.20 and 14.77 ± 5.90, respectively ($P < .05$), while the current densities of the BK channels in the three groups were 43.09 ± 4.35 pA/pF, 34.23 ± 6.07 pA/pF and 17.87 ± 4.33 pA/pF, respectively ($P < .05$). The Δ tensions (% Max) of rat coronary arteries after applying IBTX in the GF-DM rats injected with 0.9% sodium chloride (NaCl) (GF-DM + NaCl) and the GF-DM rats injected with N-acetyl-L-cysteine (NAC) (GF-DM + NAC) groups were found to be 8.86 ± 1.09 and 48.90 ± 10.85, respectively ($P < .05$). Excessive oxidative stress and the activation of protein kinase C (PKC) α and nuclear factor (NF)-κB induced by glucose fluctuations promoted the decrease of BK-β1 expression, while the inhibition of reactive oxygen species (ROS), PKCα, NF-κB and muscle ring finger protein 1 (MuRF1) reversed this effect. Glucose fluctuations aggravate BK channel dysfunction via the ROS overproduction and the PKCα/NF-κB/MuRF1 signaling pathway.

1. Introduction

Diabetes mellitus (DM), a chronic and progressive metabolic disorder, has become a major public health concern. DM contributes to the development of vascular pathology and cardiovascular diseases [1]. Recent studies have demonstrated that glucose fluctuations have a more detrimental effect on the cardiovascular system than persistent high blood glucose, and may be related with coronary diseases [2,3]. However, the precise mechanisms underlying glucose fluctuation related to vascular pathology remain elusive.

The large conductance calcium activated potassium (BK) channels function as critical regulators of membrane potential and coronary arterial smooth muscle tone [4,5]. BK channels, a tetramer composed of four α-subunits (BK-α) and auxiliary β1-subunits (BK-β1), are abundantly expressed in coronary arterial smooth muscle cells (SMCs) [6]. The BK-β1 subunit, encoded by the *KCNMB1* gene, can alter the

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sensitivity of BK-α subunits to Ca^{2+} and participate in the regulation of the electrophysiology of BK channels [7]. Accumulated evidence has demonstrated that the downregulation of BK-β1 subunits in diabetes is primarily responsible for BK channel dysfunction [8,9]. However, it remains unclear whether glucose fluctuations lead to the degradation of BK-β1 subunits.

The nuclear factor (NF)-κB belongs to a ubiquitous family of several transcription factors, including RelA (p65), RelB and c-Rel subunits [10]. In an inactive state, the p65 subunit and its inhibitory subunit (IκB) are bound together. IκB kinase (IKK) promotes the phosphorylation of IκB, enabling IκB to dissociate from p65 [11]. The muscle ring finger protein 1 (MuRF1) is a target gene regulated by NF-κB in diabetic vascular SMCs [12]. MuRF1 is known as a muscle specific E3 ligase and plays an important role in the downregulation of BK-β1 subunits in diabetic mouse arteries [13]. In addition, several studies have reported that NF-κB is activated in diabetic rats after exposure to glucose fluctuations [14,15]. However, it is unclear whether upstream signaling leads to the activation of NF-κB, in response to glucose fluctuations.

Protein kinase C (PKC) is a family of several isoenzymes, and functions as an important regulator of various types of physiological processes [16]. Chakraborti et al. [17] reported that PKCα, a subunit of PKC, was activated in response to excessive oxidative stress in pulmonary artery smooth muscle cells. Furthermore, NF-κB is activated by PKCα, and found to be involved in the regulation of inflammatory response [18].

In this study, we aimed to investigate whether glucose fluctuations aggravated BK channel dysfunction. We found that the overproduction of reactive oxygen species (ROS) and the activation of NF-κB via PKCα in diabetic rats with glucose fluctuations play a vital role in the promotion of MuRF1-induced BK-β1 degradation.

2. Methods

2.1. Experimental animal models

Sprague-Dawley rats weighing 150–200 g (n = 46) were obtained from Jiangsu Institute of Schistosomiasis Control and Prevention in China. The rats were housed under specific pathogen-free conditions with standard food and water available. The rat diabetic model was established as previously described [19]. In brief, the rats received an intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich Corp.; 60 mg/kg). One week later, after fasting for 6 h, rats with a blood glucose concentration > 16.7 mmol/L were enrolled in this study. Then, the rats were randomly divided into five groups: the controlled DM (C-DM) group (n = 12), the uncontrolled DM (U-DM) group (n = 12), the DM with glucose fluctuation (GF-DM) group (n = 12), the GF-DM rats injected with N-acetyl-L-cysteine (NAC) (GF-DM + NAC) (n = 5) group and the GF-DM rats injected with 0.9% sodium chloride (NaCl) (GF-DM + NaCl) group (n = 5). The C-DM group rats received a subcutaneous injection of long-acting insulin (insulin glargine; Sanofi-Aventis Co.; 20 IU/kg) twice a day (8:00 and 20:00) to control their blood glucose levels. The GF-DM group rats were treated with a-24 h starvation period followed by a-24 h consumption period, and an adequate amount of foods were provided or removed at 20:00 every day. During a-24 h starvation period, the rats would receive regular subcutaneous insulin (0.5 IU/kg Insulin Aspart, Novo Nordisk Corp.) to reduce blood glucose levels when it was > 5.5 mmol/L. The U-DM group rats were raised with adequate food and water without any treatments. The GF-DM + NAC group rats were treated with starvation and consumption as the same as the GF-DM group rats, and were intraperitoneally injected with NAC (150 mg/kg) at 16:00 every day. While, GF-DM + NaCl group rats were intraperitoneally injected with 0.9% NaCl as a placebo. After three weeks, the rats were sacrificed, and their coronary arteries were harvested and stored at −80 °C. The animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 8th edition, Washington (DC): National Academies Press (US); 2011). The study protocol was approved by the Institutional Animal Care of The Affiliated Wuxi People's Hospital of Nanjing Medical University.

2.2. Glucose monitoring and insulin measurement in vivo

Plasma glucose levels were measured using a blood glucose monitor (Roche Co., ACCU-Chek) between 12:00 p.m. and 1:00 p.m. every day. Three weeks later, blood samples were collected from the inferior vena cava of the diabetic rats after the last 24 h of exposure to food. Then, the plasma insulin levels were measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit (AMEKO, #AE90616Ra).

2.3. ELISA of malondialdehyde (MDA)

Blood samples were collected as mentioned above and the levels of MDA in rat plasma were measured using a MDA ELISA kit (Bryotime Co., #S0131).

2.4. Isolation of rat coronary arteries

After being anesthetized using isoflurane (2%) (Ruiwode Lift Technology Co.), the rat hearts were rapidly obtained and placed in a 4 °C physiological saline solution: 145.0 mmol/L NaCl, 4.0 mmol/L KCl, 0.05 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 10.0 mmol/L HEPES and 10.0 mmol/L glucose, and pH was adjusted to 7.4 with NaOH. Then, the secondary and tertiary branches of the right and left coronary arteries, and the septal coronary arteries were dissected carefully and freed of surrounding myocardium and connective tissue, under a microscope (Olympus SZX10 Stereo Microscope). All chemicals mentioned above were obtained from Sigma-Aldrich Corporation.

2.5. Coronary artery tension measurements

In brief, rat coronary arteries were isolated and then a length of 1.5–2.0 mm was cut out and mounted on a four-chamber wire myograph (model 620 M, Danish Myo Technology). The coronary arteries were maintained at 37 °C in a modified physiological salt solution (PSS): 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH_2PO_4, 1.17 mmol/L $MgSO_4{\cdot}7H_2O$, 14.9 mmol/L $NaHCO_3$, 5.5 mmol/L Glucose, 0.026 mmol/L EDTA and 1.6 mmol/L CaCl2, with a pH of 7.4. The modified PSS was pre-warmed at 37 °C and gassed with 95% O_2 and 5% CO_2, in order to maintain a pH of 7.4 at a resting tension of 2.5 mN. Then, the coronary artery tension was tested with 60 mmol/L KCl and referenced as the maximum contraction for contractile capacity. When the arteries achieved a stable contractile response, iberiotoxin (IBTX) was added to the bath after being washed three times to basal tension. Changes in rat coronary tension were recorded on the setting of 100 nmol/L IBTX. An identical procedure of applying 60 mmol/L KCl to reach a stable plateau, sodium nitroprusside (SNP, 10^{-8}–10^{-3} M) was applied and the concentration-response curve was created. All chemicals mentioned above were obtained from Sigma-Aldrich Corporation.

