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Sodium butyrate alleviates high-glucose-induced renal glomerular endothelial cells damage via inhibiting pyroptosis

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ABSTRACT

We recently found that Sodium butyrate (NaB) possesses anti-inflammatory effects in diabetic nephropathy (DN) mouse model and in high-glucose induced mouse glomerular mesangial cells. Pyroptosis is a programmed cell death accompanied with the release of pro-inflammatory factors. Gasdermin D (GSDMD) is a novel discovered pivotal executive protein of pyroptosis, which can be cleaved by inflammatory caspases. The aim of our study is to verify if NaB have some effects against high-glucose induces pyroptosis in renal Glomerular endothelial cells (GECs). For this aim, human GECs were cultured and exposed to high-glucose. Exogenous NaB, caspase 1 inhibitor Ac-YVAD-CMK (A-Y-C) or knockdown GSDMD by siRNA were used. We found high glucose could increase Propidium Iodide (PI) positive cells and elevate release of lactate dehydrogenase (LDH), Interleukin 1 beta (IL-1β) and Interleukin 18 (IL-18); protein levels of GSDMD, GSDMD N-terminal domain (GSDMD-N) and cleaved-caspase-1 were also elevated. Effect of NaB on LDH release and PI positive cells was further enhanced by inhibiting caspase 1-GSDMD. In addition, high glucose-induced nuclear factor kappa-B (NF-κB)/NF-κB inhibitor α (IκB-α) signaling pathway was reversed by NaB or A-Y-C administration. In conclusion, NaB could ameliorate high-glucose induced GECs via caspase1-GSDMD canonical pyroptosis pathway; and NF-κB/IκB-α signaling pathway was involved in it.

1. Introduction

The chronic micro-inflammation [1–4] and cell death [5,6] induced by high glucose are two major factors contributing to the progression of diabetic nephropathy (DN). As the first layer of the glomerular filtration barrier, glomerular endothelial cells (GECs) damage often occurs in the early stage of DN [7–9].

Pyroptosis is a highly inflammatory form of programmed cell death that is associated with pathogenesis of many chronic inflammatory diseases [10–13]. Studies found pyroptosis was accompanied with the release of pro-inflammatory factors such as interleukin-1β (IL-1β), interleukin-18 (IL-18) and lactate dehydrogenase (LDH) [14]. As both diabetes and diabetic nephropathy have been recognized as low grade inflammatory disease, the relationship between pyroptosis and DN has attracted much attention [15]. However, underlying mechanism of pyroptosis during the development of DN is still not fully understood, especially for influencing function of GECs.

Butyric acid is a short-chain fatty acid produced by the intestinal flora within the gut lumen [16]. Our previous study found that its administration can decrease high-glucose-induced expression of IL-1β in mesangial cells and ameliorate development of DN [17]; moreover, sodium butyrate (NaB) oral administration showed therapeutic effects of diabetic mice on reducing inflammatory cytokines and ameliorating kidney function [18]. Some reports had demonstrated that NaB improved aortic endothelial dysfunction in diabetic rats and decreased endothelium oxidative stress damage [19,20]. Furthermore, endothelial cells pyroptosis had been observed in both traumatic brain injury (TBI)-induced lung injury and atherosclerosis models [21,22]. However, mechanism of NaB against DN is still not fully understood. Evidence from reports and our previous findings suggests that pyroptosis may

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participate in development of DN, and NaB might ameliorate GECs and kidney function via inhibiting pyroptosis. For this aim, we designed this study. The present study may supply novel understanding and strategy on administration of DN.

2. Materials and methods

2.1. Materials

Human renal Glomerular endothelial cells (GECs) and Endothelial cell medium (ECM) were obtained from ScienCell (San Diego, USA). NaB was purchased from GBC BIOtechnology (Guangzhou, China). Glucose was purchased from Chemical reagent factory (Guangzhou, China). Mannitol was obtained from Kelun industry group (Sichuan, China). Primary antibodies against Gasdermin D (GSDMD) or GSDMD N-terminal domain (GSDMD-N) were derived from Cell Signaling Technology (Trask Lane Danvers, USA); caspase-1 was purchased from Abcam (Cambridge, England). Antibodies for nuclear factor kappa-B (NF-κB) P65, phosphorylated NF-κB (p-NF-κB), NF-κB inhibitor α (IκB-α) and phosphorylated IκB-α (p-IκB-α) were obtained from Santa Cruz (Dallas, Texas, USA), and β-actin were purchased from Beyotime Biotechnology (Shanghai, China). FITC-conjugated secondary antibody (Goat Anti-Rabbit IgG) and Cy3-conjugated secondary antibody (Goat Anti-Mouse IgG) were supplied by Boster Biotechnology Co (Wuhan, China). ELISA kits for human IL-1β and IL-18 were obtained from Andy gene (Beijing, China). LDH assay kit was obtained from Jiancheng (Nanjing, China). Caspase-1 inhibitor Ac-YVAD-CMK (A-Y-C), Propidium Iodide (PI) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich (Taufkirchen, Germany). The GSDMD small interfering RNA (siRNA) and control-siRNA were obtained from RiboBio (Guangzhou, China). All the other reagents were obtained from commercial sources.

2.2. Cell culture

GECs were incubated in ECM containing 5.6 mmol/L glucose and 5% fetal bovine serum, 1% penicillin-streptomycin and 5% endothelial Cell Growth Supplement at 37 °C and 5% CO_2 in the cell culture incubator.

GECs were exposed to high glucose to establish disease model in vitro; Mannitol (30 mM) was used as an osmotic pressure control of high glucose; exogenous sodium butyrate (5 mM) was used as an intervention reagent; and then caspase 1 inhibitor Ac-YVAD-CMK (40 μM) pre-treated or silencing GSDMD by Small interfering RNA (siRNA) was used to inhibit Caspase 1-GSDMD pyroptosis pathway on GECs.

2.3. Small interfering RNA transfection

GECs were transfected with GSDMD-siRNA (100 nM, Target sequence: GCAGGAGCTTCCACTTCTA) or control siRNA (100 nM) at 37 °C for 24 h according to the manufacturer's protocol. siRNA-mediated knockdown of GSDMD cells and control siRNA-mediated cells were then stimulated by 30 mM glucose or were incubated in 30 mM glucose and NaB co-treatment for 24 h. Finally, the cells and culture supernatant were collected for WB, ELISA or PI staining.

