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Anagliptin ameliorates high glucose- induced endothelial dysfunction via suppression of NLRP3 inflammasome activation mediated by SIRT1

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ABSTRACT

High glucose- induced endothelial dysregulation has been recognized as an initiation of vascular complications in Type 2 diabetes mellitus (T2DM). Anagliptin is a novel licensed dipeptidyl peptidase-4 (DPP-4) inhibitor for the treatment of T2DM. The effects of anagliptin in high glucose- induced endothelial dysfunction are less reported. In the current study, we found that treatment with anagliptin prevented high glucose- induced reduction of cell viability and increase in LDH release in human umbilical vein endothelial cells (HUVECs). Our results indicate that anagliptin- reduced high glucose- induced increase in mitochondrial ROS and NOX-4 expression. Additionally, anagliptin treatment inhibited high glucose- induced expressions of TXNIP in HUVECs. Importantly, anagliptin treatment downregulated high glucose- induced NLRP3 inflammasome activation, as evidenced by reducing the expressions of NLRP3, ASC, and cleaved caspase-1 (P10). Also, ELISA results demonstrate that anagliptin treatment significantly abolished high glucose- induced maturation of IL-1β and IL-18. Mechanistically, we found that anagliptin treatment restored high glucose- induced reduction of SIRT1 expression. Silencing of SIRT1 by transfection with SIRT1 siRNA abolished the inhibitory effects of anagliptin in NLRP3 inflammasome activation. These results display that anagliptin may confer protection against high glucose- induced endothelial injury via SIRT1-dependent inhibition of NLRP3 infammasome activation.

1. Introduction

Macrovascular and microvascular damage has been recognized as an important pathophysiological characteristic of type IIdiabetes mellitus (T2DM) (De Rosa et al., 2018). Under pathological condition, endothelial dysfunction is closely associated with hyperglycemia and becomes one of the early events of cardiovascular complications in T2DM (Kang et al., 2017). Hypoadiponectinemia is closely linked with impaired endothelium-dependent vasodilation. Increasing evidence has shown that high glucose level could cause high levels of intracellular ROS production and increased inflammatory conditions in endothelial cells (Hui and Yin, 2018). The aberrant activation of the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome plays pivotal roles in the pathogenesis of a diversity of diseases, including cardiovascular disorders (Peng et al., 2015). NLRP3 inflammasome has been identified as a multiprotein cytoplasmic complex composed of NLRP3, caspase-1, and apoptosis-associated speck-like protein (ASC). The endoplasmic reticulum (ER) stress associated protein thioredoxin-interacting protein (TXNIP) and mitochondrial ROS- induced the activation of NLRP3 lead to maturation of the proinflammatory cytokines to IL-1β and IL-18 (Zhou et al., 2018). A recent study demonstrates that NLRP3 inflammasome activation was responsible for diabetic vascular endothelial dysfunction by finding that silencing of NLRP3 significantly attenuated endothelial cell dysfunction, endothelial cell apoptosis, and inflammatory responses induced by high glucose (Lv et al., 2017). The regulation of NLRP3 inflammasome is complex. Interestingly, it has been recently reported that Sirtuin 1 (SIRT1), a class III histone deacetylase, plays a negative role in regulating the activation of NLRP3 inflammasome (Fu et al., 2013). Blockage of NLRP3 inflammasome activation has become an important therapeutic strategy for the

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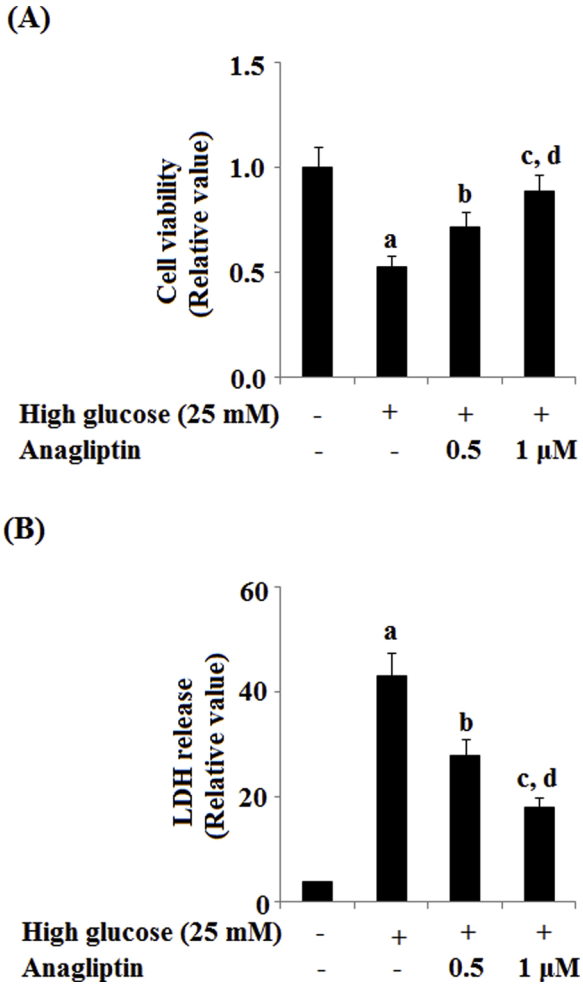

Fig. 1. The effects of Anagliptin on high glucose- induced reduction of cell viability and LDH release. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 48 h. (A). Cell viability was determined by MTT assay; (B). LDH release determined by a commercial kit (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

treatment of endothelial dysfunction in cardiovascular disease induced by high glucose.