2.6. Isolation of rat coronary arterial SMCs

In order to isolate rat coronary arterial SMCs, three dissected coronary arteries were placed in 1.0 mL of physiological saline solution containing bovine serum albumin for an 8-min incubation period, at 37 °C, in a shaking water bath, and then the vessels were transferred into another tube with 1.5 mg papain, 1.0 mg dithiothreitol and 0.1% bovine serum albuminin in 1.0 ml of saline solution for 8 min, and further digested with 1.0 mg collagenase, 1.0 mg trypsin inhibitor, 0.25 mg elastase, and 0.1% bovine serum albumin in 1.0 mL of saline solution for 8 min at 37 °C. Then, the vessels were washed three times using saline solution and placed in a saline solution at 4 °C. Coronary arterial SMCs were obtained before performing the experiments by

gently pipetting using a transferpettor until the cells were completely dissociated. All chemicals mentioned above were obtained from Sigma-Aldrich Corporation.

2.7. Whole-cell patch clamp recording

Whole-cell BK currents were recorded from freshly isolated rat coronary arterial SMCs, as we previously reported [20], using an Axopatch 200B Amplifier (Molecular Devices, Inc.), filtered at 2 kHz, and sampled at 50 kHz. All experiments were performed at room temperature (22–24 °C). Whole-cell BK currents were defined as the IBTX (100 nmol/L)-sensitive K^+ current component. Whole-cell K^+ currents were recorded from a holding potential (HP) of −60 mV with pulses of 100 ms duration to testing potentials (TPs) in +100 mV. For whole-cell BK channel recordings, the pipette solution contained 140.0 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.0 mmol/L Na_2ATP (EGTA) and 0.465 mmol/L $CaCl_2$ (~200 nmol/L free Ca^{2+}), at pH 7.35. The bath solution for whole-cell recordings contained 145.0 mmol/L NaCl, 5.6 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 0.5 mmol/L $CaCl_2$, 10.0 mmol/L HEPES, and 10.0 mmol/L glucose, at pH 7.4. All chemicals mentioned above were obtained from Sigma-Aldrich Corporation.

2.8. Glucose fluctuations model in vitro and shRNA transfection

Human coronary arterial SMCs (ATCC, #PCS-100-021) and the culture medium (ATCC, #PCS-100-042 and #PCS-100-030) were purchased from ATCC. The in vitro glucose fluctuation model was established as reported previously [21]. Cells were seeded into culture dishes (Corning, #430167) and initially incubated at 37 °C and 5% CO_2. In brief, human coronary arterial SMCs were divided into three groups: the normal glucose group (NG), the high glucose (HG) group and the glucose fluctuation (GF) group. Cells of the NG group were exposed to 5.5 mmol/L glucose, cells of the HG group were exposed to 25 mmol/L glucose, while cells of the GF group were incubated for 72 h in glucose solution alternating between 5.5 mmol/L and 25 mmol/L glucose every 12 h. In order to decrease the expression of MuRF1, the human coronary arterial SMCs were cultured in a serum-free medium and subsequently transfected with MuRF1 shRNA at a multiplicity of infection (MOI) of 50 for 12 h. The MuRF1-shRNA (LV-TRIM63-RNAi) and the scrambled control (shNC) (Hu6-MCS-Ubiquitin-EGFP-IRES-puromycin) were obtained from Genechem Corporation. When cells were treated with the ROS inhibitor NAC (Sigma-Aldrich Co. #A7250), the IKK inhibitor TPCA-1 (Sigma Co., #T1452) and the PKCα inhibitor Go6976 (Selleck Co., #S7119) these agents were added at the beginning and remained present throughout the experiment.

2.9. ROS level analysis

ROS level was detected using ROS Detection Reagents (CM-H2DCFDA; Invitrogen, C400) in human coronary arterial SMCs. The cells were incubated in a serum-free medium containing the fluorescence probe 5-(and-6)-chloromethyl-2′7′-dichlorofluoresce in diacetate at room temperature for 50 min with a final working concentration of 10 μmol/L. After being washed with PBS three times, the cells with the ROS probes were visualized under a fluorescence microscope.

2.10. Quantitative real-time (qRT) PCR

Total RNA was extracted from rat coronary arteries and was subsequently reverse-transcribed using a reverse transcription system (Takara Bio. Inc., #DRR036A). Quantitative PCR was performed using a SYBR Green PCR mix (Roche Co., #4913914001) on an ABI Prism 7500HT sequence detection system (Applied Biosystems Fisher Scientific Inc.). The cycling was as follows: step 1: 50 °C, 2 min; step 2: 95 °C, 10 min; and step 3: 95 °C, 15 s, and 60 °C, 60 s, for 40 cycles. The β-actin was used as an internal control to normalize differences in the amount of total RNA in each sample. The relative mRNA expression levels of target genes were calculated according to the $2^{-\Delta\Delta Ct}$ method. The primer pairs used for cDNA amplification were as follows: Rat BK-β1 forward: 5′-CCAACAGTGCTCCTATATCCCCA-3′, reverse: 5′-ATAAG AAGGCCACCAGTCAGCAG-3′. Rat BK-α forward: 5′-AAACAAGTAATT CCTCAAGCTGGTG-3′, reverse: 5′-CGTAAGTGCCTGGTTGTTTTGG-3′. Rat β-actin forward: 5′-AGATTACTGCCCTGGCTCCTA-3′, reverse: 5′-CCTGCTTGCTGATCCACATCT-3′.

2.11. Western blot analysis

The rat coronary arterial scraps and cultured cells were lysed in an ice-cold RIPA buffer (Pierce Co., #89900) containing protease and phosphatase inhibitors (Roche Co., #04693159001). The lysates were fractionated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences Co.). The PVDF membranes were incubated with specific primary antibodies and α-tubulin (Cell Signaling Technology Co., #2144), GAPDH (Cell Signaling Technology Co., #2118), PKCα (Cell Signaling Technology Co., #2056), NF-κB p65 (Cell Signaling Technology Co., #8242), p-NF-κB p65 (Cell Signaling Technology Co., #3033), MuRF1 (Santa Cruz Corp.; #sc-32,920), BK-α (Alomone Labs, #APC-021) and BK-β1 (Alomone Labs, #APC-036). Immunoblot bands were quantified using a densitometry along with ImageJ software (Scion Corp.). Densities were normalized to the control treatment levels and relative folds were normalized to α-tubulin or GAPDH levels.

2.12. Confocal microscopy

Human coronary arterial SMCs were seeded into 12-well plates with lysine-coated slides and exposed to glucose fluctuations for 72 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich Corp., #T9284) and blocked with 2% BSA (Santa Cruz Corp., #sc-2323). In order to assay the nucleation of NF-κB, the cells were incubated with anti-NF-κB p65 (Cell Signaling Technology Corp., #8242), washed with PBST and incubated with an anti-rabbit IgG (H + L)F(ab')2 fragment secondary antibody (Cell Signaling Technology Corp., #4412) and DAPI (Sigma-Aldrich Corp., #F6057). In order to assay the intracellular MuRF1, the cells were incubated with an anti-MuRF1 (Santa Cruz Corp., sc-32,920) antibody overnight. After being washed with PBST, the cells were incubated with a FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., 115–095-003) and DAPI. For the immunofluorescence staining of the coronary artery tissue, the rat coronary arteries were harvested and fixed using 4% paraformaldehyde. After OCT paraffin treatment, the coronary arteries were cut into 4 μm sections. The BK-β1 expression was detected in the coronary artery sections using the above mentioned antibodies. An identical procedure of permeation, blocking and incubation with antibodies was performed on coronary artery tissue.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 and SPSS 22.0 software. Data are presented as mean ± SEM. Student's *t*-test was used to compare data between two groups. One-way ANOVA with post-hoc LSD analysis was used to compare data between multiple groups. Statistical significance was defined as a p value of < 0.05.