2.4. Propidium iodide (PI) staining

GECs were cultured in 6-well plates for indicated treatments. Twenty-four hours later, cells were harvested and re-suspended in PBS containing 2.5 mg/mL PI for 20 min at room temperature. Finally, PI positive cells were detected using a flow cytometer (BD FACSAria III) and analyzed by flowjor 10.0 software (FlowJo LLC, U.S.A).

2.5. MTT assay for cell viability

GECs were seeded into 96-well cell culture plate at concentrations of 1×10^5 cells/mL. Cells were treated with different concentrations of NaB (0.1–20 mM) for 24 h. After that, ECM complete growth medium containing 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the culture system. Four hours later, 10% SDS (Sodium dodecyl sulfate) was applied to dissolve formazan crystals. Cell viability was measured at 595 nm absorbance by a plate reader (Molecular Devices, USA). ECM complete growth medium was used as normal glucose control (NG group).

2.6. Immunofluorescence

GECs were grown on coverslips in 6-well plates. After adhesion, cells were incubated as described above, fixed in 4% paraformaldehyde, and blocked with 5% bovine serum albumin (BSA). After being gently washed with PBS, the cells were incubated with the primary antibodies including GSDMD (1:200), NF-κB P65 (1:200), p-NF-κB p65 (1:200), IκB-α (1:200), and p-IκB-α (1:200) overnight at 4 °C, and then incubated with secondary antibodies conjugated with Fluorescein isothiocyanate (FITC) or cyanine dye 3 (Cy3) for 1 h at room temperature. 4′ 6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Finally, cells were observed under a confocal microscope (Leica, Germany). Relative expressions of proteins were analyzed by Image-J 6.0 software (National Institutes of Health, U.S.A).

2.7. Western blotting (WB) analysis

Total proteins from cells were lysed using RIPA buffer and the protein concentrations in the cell lysates were assayed by a protein assay dye reagent concentrate (Bio-Rad, USA). Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (pore size 0.45 μm). After being blocked with 5% BSA for 1 h, membranes were incubated with primary antibodies including GSDMD (1:1000), GSDMD-N (1:1000), caspase-1 (1:500), NF-κB P65 (1:1000), p-NF-κB p65 (1:1000), IκB-α (1:1000), p-IκB-α (1:1000) and β-actin (1:1000) at 4 °C overnight; after being washed with TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, the protein bands were visualized by ECL from Santa Cruz (Dallas, Texas, USA) and analyzed by Image-J software.

2.8. ELISA assay

GECs were seeded in 24-well plates. The levels of IL-1β, IL-18 and LDH in the supernatant were determined by kits according to the manufacturers' instruction.

2.9. Statistical analysis

Data were presented as mean ± SD and were analyzed by SPSS 19.0. Comparison between the two groups was analyzed using the student's *t*-test, the differences among multiple groups was analyzed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered as statistical significant.

3. Results

3.1. High glucose triggers pytoptosis in GECs

Pyroptosis is one of a form of cell death. To explore the relationship between high glucose and pyroptosis in GECs, expression of GSDMD, a specific marker of pytoptosis, was determined. As shown in Figs. 1A–D and 2, level of GSDMD was significantly increased in a time- and concentration-dependent manner (10, 20, 30 mM), and its expression