Anagliptin, a novel member of the gliptins family, acts as a selective inhibitor of dipeptidyl peptidase-4 (DPP-4) and has been licensed for clinical treatment of T2DM in 2012. Administration of anagliptin is able to improve glycemic control by stimulating insulin secretion, promoting the activity of glucagon-like peptide-1 (GLP-1), and suppressing excessive glucagon secretion (Kaku, 2012). In patients with type 2 diabetes, the serum triglyceride (TG) level is an important predictor of coronary heart disease (CHD). Interestingly, in addition to lowering plasma glucose, a recent clinical study demonstrated that administration of anagliptin significantly reduced total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) (Chiba et al., 2018). Anagliptin administration was also reported to prevent intimal hyperplasia formation after balloon injury by stimulating the migration of endothelial cells via the SOD-1/RhoA/JNK signaling pathway (Li et al., 2018a). Pre-incubation with anagliptin displayed an endothelial protective capacity against H_2O_2- induced oxidative stress and apoptosis by inhibiting the formation of ROS, Bax, cleave caspase-3, and Cytochrome C overexpression in human umbilical vein endothelial cells (HUVECs) (Li et al., 2018b). An *in vivo* experiment demonstrated that administration of anagliptin prevented the pathological progression of atherosclerosis in a model of apoE-deicient mice through inhibiting smooth muscle cell proliferation and inflammatory reaction (Ervinna et al., 2013). Although extensive investigations are dedicated to the pharmacological roles of agents on NLRP3 inflammasome, little information regarding the effects and the underlying mechanisms of anagliptin on NLRP3 inflammasome activation in endothelial cells is known. Therefore, in the current study, for the first time, we aimed to study the effects of anagliptin on high glucose- induced NLRP3 inflammasome activation in HUVECs and explore the underlying mechanisms.

2. Materials and methods

2.1. Cell culture, siRNA transfection, and treatment

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum(FBS), 3 ng/ml β-endothelial cells growth factor, 4 U/ml heparin, 100 units/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were incubated with normal glucose

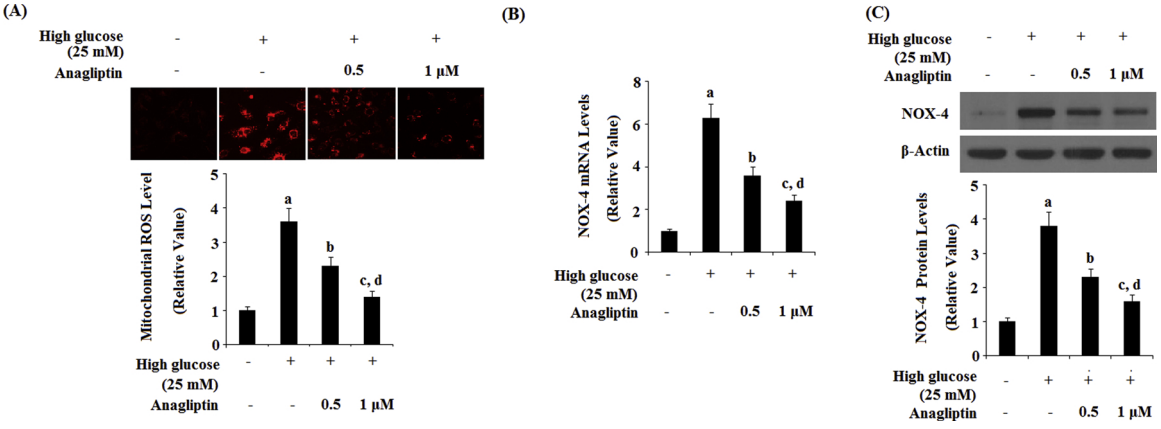

Fig. 2. Anagliptin reduced high glucose- induced generation of mitochondrial ROS and the expression of NOX4. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h. (A). Mitochondrial ROS was determined by MitoSOX; (B). Expression of NOX4 at mRNA levels were determined by real time PCR; (C). Expression of NOX4 at protein levels were determined by western blot analysis (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

(5 mM) or high-glucose (25 mM) in the presence or absence of Anagliptin (SANWA KAGAKU KENKYUSHO CO. LTD, Aichi, Japan) (0.5, 1 μM) for 24 h or 48 h. HUVECs were transfected with SIRT1 small interfering RNA (SIRT1 siRNA) (Santa cruz biotechnology, USA) using lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufactory's instructions.