3. Results

3.1. Glucose fluctuations aggravated coronary arterial dysfunction

The blood glucose levels and the body weights of STZ-induced DM rats are shown in Fig. 1. The glucose level of the C-DM group was maintained at around 5 mmol/L, while the glucose level of the U-DM

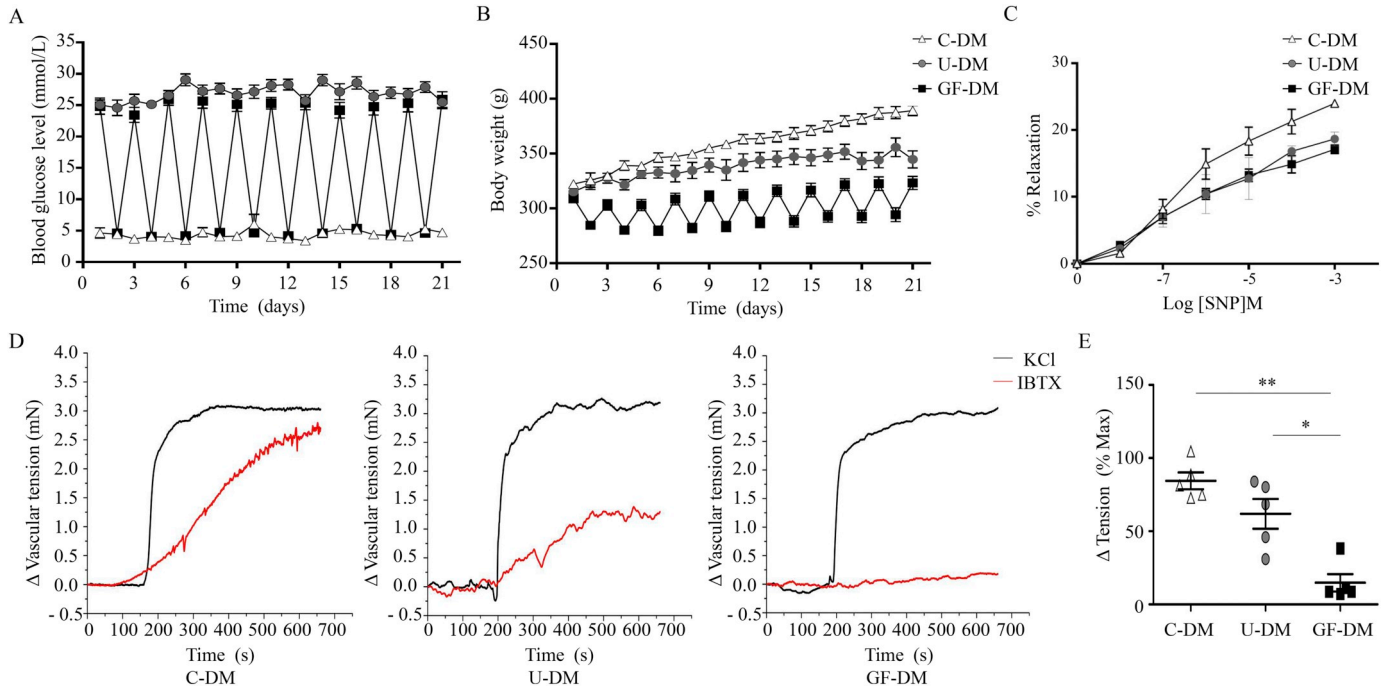

Fig. 1. Effects of glucose fluctuations on coronary artery tensions (A) Levels of blood glucose in the C-DM, U-DM and GF-DM groups, respectively (n = 12 per group). (B) Body weights of rats in three groups (n = 12 per group). (C) Concentration-response curves of SNP (10^{-8}–10^{-3} M) in the C-DM, U-DM, GF-DM groups. (D-E) Representative tracings of vascular tensions from the C-DM, U-DM and GF-DM groups after using 60 mmol/L KCl. The coronary artery tensions in three groups were increased after using 100 nmol/L IBTX (a BK channel inhibitor) (n = 5 per group). $^{*}P < .05$, $^{**}P < .01$.

group was kept > 25 mmol/L, and the glucose level of the GF-DM group fluctuated between 5 mmol/L and 25 mmol/L (Fig. 1A). The body weight gains in the GF-DM group were significantly lower than those of the C-DM group and the U-DM group. Repetitive starvation was responsible for the fluctuation in body weights in the GF-DM group (Fig. 1B). As shown in Table 1, after three weeks, the heart weights were significantly lower in the U-DM and GF-DM group rats compared with those in the C-DM group. However, there were no significant differences in heart-to-body weight ratios among the three groups. Moreover, the plasma insulin levels were extremely low in the U-DM and GF-DM groups, compared with those in the C-DM group. Fig. 1C shows the concentration-response curves of SNP (10^{-8}–10^{-3} M) in the C-DM, U-DM, GF-DM groups. The maximal relaxation percentages were lower in the U-DM and GF-DM groups, compared with the C-DM group, indicating that the sensitivity of coronary to SNP-induced relaxation was reduced in the U-DM and GF-DM groups. BK channel-mediated coronary vasodilation was examined after the application of IBTX. Fig. 1D-E show the representative traces of vascular tension in the three groups of diabetic rats. After incubation with 100 nmol/L IBTX, Δ tension (% Max) decreased in the U-DM group (61.89 ± 10.20) compared with that of the C-DM group (84.46 ± 5.75), and was more pronounced in the GF-DM group (14.77 ± 5.90), indicating that BK channel-mediated coronary vasodilation was impaired in the GF-DM group rats.

3.2. Glucose fluctuations promoted the downregulation of BK-β1 subunit levels

In order to investigate the effects of glucose fluctuations on BK channel function in vivo, whole-cell BK currents were recorded using a patch clamp technique. Fig. 2A-B show the total K^+ currents in the coronary arterial SMCs of the three groups at the baseline and after exposure to 100 nmol/L IBTX. Fig. S1 shows the statistical results of total K^+ current densities. There were reduced trends of total K^+ current densities in the U-DM and GF-DM groups compared with that in the C-DM group, however, there were no significant differences of total K^+ current densities among three groups. The current densities of BK channels decreased in the U-DM group (34.23 ± 6.07 pA/pF), compared with those in the C-DM group (43.09 ± 4.35 pA/pF), however the decrease was more pronounced in the GF-DM group (17.87 ± 4.33 pA/pF). Fig. 2C shows that BK-β1 expression detected through immunofluorescence staining decreased in the U-DM group, compared with that in the C-DM group, and to a greater extent in the GF-DM group. As shown in Fig. 2D-E, there were no differences in the mRNA levels of BK-α and BK-β1 among the three groups. There were also no differences in BK-α protein expression among the three groups. However, the protein expression of BK-β1 decreased significantly in rats with glucose fluctuations (Fig. 2F-H). These data indicated that glucose fluctuations promoted coronary BK channel dysfunction due to the downregulation of BK-β1 expression.

The blood glucose levels and the body weights of rats in the GF-DM + NaCl and GF-DM + NAC groups are shown in Fig. S2. There were no significant differences in body weights, heart weights, heart-to-body weight ratios and plasma insulin levels among the two groups (Fig. S2). As shown in Fig. 6A, the MDA level increased in the U-DM group (11.81 ± 0.84 nmol/mL) compared with that of the C-DM group (6.80 ± 0.58 nmol/mL), and was more pronounced in the GF-DM group (15.56 ± 0.38 nmol/mL). While, the MDA level decreased in GF-DM + NAC group (10.98 ± 0.79 nmol/mL). The fluorescent dye CM-H2DCFPA was applied directly to assess ROS production. Fig. 6B shows that ROS production increased significantly in human coronary SMCs of the GF group. Fig. 6C-D show the representative traces of vascular tension in rats with glucose fluctuations. After incubation with 100 nmol/L IBTX, Δ tension (% Max) increased in the GF-DM + NAC group (48.90 ± 10.85), compared with that of the GF-DM + NaCl group (8.86 ± 1.09). Fig. 6E-F show the total K^+ currents in the coronary arterial SMCs at the baseline and after exposure to 100 nmol/L IBTX. The current densities of BK channels increased in the GF-DM + NAC group (58.79 ± 9.41 pA/pF), compared with those of the GF-DM + NaCl group (26.04 ± 5.50 pA/pF). Moreover, the protein expressions of MuRF1 decreased in the GF-DM + NAC group. Meanwhile, the protein expression of BK-β1 increased in the GF-