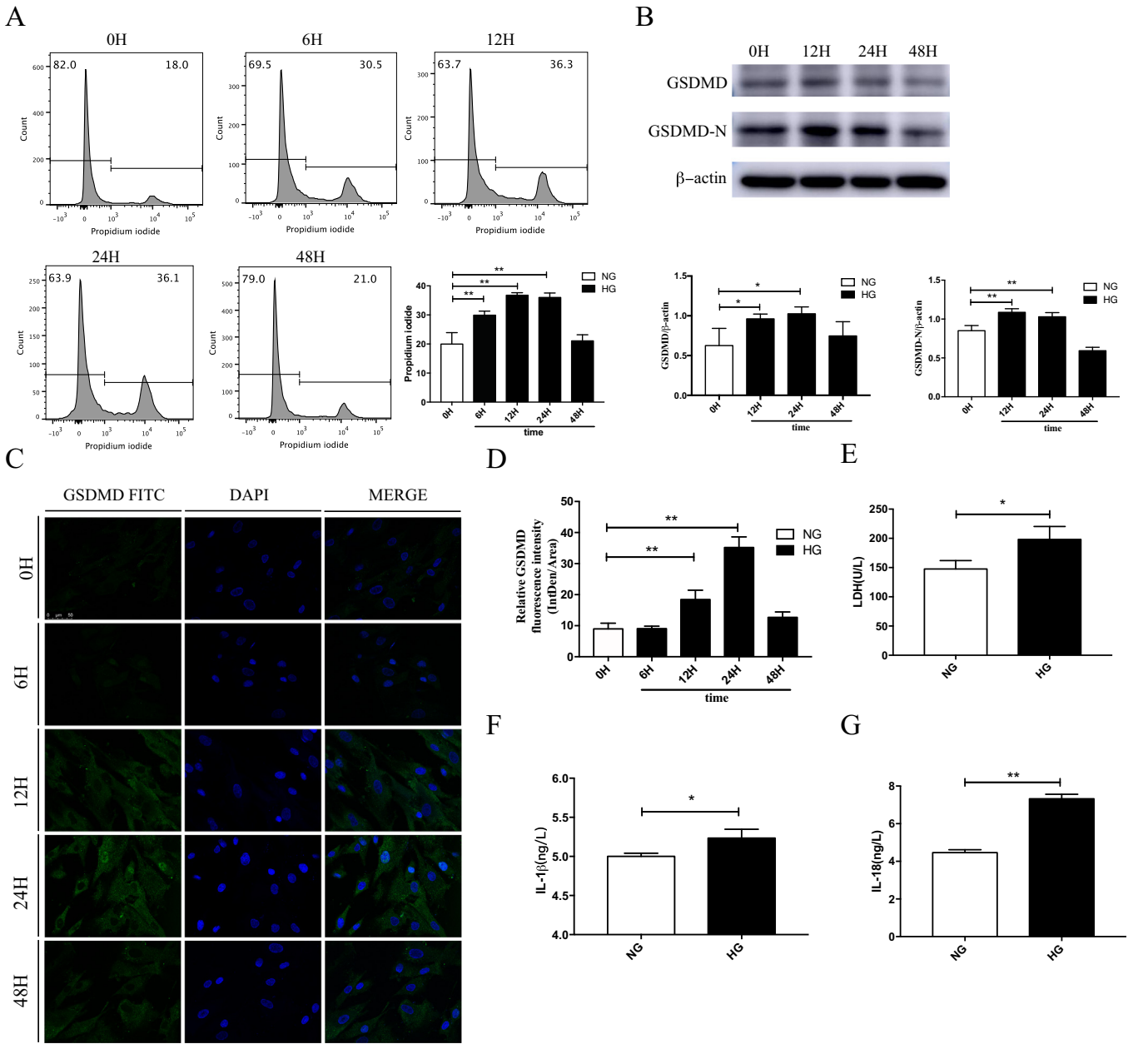

Fig. 1. High glucose-induced pytoptosis in renal glomerular endothelial cells. (A) PI-positive cells were detected by a flow cytometer in GECs were incubated with 30 mM high glucose (HG) for different times, pytoptosis were detected by (A) PI-positive, (B) western blotting, and (C–D) immunofluorescence assay (magnification: ×800). Levels of (E) LDH, (F) IL-1β and (G) IL-18 in culture supernatant were detected by ELISA method at 24 h. *$p < 0.05$, **$p < 0.01$.

reached a peak when cells were incubated with 30 mM high-glucose at 24 h. There was no difference in GSDMD expression between the 30 mM mannitol osmotic control group and the 30 mM high glucose group. Furthermore, the release of LDH, IL-1β and IL-18 were significantly increased when GECs were incubated with high-glucose (30 mM) at 24 h compared with NG group (Fig. 1E–G).

3.2. GSDMD is essential for the high glucose-induced pyroptosis in GECs

To investigate the role of GSDMD in 30 mM high-glucose-induced pyroptosis, GSDMD was knocked-down by siRNA. Our results showed that the protein levels of both GSDMD and GSDMD-N were significantly decreased by si-GSDMD compared with control (Fig. 3A–B). Meanwhile, the release of LDH (Fig. 3C), IL-1β (Fig. 3D) and IL-18 (Fig. 3E) were significantly decreased by si-GSDMD treatment. These figures suggested that GSDMD plays a pivotal role in high-glucose-induced pyroptosis in GECs.

3.3. Sodium butyrate (NaB) inhibits high glucose-induced caspase1-GSDMD canonical pyroptosis pathway in GECs

In our previous study, we found NaB, a microbiota metabolite, could ameliorate DN [23]. Here we'd like to know its role in pyroptosis. Consistent with above findings that high glucose increased GSDMD and GSDMD-N protein levels (Fig. 1B), we observed here that cleaved-caspase 1 could also be induced by high glucose at 12 h and 24 h (Fig. 5A). To initially evaluate influence of NaB on cell viability, a MTT assay was carried out. We found 0.1–5 mM NaB could promote cell viability (Fig. 5B), and decrease high-glucose induced protein expression of GSDMD in a dose-dependent manner (Fig. 5C–D). Converging from our previous report [17,23] and present observation, we applied “5 mM NaB” in the following study.

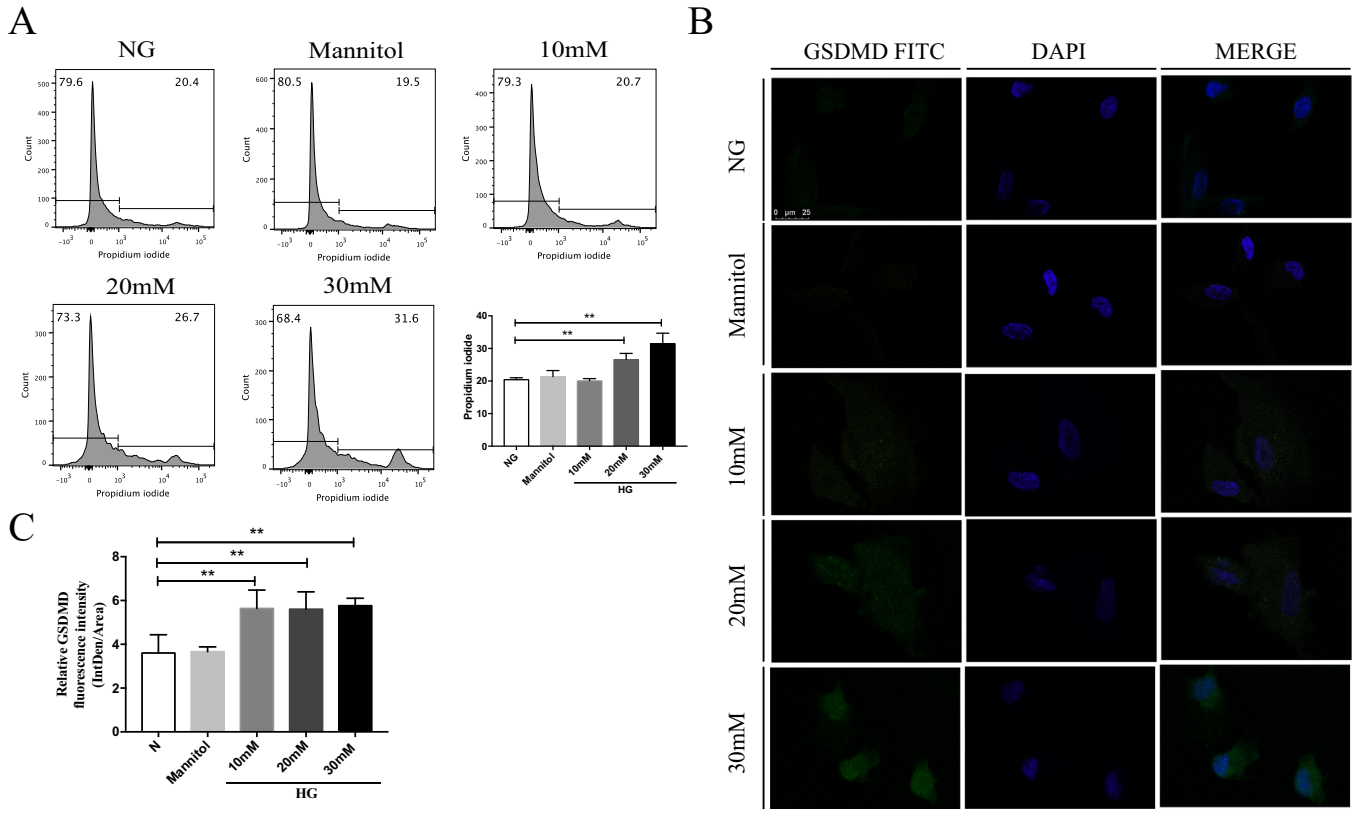

Fig. 2. GSDMD protein expression at different glucose concentrations in GECs. GECs were incubated with different concentrations of glucose for 24 h, protein expression of GSDMD were detected by (A) PI-staining, and (B–C) immunofluorescence assay (magnification: ×800). $^{**}p < 0.01$.