2.2. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

Cell viability of HUVECs was evaluated by MTT (Sigma-Adrich, USA) assay (Sheng et al., 2009). Cells were incubated with normal glucose (5 mM) or high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 48 h. MTT solution was added into each well at a final concentration of 1 mg/mL to incubate for 4 h. 100 μl dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. Signals were recorded at 570 nm using a scanning multi-well spectrophotometer.

2.3. Assessment of lactate dehydrogenase (LDH) release

HUVECs were incubated with normal glucose (5 mM) or high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h or 48 h. Released LDH in the culture supernatant was measured using the LDH kit (Thermo Fisher Scientific, USA). Briefly, 50 μl culture medium was mixed with equal volume substrate in a 96-well plate to incubate for 30 min. Signals were recorded at 490 nm using a scanning multi-well spectrophotometer.

2.4. Real time PCR analysis

After incubation, total intracellular RNA was isolated from HUVECs with Qiazol (Qiagen, Germany). 2 μg purified RNA was used to synthesize cDNA using the Trans Script cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China). Real time PCR analysis was performed to determine the expressions of target genes using SYBR Green Master Mix in 20 μl reactions (Thermo Fisher Scientific, USA). The following protocol was used for real time PCR: 40 cycles (30 s at 95 ℃ and 10 s at 52 ℃) after an initial activation step for 10 min at 95 ℃.

2.5. Western blot analysis

After incubation, HUVECs were lysed using RIPA buffer (Thermo Fisher Scientific, USA) supplemented with cocktail inhibitors (Thermo Fisher Scientific, USA). Lysates were centrifuged at 12,000 ×g for 15 min at 4 ℃. Concentration of protein was evaluated using a Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich, USA). Samples in each group were run on SDS polyacrylamide electrophoresis. Separated proteins were transferred to 0.45 μM PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk, followed by being probed with primary antibodies overnight at 4 ℃and HRP-conjugated secondary antibody at RT for 2 h. Bands were visualized using enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, USA) and quantized with Image-Pro Plus software (Version 6.0). The following antibodies were used in this study: Rabbit monoclonal antibody (mab) against NOX-4 (#ab133303, Abcam, USA); Mouse mab against β-actin (#ab8226, Abcam, USA); Rabbit mab against TXNIP (#ab188865, USA); Rabbit polyclonal antibody (pab) against NLRP3 (#ab214185, Abcam, USA); Rabbit polyclonal antibody (pab) against ASC1 (#ab70627, Abcam, USA); Mouse mab against p10 (#ab2553, Abcam, USA); Mouse mab against SIRT1 (#ab110304, Abcam, USA); Rabbit anti-mouse IgG (#ab6728, Abcam, USA).

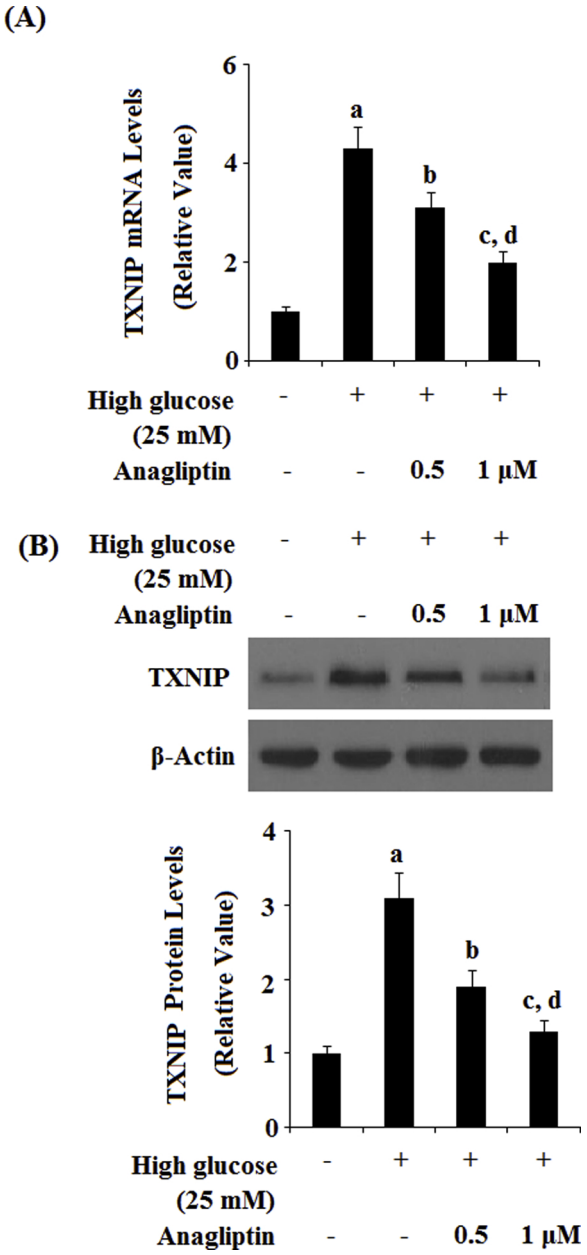

Fig. 3. Anagliptin reduced high glucose- induced expression of TXNIP. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h. (A). Expression of TXNIP at mRNA levels were determined by real time PCR; (B). Expression of TXNIP at protein levels were determined by western blot analysis (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