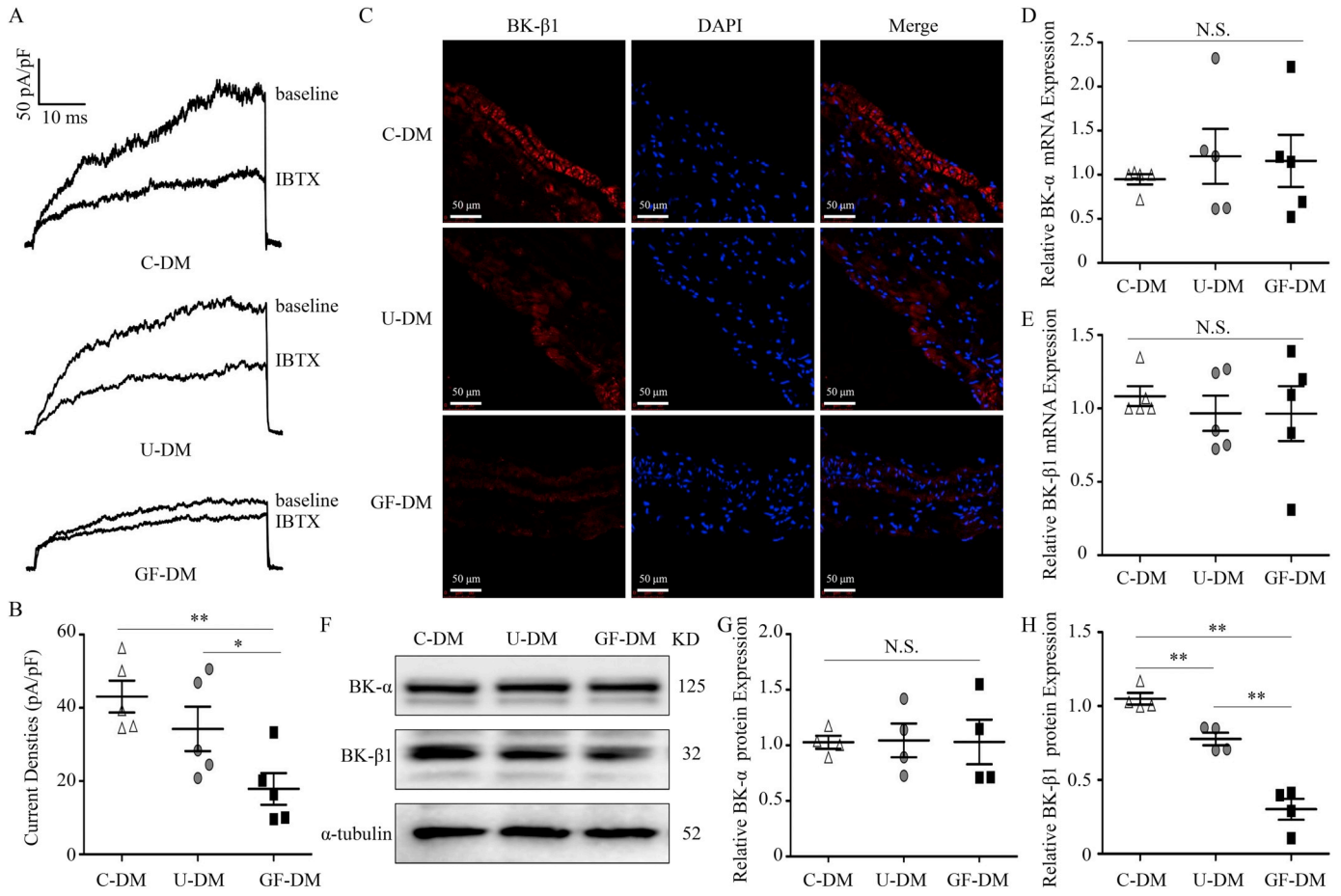

Fig. 2. Impairments of BK channels due to glucose fluctuations (A-B) Representative tracings showing current density-voltage relationship of BK channels in rat coronary arterial SMCs of the C-DM, U-DM and GF-DM groups, respectively (n = 5 per group). (C) Representative immunofluorescence staining of BK-β1 subunits in rat coronary arteries of three groups (n = 3 per group) (Scale bar = 50 μm). (D-E) The mRNA levels of BK-α and BK-β1, β-actin was used as an internal control to normalize differences in the amount of total RNA in each sample (n = 5 per group). (F-—H) The protein expressions of BK-α and BK-β1 in rat coronary arteries of three groups (n = 4 per group). Western data were normalized to α-tubulin protein expression levels. $^{*}P < .05$, $^{**}P < .01$.

DM + NaCl group. In order to further determine the effect of ROS on glucose-fluctuation-induced BK-β1 degradation, NAC (1 mmol/L) was applied in human coronary arterial SMCs. After the addition of NAC, the nucleation of p65 and the MuRF1 expression detected through immunofluorescence staining decreased in the GF + NAC group, and the protein expression of BK-β1 increased in the three groups, respectively (Fig. 6J-M).

3.3. Regulation of BK-β1 expression by MuRF1 in cultured human coronary arterial SMCs with glucose fluctuations

There were no significant differences in BK-α protein expression among the three groups; however, BK-β1 protein expression decreased significantly in human coronary arterial SMCs after exposure to glucose fluctuations (Fig. 3A-C). Fig. 3D-E show that the MuRF1 expression increased in the HG group compared with that in the NG group and to a greater extent in the GF group. As shown in Fig. 3F, the immunofluorescence staining of MuRF1 in human coronary arterial SMCs was mostly nuclear and faint cytoplasmic located. The expression of MuRF1 increased significantly in the GF group, together with a slight increase in the cytosol. In order to further elucidate the role of MuRF1 in the regulation of BK-β1 expression, MuRF1 shRNA was transfected into the human coronary arterial SMCs. After transfection, BK-β1 protein expression increased in the three groups of human coronary arterial SMCs (Fig. 3G-I).

Table 1
Physiological findings and Insulin levels.

	C-DM	U-DM	GF-DM
BW (g)	397.83 ± 7.90	327.83 ± 6.00**	308.33 ± 2.93**‡
HW (g)	1.30 ± 0.03	1.09 ± 0.03**	1.01 ± 0.03**
HW/BW (mg/g)	3.29 ± 0.04	3.31 ± 0.08	3.29 ± 0.08
Insulin (pg/mL)	273.40 ± 45.00	31.54 ± 5.69**	31.76 ± 11.21**

n = 6 for each group. Data are presented as mean ± SEM.
BW, body weight; HW, heart weight; HW/BW, heart-to-body weight ratio.
$^{*}P < .05$ vs. C-DM. $^{**}P < .01$ vs. C-DM. $^{\dagger}P < .05$ vs. U-DM. $^{\ddagger}P < .01$ vs. U-DM.

3.4. Regulation of BK-β1 and MuRF1 expression by NF-κB in cultured coronary arterial SMCs with glucose fluctuations

In order to determine the role of glucose fluctuations in the activation of NF-κB, we examined phosphorylated NF-κB/p65 expression in human coronary arterial SMCs. Compared with the NG group, phosphorylated NF-κB/p65 expression increased in the HG group, and was more pronounced in the GF group (Fig. 4A-B). In order to evaluate the quantity of NF-κB p65 entering the nucleus, immunofluorescence assays were performed. As shown in Fig. 4C, the nucleation of NF-κB p65 was obvious in human coronary arterial SMCs with fluctuations in glucose concentrations. In order to further investigate the role of NF-κB in glucose fluctuation-induced BK-β1 degradation, the IKK inhibitor,