GSDMD is a substrate for caspase 1 in canonical pyroptosis pathway [24]. Accordingly, caspase 1 inhibitor Ac-YVAD-CMK (A-Y-C, 40 μM) was used in the present study. We verified that A-Y-C could not alleviate 30 mM high glucose-induced apoptosis (Fig. 4). By WB analysis, we found protein levels of cleaved-caspase 1, GSDMD and GSDMD-N were significantly reduced on blockade of caspase 1 by A-Y-C; furthermore, administration with NaB was shown with similar effect with that of A-Y-C (Fig. 5E–H). This was further demonstrated in our study by PI staining and ELISA assay that NaB inhibited pyroptosis and reduced LDH/IL-1β/IL-18 release from cells (Fig. 5I–M). These findings

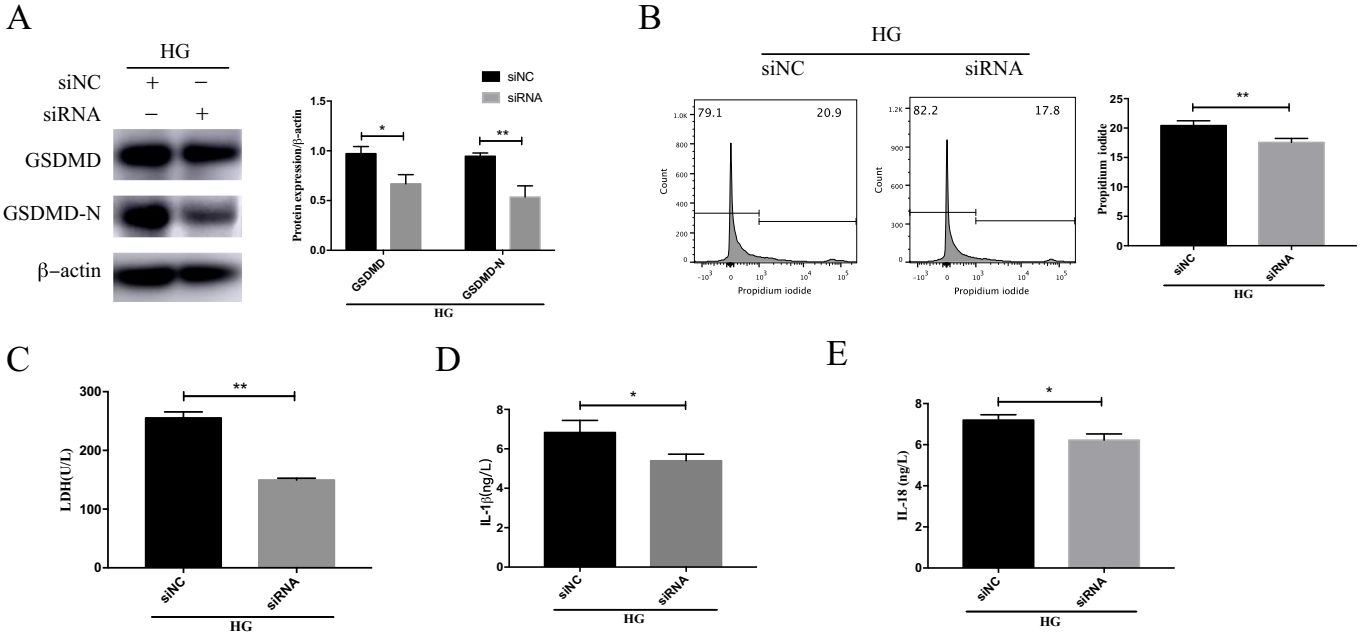

Fig. 3. GSDMD silencing inhibited high glucose-induced pytoptosis in GECs. GECs were pre-treated by control-siRNA or siRNA of GSDMD, and then were further cultured with high glucose (30 mM) for 24 h. (A) Protein expression of GSDMD and GSDMD-N were detected by WB. (B) PI-positive cells were detected by a flow cytometer. (C) LDH, (D) IL-1β and (E) IL-18 levels in culture medium were detected by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$.