2.6. Determination of mitochondrial ROS

Mitochondrial ROS was measured by MitoSOX™ red mitochondrial superoxide indicator (Thermo Fisher Scientific, USA) staining. Briefly, approximately 1×10^5 HUVECs were seeded in a 60 mm culture dish. 12 h later, cells were incubated with normal glucose (5 mM) or high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h or 48 h. Cells were then loaded with the MitoSOX Red dye at a

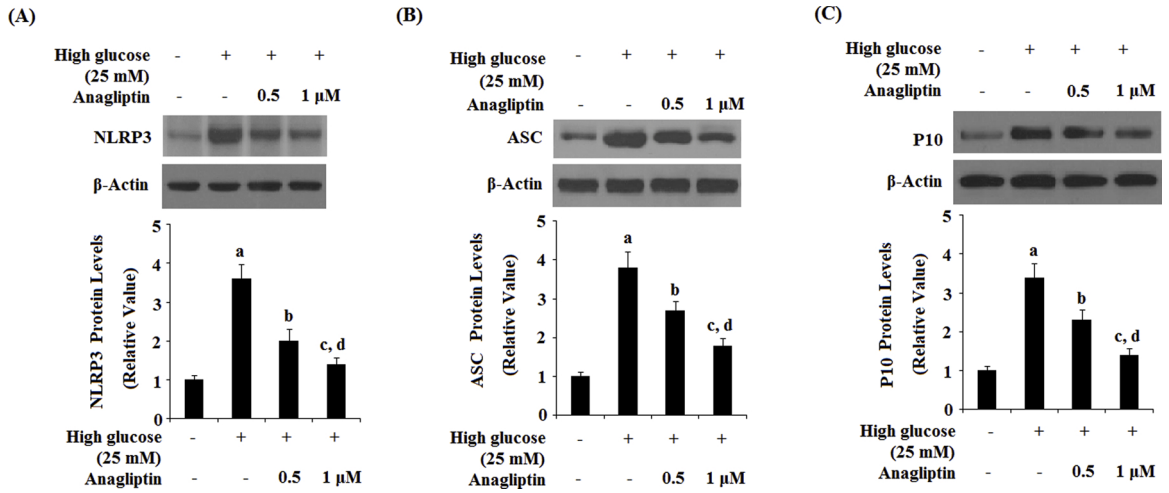

Fig. 4. Anagliptin inhibits high glucose- induced the activation of NLRP3 inflammasome in HUVECs. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h. (A). Expression of NLRP3 was determined by western blot analysis; (B). Western blot analysis of ASC; (C). Expression of cleaved caspase 1 (P10) (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

final concentration of 5 μM for 30 min. Fluorescent signals were visualized using a fluorescent microscope.

2.7. ELISA assay for IL-1β and IL-18

5×10^4 HUVECs were seeded in each well in 6-well plates. 12 h later, cells were incubated with normal glucose (5 mM) or high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h or 48 h. Concentrations of secreted IL-1β and IL-18 in the culture supernatants were measured and quantified by commercial ELISA Kits from R &D systems: human IL-8 quantikine ELISA Kit (#D8000C, R&D systems, USA); human IL-1β quantikine ELISA Kit (#DLB50, R&D systems, USA).

2.8. Statistics

Experimental data were presented as means ± S.D. (standard deviation). Significant diff ;erences between different groups were analyzed by analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. A value of P less than 0.05 was considered statistically significant.

3. Results

Firstly, we determined the effects of anagliptin in high glucose-induced reduction of cell viability and increase in LDH release. Cell viability of HUVECs was assessed by the MTT assay. Results in Fig. 1A indicate that high glucose (25 mM)- induced reduction of cell viability was prevented by treatment with anagliptin in a dose dependent manner. As shown in Fig. 1B, high glucose exposed cells exhibited obviously augmented LDH release into the medium as compared to the controls. However, high glucose-induced cell death was effectively prevented by anagliptin treatment in a dose dependent manner.