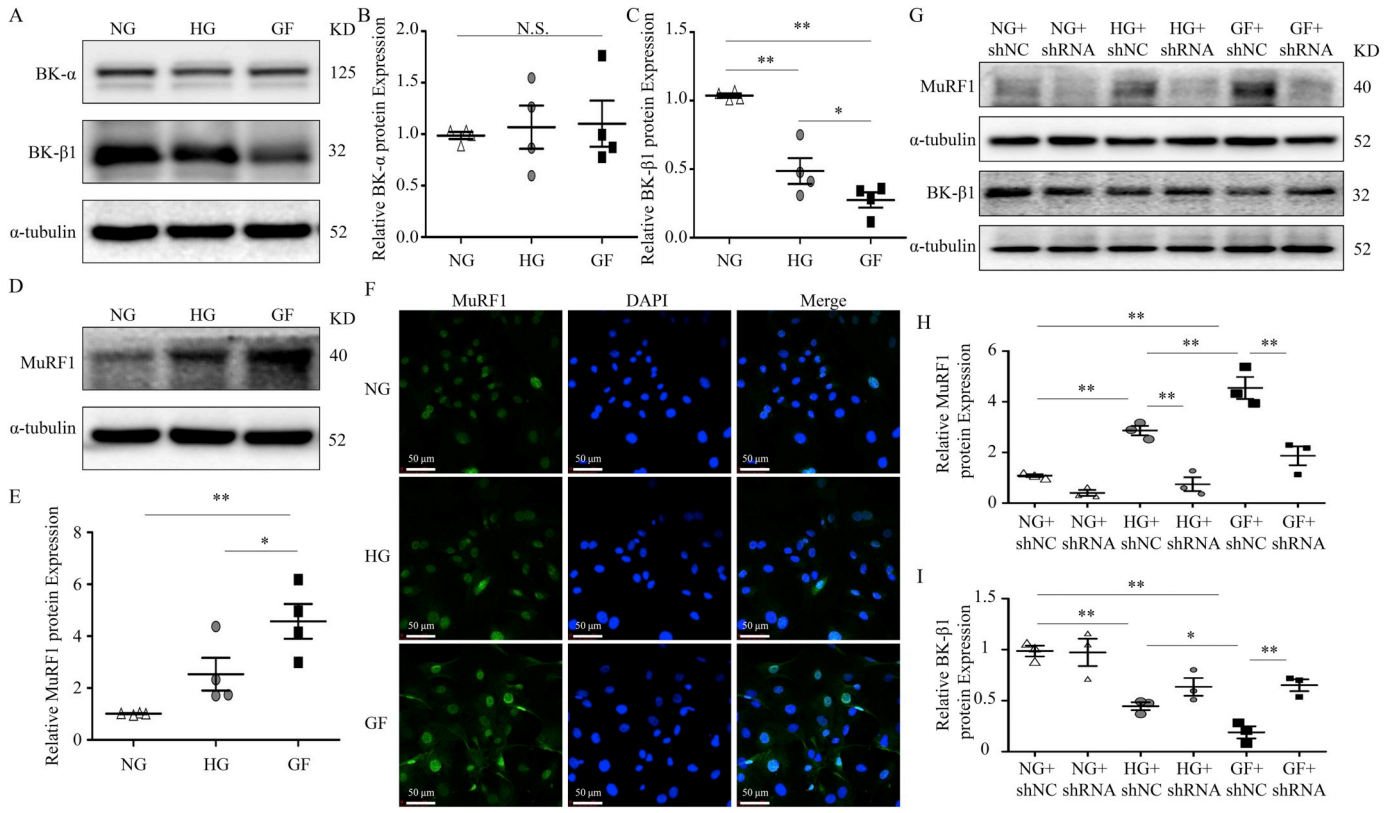

Fig. 3. Regulation of MuRF1 in glucose-fluctuations-mediated BK-β1 degradation in vitro (A-C) The protein expressions of BK-α and BK-β1 in human coronary arterial SMCs in the NG, HG and GF groups (n = 4 per group). (D-E) The protein expression of MuRF1 in human coronary arterial SMCs of three groups (n = 4 per group). (F) The immunofluorescence staining of intracellular MuRF1 in human coronary arterial SMCs visualized by confocal microscopy (n = 3 per group) (Scale bar = 50 μm). (G-I) The protein expressions of BK-β1 and MuRF1 were measured after the human coronary arterial SMCs were transfected with shRNA targeting MuRF1 (MOI = 50), and cultured with DMEM of normal glucose concentration or high glucose concentration or normal/high glucose concentrations for 72 h (n = 3 per group). Western data were normalized to α-tubulin protein expression levels. $^{*}P < .05$, $^{**}P < .01$.

TPCA-1 (0.5 μmol/L), was used. We found that the protein expression of phosphorylated NF-κB/p65 and MuRF1 decreased in the three groups of human coronary arterial SMCs after the application of TPCA-1, while the protein expression of BK-β1 increased in the three groups (Fig. 4 D-G).

3.5. Regulation of BK-β1, MuRF1 and NF-κB expressions by PKCα in cultured human coronary arterial SMCs with glucose fluctuations

Fig. 5A-B show that PKCα protein expression increased significantly in human coronary arterial SMCs with glucose fluctuations. In order to further investigate the effect of PKCα on glucose fluctuation-induced BK channel dysfunction, Go6976 (0.5 μmol/L), a PKCα inhibitor, was used. As shown in Fig. 5C-I, the application of Go6976 decreased PKC expression, and the protein expressions of phosphorylated NF-κB/p65 and MuRF1 decreased in the three groups of human coronary arterial SMCs after the application of Go6976. Meanwhile, the protein expression of BK-β1 increased in the three groups.

3.6. Regulation of BK-β1, MuRF1, NF-κB expressions by ROS

The blood glucose levels and the body weights of rats in the GF-DM + NaCl and GF-DM + NAC groups are shown in Fig. S2. There were no significant differences in body weights, heart weights, heart-to-body weight ratios and plasma insulin levels among the two groups (Fig. S2). As shown in Fig. 6A, the MDA level increased in the U-DM group (11.81 ± 0.84 nmol/mL) compared with that of the C-DM group (6.80 ± 0.58 nmol/mL), and was more pronounced in the GF-DM group (15.56 ± 0.38 nmol/mL). While, the MDA level decreased in GF-DM + NAC group (10.98 ± 0.79 nmol/mL). The fluorescent dye CM-H2DCFPA was applied directly to assess ROS production. Fig. 6B shows that ROS production increased significantly in human coronary SMCs of the GF group. Fig. 6C-D show the representative traces of vascular tension in rats with glucose fluctuations. After incubation with 100 nmol/L IBTX, Δ tension (% Max) increased in the GF-DM + NAC group (48.90 ± 10.85), compared with that of the GF-DM + NaCl group (8.86 ± 1.09). Fig. 6E-F show the total K^+ currents in the coronary arterial SMCs at the baseline and after exposure to 100 nmol/L IBTX. The current densities of BK channels increased in the GF-DM + NAC group (58.79 ± 9.41 pA/pF), compared with those of the GF-DM + NaCl group (26.04 ± 5.50 pA/pF). Moreover, the protein expressions of MuRF1 decreased in the GF-DM + NAC group. Meanwhile, the protein expression of BK-β1 increased in the GF-DM + NaCl group. In order to further determine the effect of ROS on glucose-fluctuation-induced BK-β1 degradation, NAC (1 mmol/L) was applied in human coronary arterial SMCs. After the addition of NAC, the nucleation of p65 and the MuRF1 expression detected through immunofluorescence staining decreased in the GF + NAC group, and the protein expression of BK-β1 increased in the three groups, respectively (Fig. 6J-M).

4. Discussion

4.1. Main findings

Diabetes mellitus has two main kinds of diabetes, type 1 DM (T1DM) and type 2 DM (T2DM). T1DM accounts for 5% to 10% of all cases of diabetes, while T2DM accounts for 90% [22]. Both T1DM and