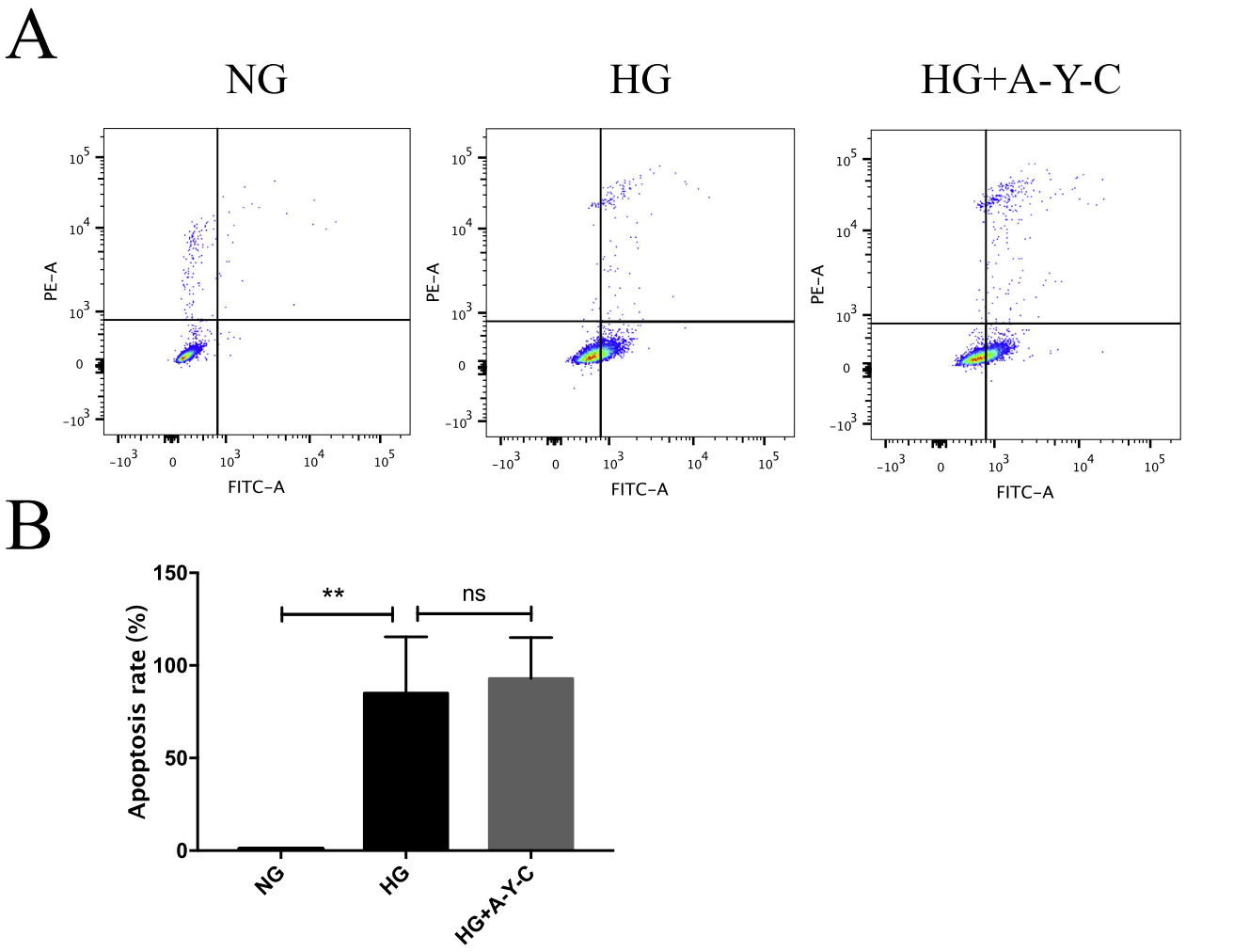

Fig. 4. Caspase 1 inhibitor Ac-YVAD-cmk could not alleviate 30 mM high glucose-induced apoptosis. GECs were incubated with normal glucose (NG), 30 mM glucose (HG) and 40 μM Ac-YVAD-cmk pretreatment adding in 30 mM glucose (HG + A-Y-C) for 24 h. The apoptosis rates of GECs were detected by apoptotic kit. $^{**}p < 0.01$.

suggested that high-glucose induced pyroptosis in GECs is caspase 1-GSDMD dependent and can be ameliorated by 5 mM NaB administration.

3.4. NaB inhibited NF-κB/IκB-α signaling in high-glucose induced GECs pyroptosis

NF-κB/IκB-α signaling is an important pathway that plays important role in development of DN. Our study showed that high-glucose significantly up-regulated and activated NF-κB p65 signaling proteins, and 5 mM NaB or 40 μM A-Y-C pretreatment reduced their expression and nucleus translocation (Fig. 6A–C). Moreover, 30 mM high glucose-induced inhibition of IκB-α was reversed by NaB/A-Y-C administration (Fig. 6D–G). Accordingly, 30 mM high glucose-induced the nucleus translocation of p-IκB-α was blocked when GECs were co-incubated with NaB or A-Y-C (Fig. 6D–E). Taken together, NF-κB/IκB-α signaling was involved in 5 mM NaB ameliorated 30 mM high-glucose induced GECs pyroptosis.

3.5. Reduction of caspase 1-GSDMD discounted effect of NaB on pyroptosis

To further verify the mechanism of NaB on inhibiting pyroptosis in GECs, expression of GSDMD and GSDMD-N were determined after si-GSDMD treatment (Fig. 7A). We found the effect of NaB on LDH release and PI positive cells was further enhanced by si-GSDMD (Fig. 7B–C); moreover, the effect of NaB on PI positive cells (Fig. 7D) and LDH (Fig. 7E) was significantly enhanced by A-Y-C pretreatment. This implies that NaB-mediated anti-pyroptosis effect was facilitated by reduction of caspase 1-GSDMD.

4. Discussion

Diabetic nephropathy is a microvascular disease and high glucose is the initial factor [25]. Glomerular endothelial cells damage is often occurred earlier, even at the normal urine protein stage [9]. Our study demonstrated that high glucose promoted the activation of caspase1-GSDMD canonical pyroptosis pathway in glomerular endothelial cells, and exogenous sodium butyrate (NaB) supplementation reduced the number of GECs with pyroptosis markers.

Pyroptosis is essential in eliminating innate immune cells infected with intracellular bacteria [26,27]. However, excessive pyroptosis will induce massive inflammatory and cell damage [28]. Currently, the NLRP3-caspase 1 canonical inflammasome is known to be closely related to DN [2,29-30], but the downstream mechanism still needs to be further clarified. It is demonstrated that GSDMD is the executor of pyroptosis and is a substrate of caspase-1 [31,32]. GSDMD is cleaved by caspase-1 to the N-terminal proteolytic fragment of GSDMD (GSDMD-N), forming pores in the cell membrane and gradually trigger cell death, which release amount of pro-inflammatory cytokines such as IL-1β and activate a strong inflammatory response [24,32]. Previous studies have