Then the fluorescence probe MitoSOX was used to evaluate the production of mitochondrial ROS in HUVECs. Mitochondrial ROS in high glucose (25 mM) exposed cells is significantly higher than that in controls (Fig. 2A), which can be suppressed by treatment with anagliptin in a concentration dependent manner. NOX4 is the major isoform of NADPH oxidases presented in mitochondria in endothelial cells and predominantly produces mitochondrial ROS. Our findings demonstrate that high glucose significantly increased expression of NOX4 at both the gene (Fig. 2B) and protein levels (Fig. 2C), which could be inhibited by anagliptin in a concentration-dependent manner. Increased production of ROS is a manifestation of ER stress, leading to the expression of TXNIP. As expected, real time PCR results in Fig.3A indicate that high glucose (25 mM)- induced gene expression of TXNIP was suppressed by anagliptin. Consistently, western blot analysis revealed this finding at the protein level, shown in Fig. 3B.

Both mitochondrial ROS production and expression of TXNIP contribute to NLRP3 inflammasome activation. Therefore, we investigated whether anagliptin had an influence in high-glucose- induced NLRP3

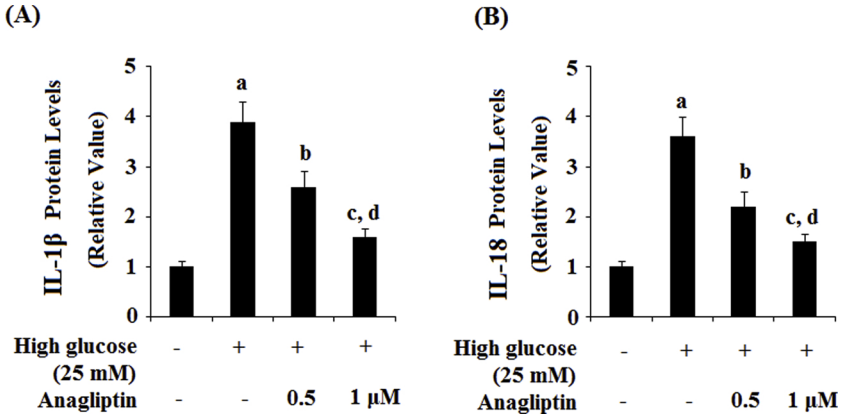

Fig. 5. Anagliptin inhibits high glucose- induced expression of IL-1β and IL-18. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h. (A). Secretion of IL-1β determined by ELISA analysis; (B). Secreted IL-18 determined by the ELISA assay (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

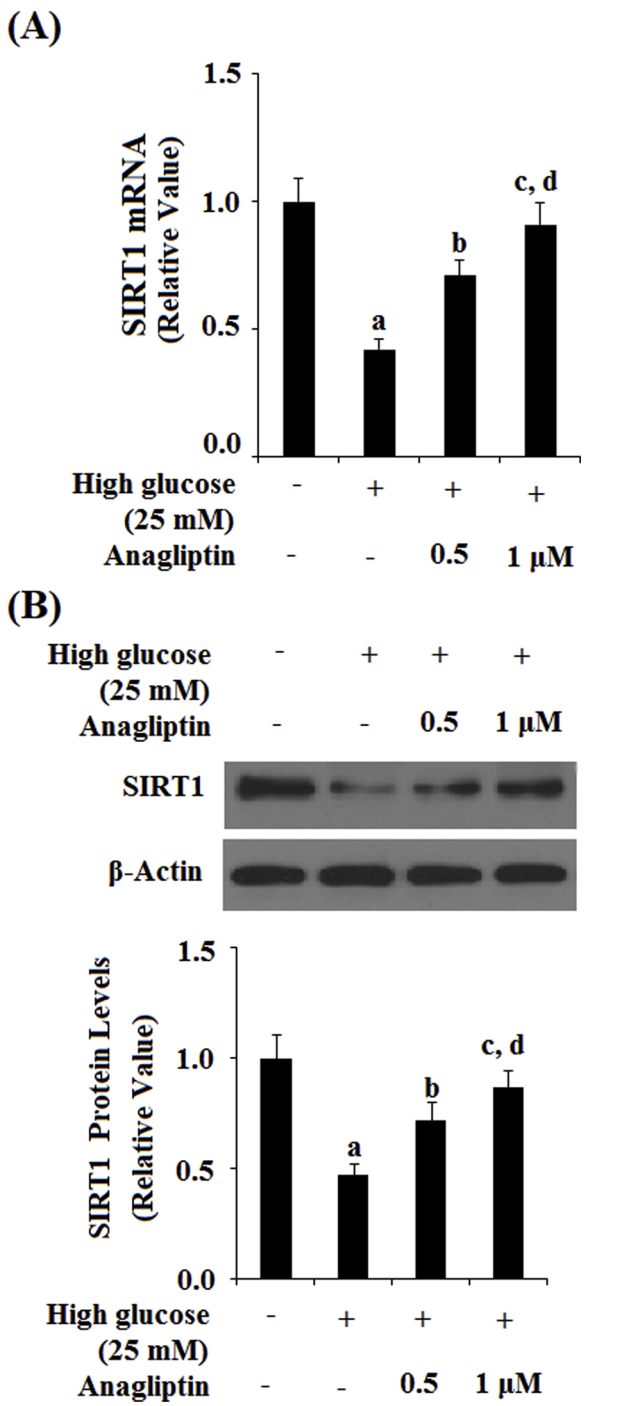

Fig. 6. Anagliptin inhibits high glucose- induced reduction of SIRT1. HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (0.5, 1 μM) for 24 h. (A). mRNA expression of SIRT1; (B). Western blot analysis of SIRT1 (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 0.5 μM Anagliptin group; d, $P < 0.001$ vs. high glucose group; n = 5–6).