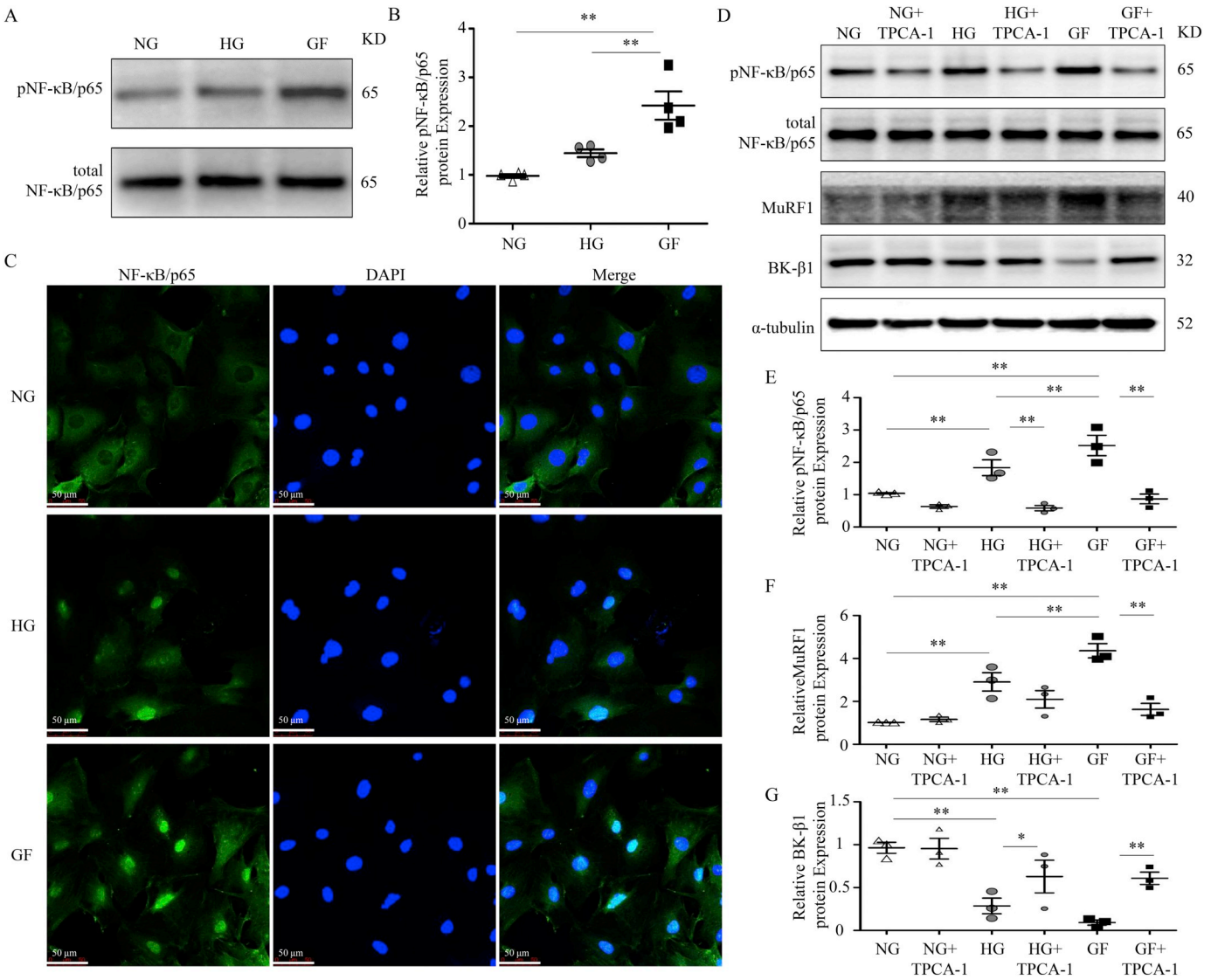

Fig. 4. Regulation of NF-κB in glucose-fluctuations-mediated MuRF1-induced BK-β1 degradation (A-B) The phosphorylation levels of NF-κB/p65 in human coronary arterial SMCs of three groups were normalized to total NF-κB/p65 protein levels (n = 4 per group). (C) The immunofluorescence staining of NF-κB/p65 nucleation in human coronary arterial SMCs of three groups (n = 3 per group) (Scale bar = 50 μm). (D-G) The phosphorylation level of NF-κB/p65 and the protein expressions of BK-β1 and MuRF1 were measured after human coronary arterial SMCs were incubated for 72 h in DMEM containing 5.5 mmol/L glucose, 25 mmol/L glucose, or 5.5 mmol/L glucose alternating with 25 mmol/L glucose in the absence or presence of TPCA-1 (0.5 μmol/L) (n = 3 per group). TPCA-1 was added at the beginning and remained throughout the experiments. Western data were normalized to α-tubulin protein expression levels. $^{*}P < .05$, $^{**}P < .01$.

T2DM contribute to a growing incidence of cardiovascular complications, such as: atrial fibrillation, sudden cardiac death, coronary heart disease and heart failure [23]. Recently, more and more studies have been reported that glucose fluctuation may be an important risk factor which needs more attention leading to the development of coronary heart diseases in diabetes [24,25]. Furthermore, a number of studies have been conducted to explore various mechanisms underlying the negative effects of glucose fluctuation on both animal and cell models with T1DM and T2DM. It has been reported that glucose fluctuation in T2DM aggravates inflammatory and oxidative stress, causing vascular endothelial injury, vasculopathy, and other diabetic complications [26,27].

Blood glucose fluctuations have been reported to be related to coronary diseases [2,28]. However, the precise molecular mechanisms are not fully elucidated. To the best of our knowledge, this is the first study that has investigated the molecular mechanisms of BK channel dysfunction resulting from glucose fluctuations in T1DM rats. The main findings of this study are: (i) glucose fluctuations aggravate coronary arterial dysregulation caused by BK channel impairment; (ii) glucose fluctuations aggravate BK channel dysfunction via the ROS overproduction and PKCα/NF-κB/MuRF1 signaling pathway.

4.2. Glucose fluctuations aggravate the impairment of BK channels due to MuRF1-induced BK-β1 degradation

BK channels are widely expressed in coronary arteries and play an important role in the regulation of coronary arterial SMC membrane potentials and smooth muscle tone [29]. However, a large body of research has demonstrated that vascular BK channel function is impaired along with a decrease in BK-β1 expression in diabetes [19,30]. In this study, we found that coronary artery function was significantly impaired along with a reduction of BK channel current density in diabetic rats after three weeks of glucose fluctuations. Furthermore, BK-β1 protein expression decreased in response to glucose fluctuations both in vivo and in vitro. These results indicated that the decrease in BK-β1 protein expression was responsible for the reduction in BK channel