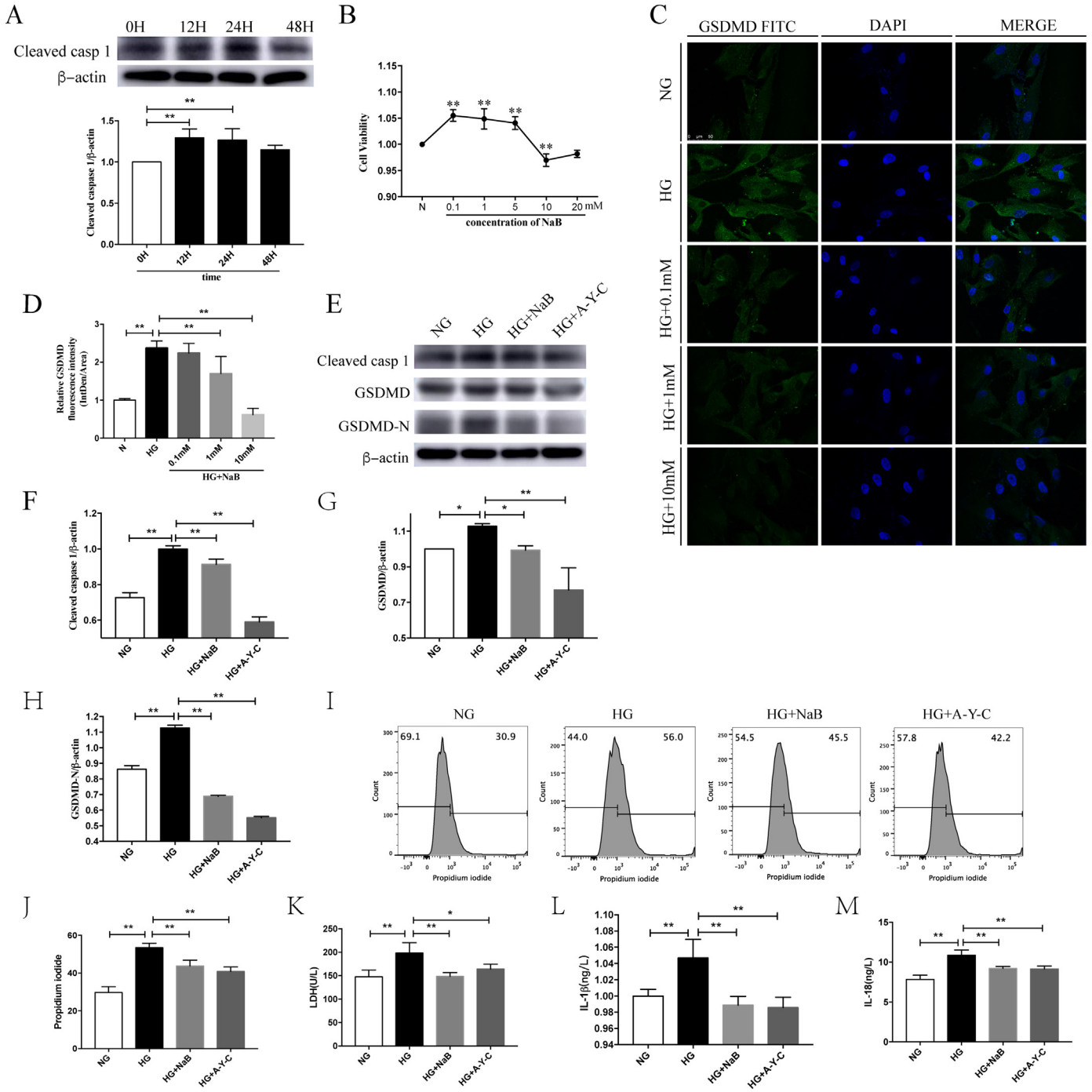

Fig. 5. Sodium butyrate inhibited the high-glucose induced pyroptosis in GECs. (A) Protein expression of cleaved-caspase 1 of cells under 30 mM high glucose (HG) was detected by WB at different time course. (B) Viability of cells after different concentrations of NaB (0.1–10 mM) was evaluated by MTT. (C and D) Protein expression of GSDMD was determined by immunofluorescence (magnification: × 800). (E–H) Protein expression of cleaved-caspase 1, GSDMD, and GSDMD-N were determined by WB. (I and J) PI-positive cells were detected by a flow cytometer. (K) LDH, (L) IL-1β and (M) IL-18 levels in culture medium was detected by ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$.

shown that pyroptosis was increased in diabetic cardiomyocytes [12,33] and renal tubular epithelial cells in kidney injury models [34], but it is still not clear whether high glucose can also cause pyroptosis in GECs.

GECs are an important part of glomerular filtration barrier and are vulnerable to damage of circulating substances, such as blood glucose and inflammatory factors. GECs damage could alter the permeability of the glomerulus and lead to chronic kidney disease (CKD) [35]. It has been demonstrated that apoptosis of GECs elevation of inflammatory factors induced by high glucose will result in endothelial damage [36]. However, role of pyroptosis in GECs damage under diabetic settings still needs to be clarified. Our study demonstrated that high-glucose could induce GECs pyroptosis, which was consistent with findings in a recent study [37]. Previous studies in pyroptosis mainly focused on NLRP3 inflammasome or inflammatory caspase (caspase 1 or caspase 4/5/11) or their upstream targets [38-42]. However, inhibiting NLRP3 or inflammatory caspase alone is limited, because GSDMD is the ultimate executor of pyroptosis and is critical for the secretion of mature IL-1β