inflammasome activation. Results in Fig. 4A indicate that NLRP3 expression was increased when HUVECs exposed to high glucose (25 mM) stimulation. Notably, anagliptin treatment downregulated high glucose-induced NLRP3 expression in a dose- dependent manner. Consistently, anagliptin treatment reduced high glucose- induced increase in ASC expression (Fig. 4B). NLRP3 can promote IL-1β and IL-18 induction by cleavage of caspase-1. As expected, we found that the cleaved caspase-1 (P10) was increased in response to high glucose treatment, which was suppressed by treatment with anagliptin at the concentration of 0.5 and 1 μM. Importantly, ELISA in Fig. 5A and Fig. 5B indicate that anagliptin treatment significantly abolished high glucose- induced expression of IL-1β and IL-18 respectively. These findings demonstrate that anagliptin treatment had a suppressive role in high-glucose induced NLRP3 inflammasome activation and its downstream protein expression.

SIRT1 is a member of the statins family, which has an anti-inflammatory property against endothelial dysfunction (Zhang et al., 2017). Previous studies have shown that SIRT1 is negatively associated with the NLRP3 inflammasome activation. We then explored whether SIRT1 participated in mediating the inhibitory effect of anagliptin on NLRP3 inflammasome activation. Results in Fig. 6 indicate that high glucose significantly reduced the expression SIRT1 at both the mRNA level and protein levels, which were prevented by anagliptin in a dose dependent manner. To confirm the involvement of SIRT1 in this process, the expression of SIRT1 was knocked down by transfection with SIRT1 siRNA. Successful knockdown of SIRT1 is shown in Fig. 7A. Importantly, we found that silencing of SIRT1 abolished the inhibitory effects of anagliptin on the expression of NLRP3, ASC, and the cleaved caspase-1 (P10) (Fig. 7B) as well as the maturation of IL-1β and IL-18 (Fig. 7C). These results suggest that regulation of NLRP3 expression by anagliptin depends on SIRT1-dependent pathway.

4. Discussion

Sustained over-nutrition and impaired insulin secretion and sensitivity are important factors for T2DM. Irregular glucose metabolism causes higher risk of cardiovascular diseases in T2DM patients. These diseases include atherosclerosis and coronary diseases (Triggle and Ding, 2010). Pro-longed exposure to hyperglycaemia leads to endothelial dysfunction associated with macrovascular and microvascular complications. Strategies to protect and recover dysregulated endothelium in vascular diseases of T2DM patients have attracted more and more attentions in recent years (Eriksson and Nyström, 2015). Dipeptidyl-peptidase 4 (DPP4) inhibitors are a family of incretin-based therapeutic agents (Bistola et al., 2018). In addition to decreasing blood glucose, those classes of drugs are able to exert diverse cardioprotective effects. They have displayed positive influences on some risk regulators for cardiovascular diseases (CVD) by improving endothelial function and affecting cardiac function (Avogaro et al., 2014). Anagliptin is a newly DPP4 inhibitor developed for the treatment of T2DM. The protective effects of anagliptin and the underlying mechanisms are less investigated (Sato et al., 2016). Recently, the protective potential of anagliptin has begun to emerge. However, the underlying mechanisms are still unknown. In the current study, we reported for the first time, to the best of our knowledge, that anagliptin suppressed high glucose-induced the activation of NLRP3 inflammasome in HUVECs. Treatment with anagliptin not only reduced the expressions of NLRP3, ASC, and cleaved caspase-1, but also increased IL-1β and IL-18 maturation in cultured cells. Notably, our findings indicate that the inhibitory effects of anagliptin on NLRP3 inflammasome activation might be dependent on the up-regulation of SIRT1 and suppression of ROS. A graphic abstract of underlying molecular mechanism is shown in Fig. 8. Interestingly, we found that treatment with anagliptin alone at the concentrations of 0.5 or 1 μM didn't have a significant influence in the expression of NLRP3 inflammasome and SIRT1 in HUVECs (data not shown). Our findings suggested that anagliptin displayed anti-oxidative and anti-inflammatory activities under stressed circumstance to protect endothelial cells against high-glucose- induced damages and maintain normal endothelial function.