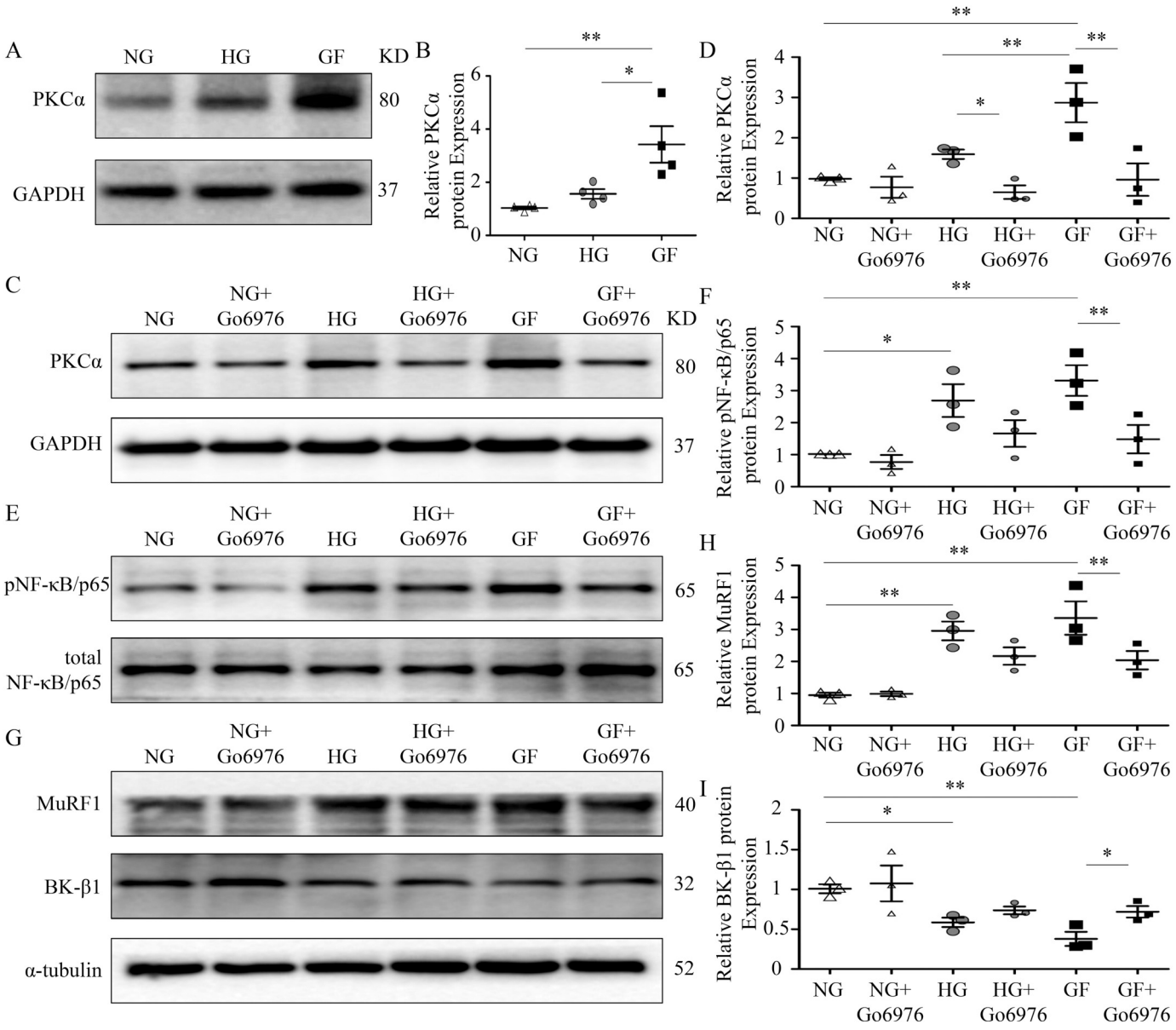

Fig. 5. Regulation of PKCα in NF-κB-involved MuRF1-induced BK-β1 degradation (A-B) The protein expression of PKCα in three groups of human coronary arterial SMCs (n = 4 per group). (C-—I) Human coronary arterial SMCs were incubated for 72 h in DMEM containing 5.5 mmol/L glucose, 25 mmol/L glucose, or 5.5 mmol/L glucose alternating with 25 mmol/L glucose in absence or presence of Go6976 (0.5 μmol/L). Go6976 was added at the beginning and remained throughout the experiments. Then, the phosphorylation level of NF-κB/p65 and the protein expressions of BK-β1 and MuRF1 were measured (n = 3 per group). Western data were normalized to α-tubulin or GAPDH protein expression levels. $^{*}P < .05$, $^{**}P < .01$.

current densities, which finally led to coronary artery dysfunction in diabetic rats with glucose fluctuations.

There is a balance in BK-β1 subunits maintained by BK-β1 protein synthesis and degradation [30]. It has been reported that MuRF1, a muscle specific E3 ligase, plays a pivotal role in BK-β1 degradation in high fat diet-induced diabetic mice [13]. In addition, the upregulation of MuRF1 contributes to BK-β1 protein degradation via the ubiquitin-proteasome system (UPS) in diabetic vessels [31]. The UPS, which includes ubiquitin-activation enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-protein ligase E3, functions as an important regulator of various types of cellular processes [32]. In this study, we found that BK-β1 protein expression decreased together with the increase in MuRF1 protein expression. As shown in immunofluorescence staining of MuRF1, there was a slight increase of MuRF1 expression in the cytosol. In addition, after the transfection of MuRF1 shRNA, the negative effects on BK-β1 were reversed. However, the mRNA level of BK-β1 was not altered after exposure to different glucose concentrations. These results indicated that there may be an underlying mechanism by which glucose fluctuations decrease the expression of BK-β1 through the activating MuRF1 and UPS-involved degradation.

4.3. NF-κB plays an important role in glucose-fluctuation-related MuRF1-induced decrease in BK-β1 expression

NF-κB belongs to a ubiquitous family of several transcription factors and participates in the regulation of cellular functions [10]. Li et al. [33] demonstrated that the activation of NF-κB could be the upstream signaling mechanism that causes the upregulation of MuRF1 expression, which leads to BK-β1 degradation in coronary arterial SMCs. In addition, previous studies have reported that the activation of NF-κB is obviously enhanced in rats with glucose fluctuations [2,15]. In this study, we found that the phosphorylation level of NF-κB/p65 increased