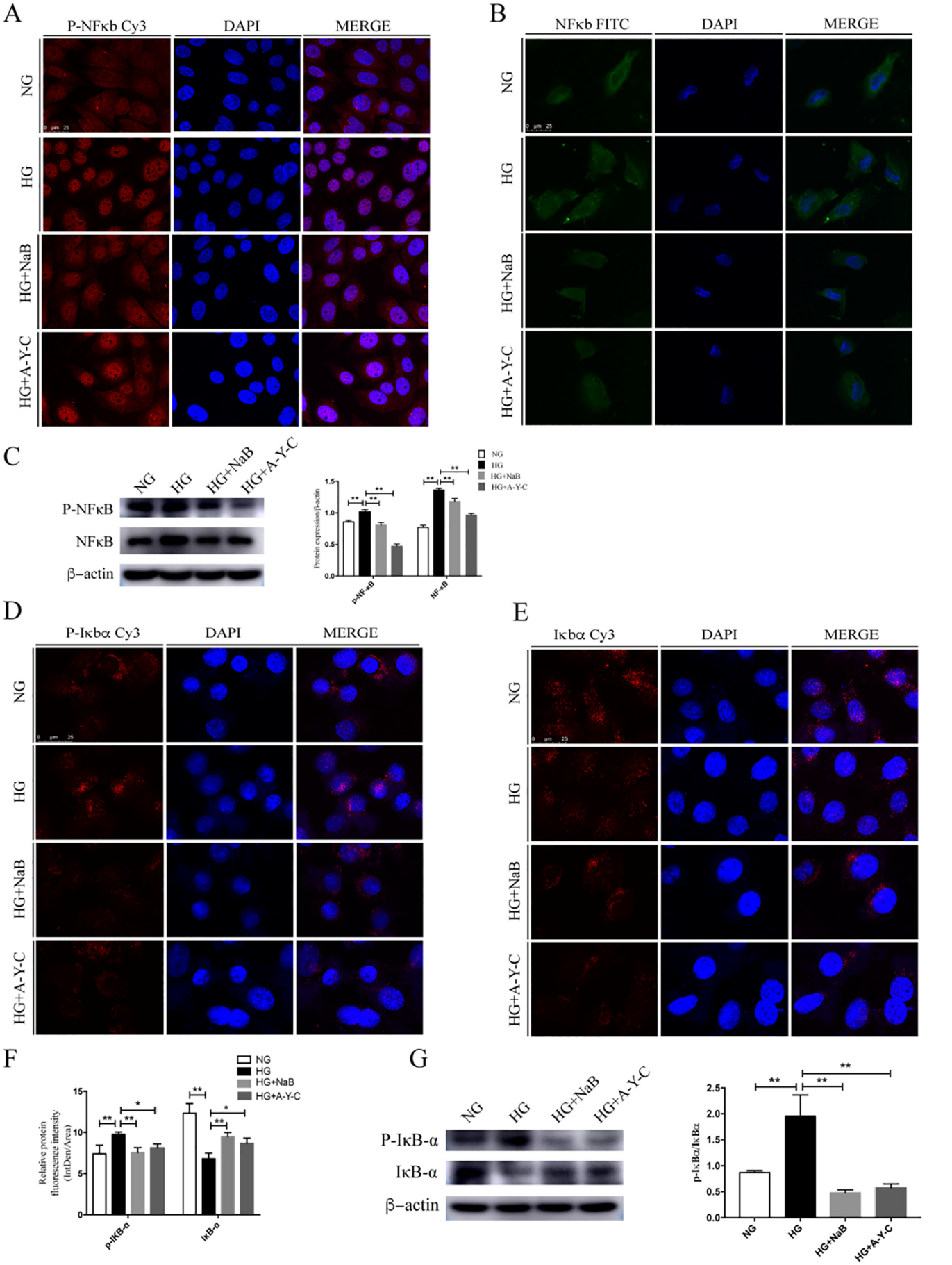

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Fig. 6. Sodium butyrate blocked 30 mM high-glucose induced NF-κB/IκB-α signaling pathway in GECs. (A) Protein expression and activation of p-NF-κB p65 (magnification: ×1200) or (B) total-NFκB p65 (magnification: ×800) were observed by confocal microscope or (C) WB. (D) The protein expression and activation of p-IκB-α and (E) IκB-α were detected by confocal microscope (Magnification: ×1200), and (F) their relative expression were analyzed. (G) The protein expression of p-IκB-α and IκB-α were detected by WB and ratio between p-IκB-α and IκB-α was analyzed. $*p < 0.05$, $**p < 0.01$.

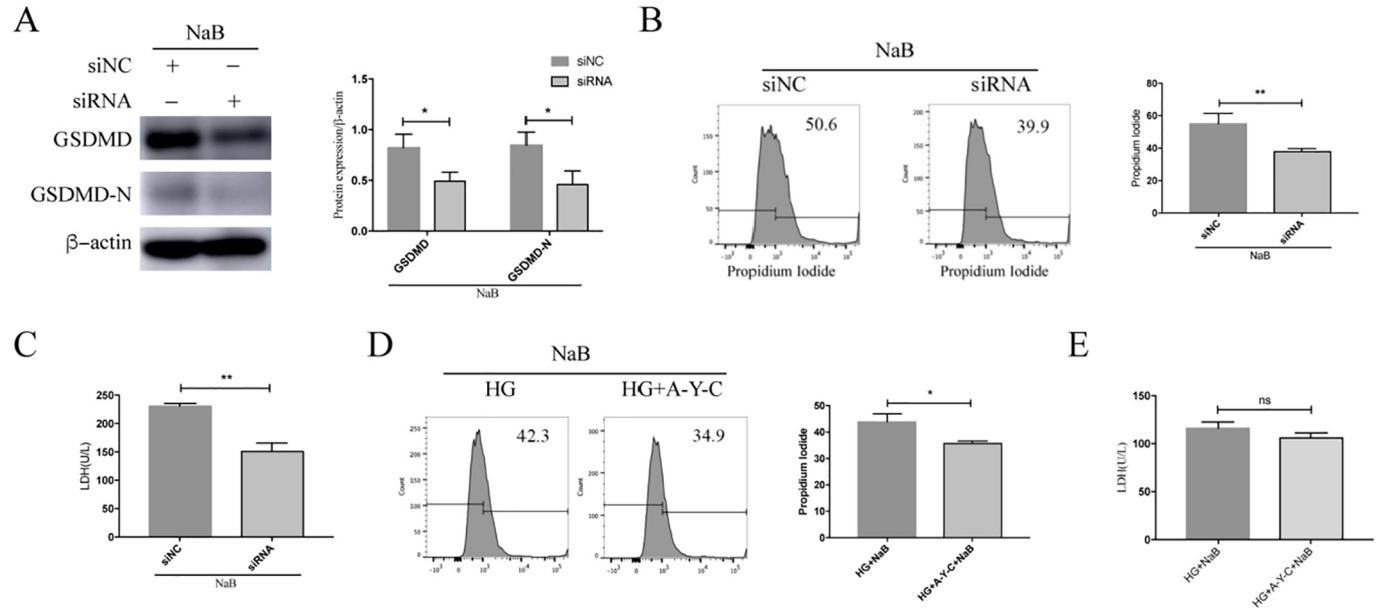

Fig. 7. Sodium butyrate-mediated anti-pyroptosis effect was facilitated by the inhibition of caspase 1-GSDMD pathway. GECs was pre-treated by control-siRNA or siRNA-GSDMD or 40 μM A-Y-C and were cultured with sodium butyrate and 30 mM high glucose for 24 h. (A) Protein expression of GSDMD and GSDMD-N were detected by WB. (B) PI-positive cells were detected by a flow cytometer. (C) LDH levels in culture medium was detected by ELISA. (D) PI-positive cells were detected by a flow cytometer. (E) LDH levels in culture medium was detected by ELISA. $*p < 0.05$, $**p < 0.01$.