Emerging evidence shows that the activation of NLRP3 inflammasome has been associated with the pathological development of T2DM (Sepehri et al., 2017). Hyperglycemia-induced mitochondrial ROS generation acts as an important contributor of chronic low-grade inflammation in endothelial cells (Luo et al., 2017). NLRP3-dependent pyroptosis and maturation of pro-inflammatory cytokines such as IL-1β

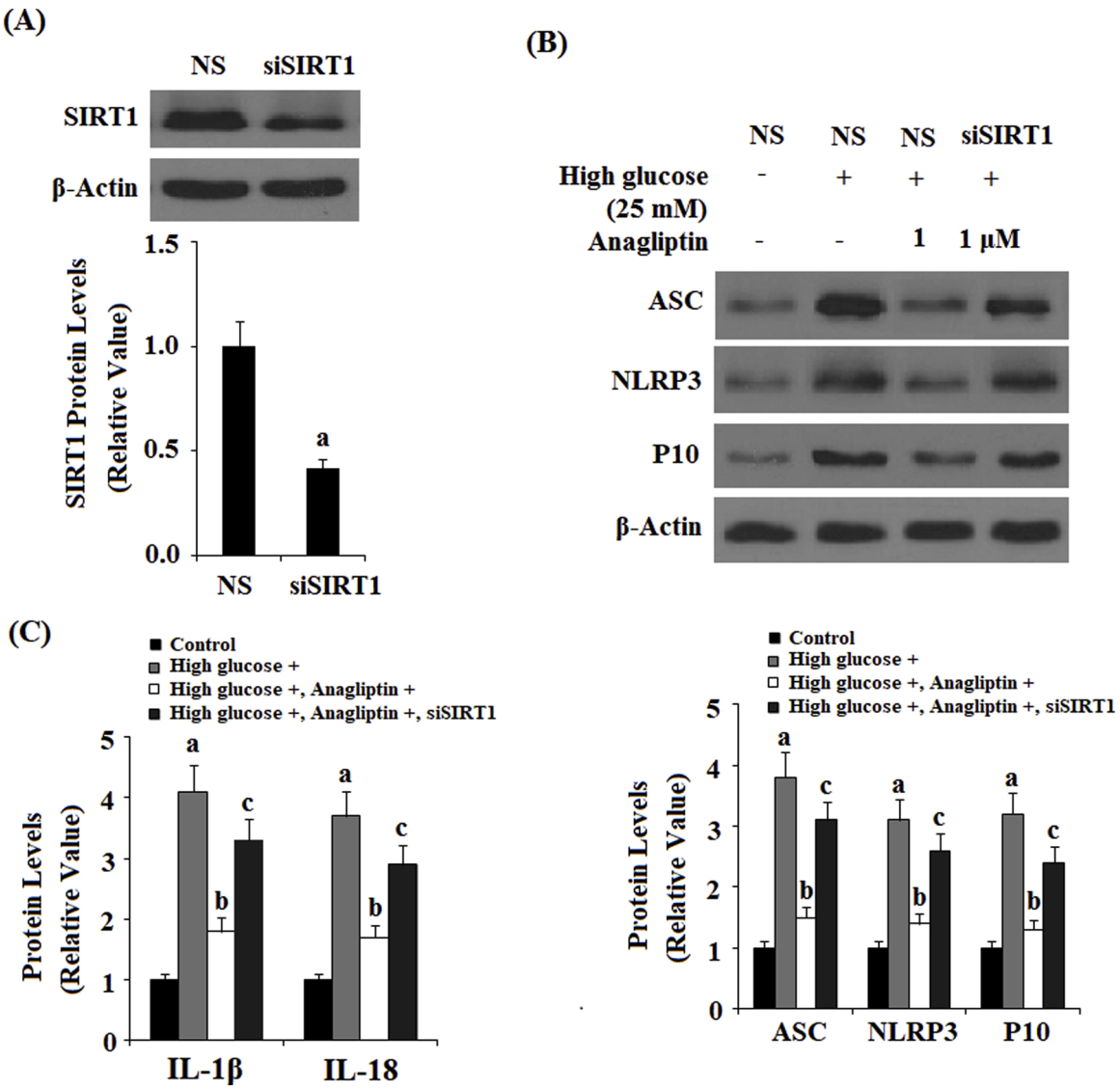

Fig. 7. Silencing of SIRT1 abolished the inhibitory effects of anagliptin on the activation of NLRP3 inflammasome. HUVECs were transfected with SIRT1 siRNA. 12 h post transfection, HUVECs were treated with high glucose (25 mM) in the presence or absence of Anagliptin (1 μM) for 24 h. NS, non-specific siRNA; siSIRT1, SIRT1 siRNA. (A). Western blot analysis revealed the successful knockdown of SIRT1 (a, $P < 0.01$ vs. NS); (B). Knockdown of SIRT1 abolished he inhibitory effects of Anagliptin on the expressions of NLRP3, ASC, and cleaved caspase 1 (P10) (a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 1 μM anagliptin group, n = 5–6); (C). Knockdown of SIRT1 abolished he inhibitory effects of Anagliptin on IL-1β and IL-18 secretion(a, $P < 0.01$ vs. vehicle group; b, $P < 0.01$ vs. high glucose group; c, $P < 0.01$ vs. high glucose + 1 μM anagliptin group, n = 5–6).