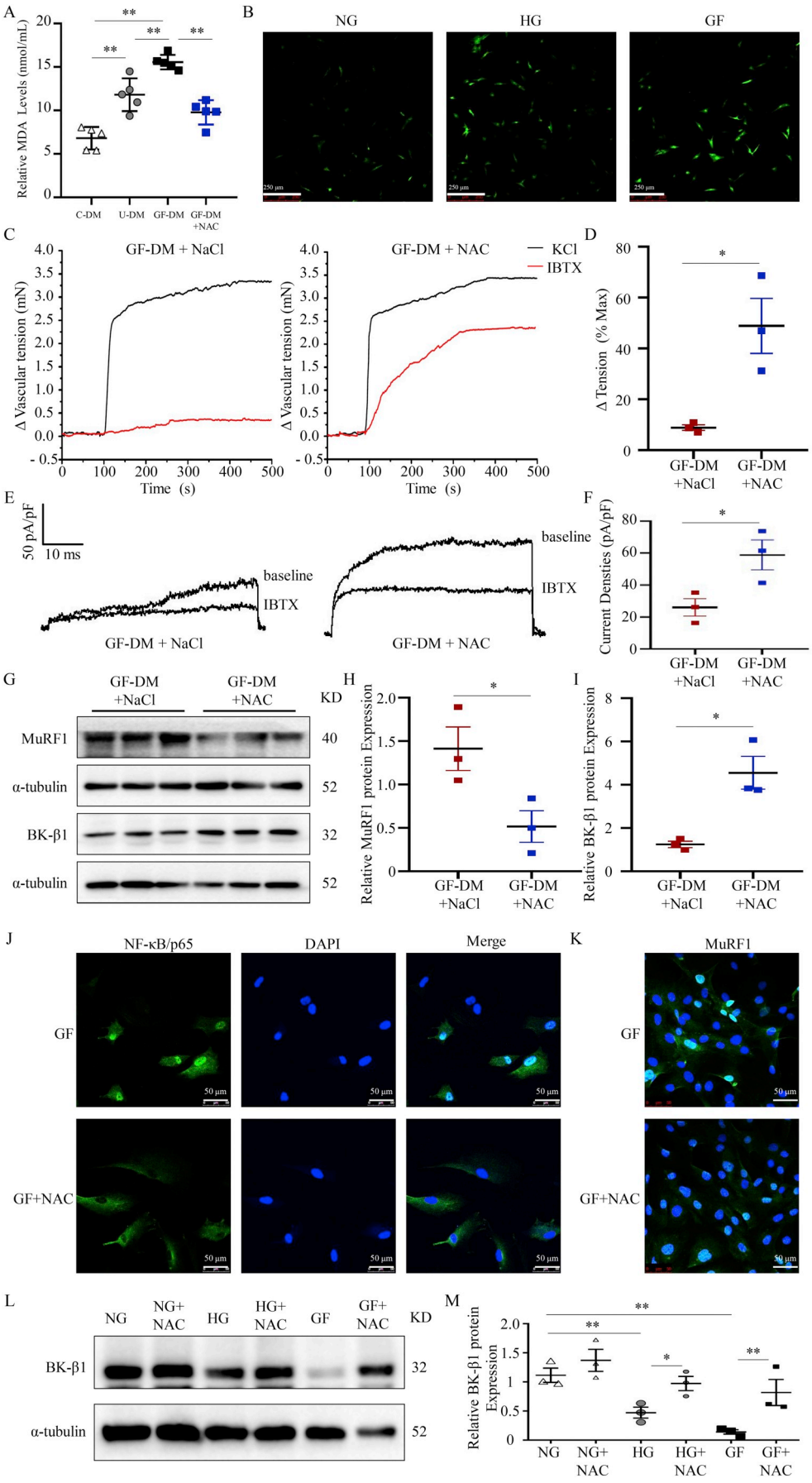

Fig. 6. Regulation of ROS in glucose-fluctuation-mediated MuRF1-induced BK-β1 degradation (A) Relative rat plasma MDA levels (nmol/mL) in the C-DM, U-DM, GF-DM and G-DM + NAC groups, respectively (n = 5 per group). (B) The ROS levels in the human coronary arterial SMCs incubated for 72 h in DMEM containing 5.5 mmol/L glucose, 25 mmol/L glucose, or 5.5 mmol/L glucose alternating with 25 mmol/L glucose (n = 3 per group) (Scale bar = 250 μm). (C-D) Representative tracings of vascular tensions from the GF-DM + NaCl and GF-DM + NAC groups after using 60 mmol/L KCl. Coronary artery tensions in the two groups were increased after using 100 nmol/L IBTX (n = 3 per group). (E-F) Representative tracings showing current density-voltage relationship of BK channels in rat coronary arterial SMCs of the GF-DM + NaCl and GF-DM + NAC groups (n = 3 per group). (G-I) The protein expressions of MuRF1 and BK-β1 in rat coronary arteries of the GF-DM + NaCl and GF-DM + NAC groups (n = 3 per group). (J) The immunofluorescence staining of NF-κB/p65 nucleation in human coronary arterial SMCs of the GF and GF + NAC groups (n = 3 per group) (Scale bar = 50 μm). (K) The immunofluorescence staining of intracellular MuRF1 in human coronary arterial SMCs of the GF and GF + NAC groups (n = 3 per group) (Scale bar = 50 μm). (L-M) Human coronary arterial SMCs were incubated for 72 h in DMEM containing 5.5 mmol/L glucose, 25 mmol/L glucose, or 5.5 mmol/L glucose alternating with 25 mmol/L glucose in absence or presence of NAC (1 mmol/L). NAC was added at the beginning and remained throughout the experiments. Then, the protein expression of BK-β1 was measured (n = 3 per group). Western data were normalized to α-tubulin protein expression levels. $^{*}P < .05$, $^{**}P < .01$.

in human coronary arterial SMCs of the HG group compared with that of the NG group, and was even more pronounced in the GF group. Moreover, immunofluorescence staining and confocal microscopy results showed that NF-κB/p65 nucleation increased significantly in human coronary arterial SMCs cultured under normal/high glucose concentrations. In order to determine the role of NF-κB in the glucose fluctuation-mediated reduction of BK-β1 expression, TPCA-1 was used. TPCA-1, an inhibitor of IKK-2, inhibits NF-κB/p65 nucleation by preventing IκB dissociating from NF-κB/p65, decreasing the phosphorylation of IκB. Our results showed that inhibition of NF-κB/p65 decreased MuRF1 expression and reversed BK-β1 downregulation, indicating that NF-κB is involved in the regulation of glucose fluctuation-mediated MuRF1-induced BK-β1 degradation.

4.4. Glucose fluctuations aggravate NF-κB related MuRF1-induced BK-β1 degradation by activating PKCα

The PKC family consists of at least eleven isoenzymes, including conventional PKCs (α, β1, β2, γ), and are involved in the regulation of cellular functions [16]. Moreover, PKCα has been reported to be activated in response to excessive oxidative stress and regulates the activation of NF-κB [18]. In order to further clarify the role of PKCα in BK channel dysfunction induced by glucose fluctuation, the inhibition of PKCα was found to have caused a decline in PKCα and MuRF1 expressions and was reversed the reduction in BK-β1 expression. Collectively, our study suggested that PKCα was involved in the regulation of glucose fluctuation-mediated MuRF1-induced BK-β1 degradation.

4.5. ROS plays an important role in glucose-fluctuation-related MuRF1-induced decrease in BK-β1 expression

The major sources of ROS production are regarded as including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, uncoupled nitric oxide synthases, xanthine oxidase, lipoxygenase and cyclooxygenase [34]. Excessive oxidative stress as a result of ROS overproduction is involved in various pathological processes by enhancing the oxidation of DNA, lipid membranes and proteins [35]. It has been reported that ROS generation increased significantly when blood glucose fluctuated [36]. MDA is a widely accepted biomarker of lipid peroxidation caused by ROS overproduction [37]. Consistent with previous studies, our results showed that the MDA level increased significantly in the GF-DM group rats and the ROS generation enhanced significantly in the GF group as shown by the results of the fluorescence assay. In order to further explore the effects of ROS in BK channel dysfunction induced by glucose fluctuations, the ROS inhibitor NAC was used both in vivo and in vitro. Vasodilation dysfunction and BK channel dysfunction were reversed in the GF + NAC group rats, compared with those in the GF + NaCl group rats. Moreover, after the application of NAC in human coronary arterial SMCs treated with fluctuated glucose concentrations, the quantity of NF-κB p65 entering the nucleus decreased, while the MuRF1-involved BK-β1 degradation was reversed.

NADPH oxidase is considered as a complex protein composed of multisubunits. NOX, as the catalytic subunit of NADPH oxidase, binds with membrane-bound p22phox to a stable state and can be activated by assembling with cytosolic components [38]. Cytosolic components have been reported to be phosphorylated by several kinases including PKC (α, β2, δ, σ), p38 MAPK, and ERK1/2, subsequently leading to the activation of the oxidase and ROS overproduction [39]. Furthermore, excessive ROS generation can be a vital trigger factor in the activation of various kinds of downstream signaling pathways, including PKC [34,40]. Collectively, there are complex feedback mechanisms between the activation of PKC and the generation of ROS. In our study, glucose fluctuations resulted in increased ROS generation and PKCα expression. However, the relationship between ROS and PKCα and their mediated regulation on BK channel function in response to glucose fluctuations need further studies.

4.6. The limitations of the study

There are at least three limitations in this study. First, this study was only performed in STZ-induced T1DM rats. It is not clear whether these findings can be extrapolated to T2DM which account for 90% of the clinically seen diabetes in patients, which is the most significant limitation and more experimental studies should be conducted on T2DM in the future. Second, in this study, we detected the ROS generation and the PKCα expression without clarifying the relationship between them and their mediated regulation on BK channel function under glucose fluctuated condition, which is also an important limitation and needs further studies. Third, our results demonstrate that there were significant differences in the sensitivity of coronary to IBTX-induced relaxation between the U-DM and GF-DM groups, without any differences in the sensitivity to SNP. As is well known, SNP functions as a vasodilator to cause the relaxation of artery via a release of nitric oxide [41]. Moreover, several studies have reported that the vasodilatation effects of SNP can be weakened by BK channel inhibitor IBTX, which indicates BK channel may influence the SNP induced relaxation of the vascular smooth muscle [42]. Thus, the differences between the coronary sensitivity to SNP and IBTX may be regulated by many other factors, and which needs to be further clarified. This is also a limitation in our study.

4.7. The clinical significances of the study

Recent clinical studies have been verified that glucose fluctuations are more harmful than constant hyperglycemia in both T1DM and T2DM patients, contributing to various kinds of microvascular and macrovascular complications. Glucose fluctuation has been reported to increase the incidence of chronic kidney diseases by promoting renal fibrosis and thickening capillary basal membrane [43], accelerate the development of diabetic retinopathy by aggravating the neurodegeneration and retina structural damage [44], and lead to serious coronary heart diseases by gradually triggering coronary plaque accumulation rupture [45]. Our study has demonstrated that blood glucose fluctuations aggravated the dysfunction of coronary vasodilatation by promoting BK channel dysregulation in T1DM rats, thus better monitoring and controlling of blood glucose in diabetic patients seems to be an important part in daily clinical treatment. Continuous glucose monitoring systems have been increasingly used to monitor glucose fluctuations in insulin-requiring diabetic patients [46], and help patients in regulating their lifestyle and medication adjustments [47], which improves glycaemic stability for patients with T1DM and T2DM [48,49]. Moreover, our study demonstrated that glucose fluctuations aggravated BK channel dysfunction via ROS overproduction and PKCα/NF-κB/MuRF1 signaling, thus targeting on the signaling pathway may provide a new potential treatment strategy in the future.

5. Conclusion

In conclusion, our study demonstrated that glucose fluctuations promoted NF-κB-related MuRF1-induced BK channel dysfunction through the activation of PKCα and excessive oxidative stress. Based on these results, glucose fluctuations may be a highly important risk factor for the development and progression of coronary diseases. Thus, better monitoring and controlling of blood glucose as well as targeting on the signaling pathway may provide a novel theory and treatment strategy to prevent and treat diabetic coronary diseases.

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Author contributions

R-XW was involved in the experiment design. Z-YZ, NW, L-FM, S-PD

and YW performed the experiments. L-LQ, X-YL and X-YL analyzed the data. R-XW and Z-YZ wrote the manuscript. XM, QC, MP and T-YL edited the manuscript. All authors read and approved the final manuscript.

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Declaration of Competing Interest

None declared.

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