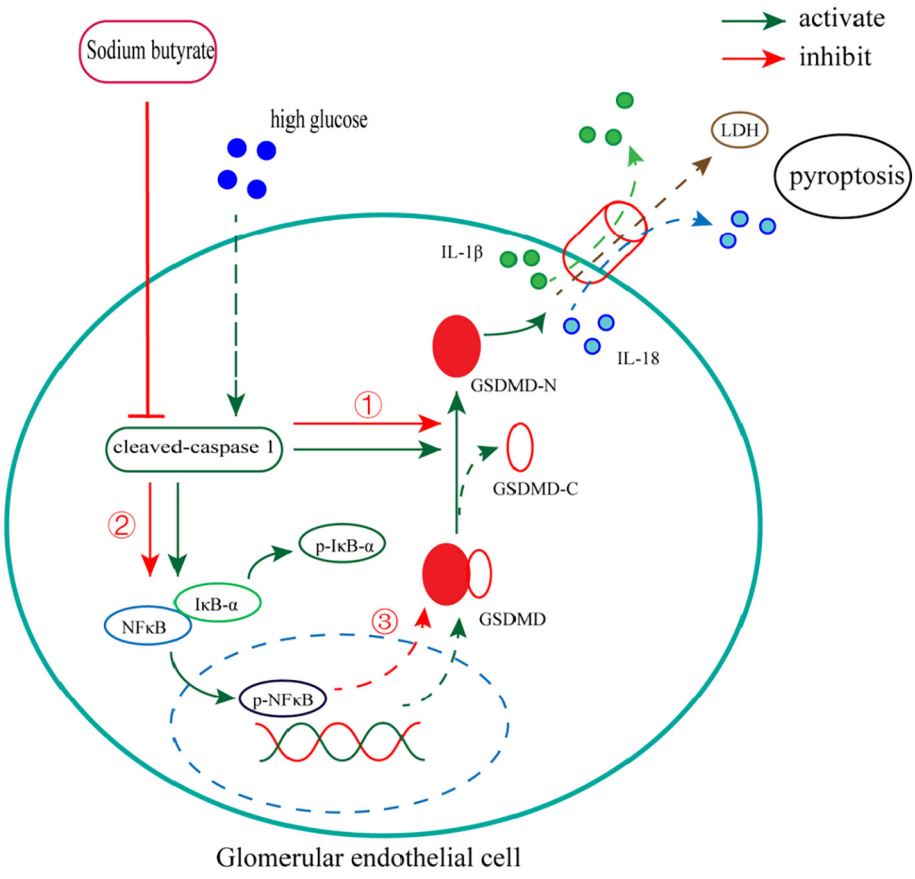

Fig. 8. Proposed mechanism of NaB against pyroptosis in GECs. (1) NaB (5 mM) reduced the expression of GSDMD-N via inhibiting caspase1-GSDMD canonical pyroptosis pathway induced by high glucose (30 mM), thus alleviating GECs damage and release of pro-inflammatory factors. (2) NaB (5 mM) inhibited high glucose-induced activation of the NF-κB/IκB-α pathway. (3) NF-κB/IκB-α signaling pathway might be involved in NaB (5 mM) inhibited 30 mM high glucose-induced protein expression of GSDMD increase.

and IL-18 to extracellular environment [43]. Therefore, our experiment clarified high-glucose induced GECs pyroptosis targeting GSDMD for the first time.

Sodium butyrate (NaB), a short-chain fatty acid, has been shown to be associated with cell proliferation, differentiation and apoptosis [44]. However, the association between NaB and pyroptosis was not reported yet. Our previous study found that 5 mM NaB reduced high glucose or LPS induced secretion of IL-1β and NF-κB signaling activation in mesangial cells [23], which was consistent with the present observations in GECs. Current understanding on the anti-inflammatory mechanisms of NaB mainly focused on receptor pathway, NLRP3 inflammasome or NF-κB signaling pathway [23,45-47]. It is reported that NLRP3 and NF-κB signaling pathway are associated with development of pyroptosis [10,11,49]; in this sense, we postulated that NaB might reduce endothelial cell damage via caspase 1-GSDMD pathway. Expectedly, our results showed that NaB not only blocked high glucose-induced cleaved-caspase 1 increase, but also inhibited expressions of GSDMD and GSDMD-N; meanwhile, GECs damage was reversed by NaB supplementation. To our knowledge, this was the first report that demonstrated the effect of NaB against pyroptosis in GECs.

There was a previous report that NaB could inhibit expression of caspase 1 in coronary endothelial cells [47]. Our study suggested that NaB could also inhibit the activation of caspase 1 in GECs. Moreover, we also suggested that NaB could decrease total-GSDMD and GSDMD-N protein levels. As GSDMD can be cleaved by active-caspase 1 into active GSDMD-N, NaB inhibited activity of GSDMD should be via inhibiting of caspase 1. We observed that blockade of caspase 1-GSDMD canonical involved pyroptosis further enhanced effect of NaB on protecting GECs from high-glucose induced damage, suggesting NaB ameliorated GECs damage was mediated via regulating caspase 1-GSDMD canonical pyroptosis pathway (Fig. 8). It is reported that NF-κB/IκB-α signaling pathway plays a pivotal role in the inflammatory process of DN [48]. Our previous study showed that NaB inhibited NF-κB activation both in vivo and in vitro [23]. In the present study, we found NaB could inhibit high-glucose induced activation of NF-κB/IκB-α signaling pathway, and these effects could be reversed by caspase 1 inhibitor. In a study concerning nonalcoholic fatty liver, NF-κB was found to possess effects on up-regulating GSDMD transcription by binding to two proximal binding sites at the upstream of GSDMD promoter region [49]. In this sense, NF-κB/IκB-α signaling pathway should play a pivotal role in NaB ameliorated high-glucose induced GECs pyroptosis (Fig. 8).

In conclusion, our study demonstrated that sodium butyrate could protect GECs against high-glucose induced pyroptosis via modulating expression of caspase 1-GSDMD. Our findings supply new strategy on administration of DN.

Acknowledgements

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Declaration of competing interest

All authors declare no conflict of interests.

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