and IL-18 induced by ROS could contribute to pathogenesis of cardiovascular diseases (Liu et al., 2017). In this study, we found that anagliptin treatment could reduce the expression of NOX4 and mitochondrial ROS production. Consistently, it has been recently reported that anagliptin treatment prevents apoptosis of HUVECs by modulating NOX4 signaling pathways (Li et al., 2018c). NOX4 is a major source of cellular superoxide anions and possesses a diversity of physiological functions. NOX4 plays an important role in inflammation and the pathological progression of human metabolic diseases, including diabetes. Importantly, inhibition of NOX4 using its specific inhibitor attenuated NLRP3 inflammasome activation and reduced caspase-1 activation as well as IL-1β and IL-18 production (Moon et al., 2016). TXNIP is a redox signaling mediator, the expression of which is increased in response to hyperglycemia (Tseng et al., 2016). Hyperglycemia increased the production of ROS, leading to an increase in free TXNIP, which will bind to NLRP3 and lead to activation of NLRP3 inflammasome. Here, we found that high glucose- induced up-regulation of TXNIP was prevented by anagliptin. These results suggest that anagliptin could regulate Nox4-ROS-TXNIP-NLRP3 signaling and endothelial dysfunction in high glucose challenge.

Sirt1, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is an important regulator in various intracellular processes (D'Onofrio and Servillo, 2018). Importantly, SIRT1 has been considered to be involved in the inflammatory response of endothelial cells (Winnik et al., 2012). Hence, we further examined whether SIRT1 was involved in the inhibitory effect of anagliptin in NLRP3 inflammasome activation. Here, we found that high glucose- induced reduction of SIRT1 was prevented by anagliptin. Importantly, silencing of SIRT1 abolished the inhibitory effects of anagliptin in NLRP3 inflammasome activation and IL-1β/IL-18 secretion. These results suggested that the effects of anagliptin in NLRP3 inflammasome activation are mediated by SIRT1. Notably, in addition to negatively regulating NLRP3 inflammasome activation, SIRT1 possesses a series of cellular functions in endothelial cells. The effects of anagliptin on SIRT1 expression suggest

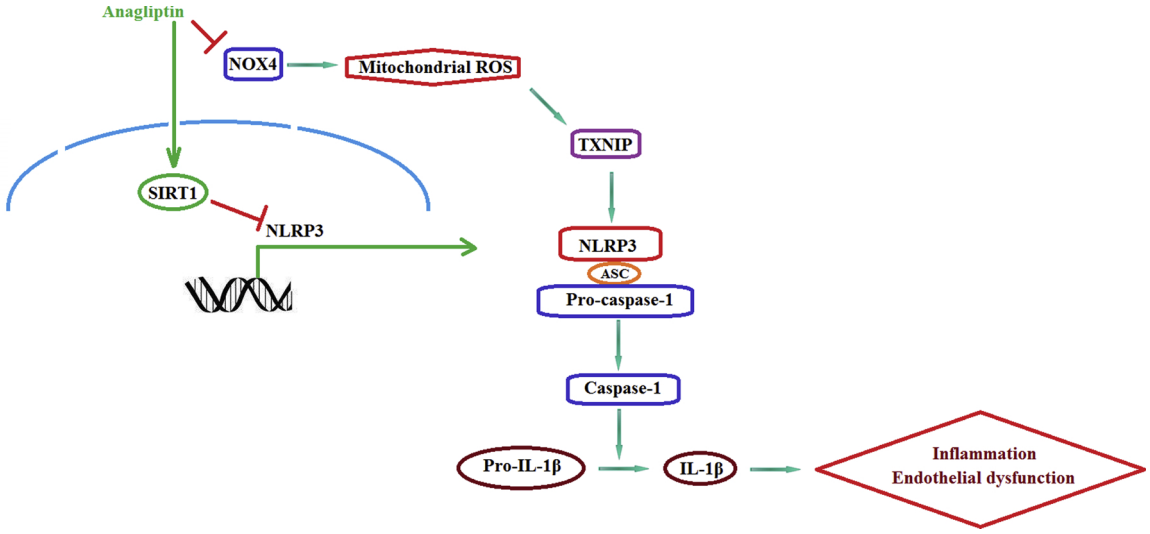

Fig. 8. Graphic abstract of underlying molecular mechanism.

that anagliptin might have a wide range of pharmacological roles in cardiovascular diseases and other metabolic diseases. Future investigations will provide us with a complete picture.

Compete interest statement

None author of this article declared he had any conflict interest need to be exposure.

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