Dipeptidyl peptidase-4 inhibitor sitagliptin prevents high glucose-induced apoptosis via activation of AMP-activated protein kinase in endothelial cells

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Abstract. Diabetes mellitus (DM), which is a chronic metabolic disorder, is the primary risk factor of life-threatening vascular complications. Endothelial apoptosis is important in the development of the initial vascular lesion preceding the diabetic disease. Sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor and extensively used in the clinical treatment of DM. DPP-4 inhibitors have been demonstrated to be beneficial in the improvement of endothelial homeostasis, however the molecular mechanism by which they exhibit these effects remains to be elucidated. The effect of sitagliptin on endothelial apoptosis was examined in cultured human umbilical vein endothelial cells (HUVECs) incubated with high glucose (HG). The present study demonstrated that treatment of HUVECs with HG increased reactive oxygen species (ROS) production, stimulated mitochondrial depolarization and resulted in cell apoptosis. Pretreatment of HUVECs with sitagliptin significantly prevented HG-induced endothelial apoptosis. It was further demonstrated that sitagliptin effectively inhibited ROS generation and mitochondrial membrane potential collapse. Similarly, adenosine monophosphate-activated protein kinase (AMPK) activation by sitagliptin protected against HG-induced ROS production, mitochondrial membrane potential collapse and endothelial cell apoptosis, as detected via western blotting and flow cytometry analysis. The present study therefore revealed a novel mechanism of sitagliptin-mediated AMPK activation in preventing endothelial apoptosis and indicated the therapeutic potential of sitagliptin in vascular complications associated with endothelial apoptosis.

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Abbreviations: DPP-4, dipeptidyl peptidase-4; ROS, reactive oxygen species; DM, diabetes mellitus; HG, high glucose; HUVECs, human umbilical vein endothelial cells; $\Delta\Psi m$, mitochondrial membrane potential; AMPK, adenosine monophosphate-activated protein kinase

Key words: dipeptidyl peptidase-4 inhibitor, apoptosis, adenosine monophosphate-activated protein kinase, endothelial cell, reactive oxygen species

Introduction

Diabetes mellitus (DM) is an increasingly prevalent worldwide disease that is challenging human health and is currently of primary concern. As one of the major risk factors for cardiovascular diseases, type 2 diabetes contributes greatly to the occurrence of disabilities in later life and to mortality (1). Over 50% of mortality events resulting from type 2 diabetes are attributable to cardiovascular diseases (2), including stroke and myocardial infarction. Microvascular endothelial apoptosis is important in the development of the initial vessel lesions of vascular complications in DM (3).

Dipeptidyl peptidase-4 (DPP-4) inhibitors are of a class of oral hypoglycemic agents, which reduce blood glucose levels with a low risk of hypoglycemia and weight gain. DPP-4 is an enzyme expressed on blood vessels, myocardium and myeloid cells and is responsible for the inactivation of endogenous glucoregulatory peptides, termed incretins (4). Glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide are two well-studied incretins. GLP-1 prolongs gastric emptying, reduces appetite, inhibits glucagon release and stimulates insulin in a glucose-dependent manner. GLP-1 receptor (GLP-1R) agonists have been used in the treatment of patients with type 2 diabetes (5). Sitagliptin was the first clinically used DPP-4 inhibitor and was approved by the US Food and Drug Administration for the treatment of type 2 diabetes in 2006 (6). Recent studies in apolipoprotein E-deficient mice revealed that sitagliptin improved endothelial dysfunction, enhanced endothelial nitric oxide synthase (eNOS) phosphorylation (7) and reduced the atherosclerotic plaque area (8), suggesting that DPP-4 inhibitors may have further potential therapeutic effects beyond the incretin-dependent hypoglycemic action. DPP-4 inhibitors have been demonstrated to

exhibit cardiovascular protective functions, however their effect on endothelial apoptosis and the underlying mechanism in diabetes remains to be fully elucidated.

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy and stress sensor (9). In diabetes, AMPK has been observed to be dephosphorylated and inactive (10). A previous study revealed that AMPK activation significantly prevents the oxidative stress-induced apoptosis of human umbilical vein endothelial cells (HUVECs) (11). Various studies have additionally demonstrated that AMPK prevents apoptosis via inhibiting reactive oxygen species (ROS) generation by mitochondria (12) and nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (13). The molecular mechanism regarding how the DPP-4 inhibitor regulates endothelial homeostasis and the associated functional role of AMPK remains to be elucidated. The present study aimed to verify the mechanism by which the DPP-4 inhibitor sitagliptin protects against high glucose (HG)-induced vascular endothelial cell apoptosis and examine if AMPK is involved in this regulatory process.

Materials and methods

Reagents. Sitagliptin (phosphate) was provided by Cayman Chemical Company (Ann Arbor, MI, USA) and 5-aminoimidazole-4-carboxamide riboside (AICAR) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Compound C was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Monoclonal rabbit anti-phospho-AMPKα antibody (catalog no. 2535p) and anti-AMPK antibody (catalog no. 2603p) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The following antibodies were also used: Monoclonal mouse anti-β-actin antibody (catalog no. sc-47778) and horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibody (catalog no. sc-2004/sc-2005) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. HUVECs were isolated by collagenase digestion from fresh cord umbilical veins, as previously described (14). The flesh cord umbilical veins were obtained from normal cesarean section surgery. This was approved by Air Force General Hospital ethics committee with informed written consent. HUVECs between passages 3 and 6 were cultured in endothelial cell medium (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) containing basal medium, supplemented with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% endothelial cell growth supplement with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate). This was conducted in a humidified atmosphere containing 5% CO_2, at 37˚C.

Western blotting. To determine the effect of sitagliptin on AMPK activation, the HUVECs were treated with 1 μM sitagliptin for 0.5, 1, 2 and 4 h or 100 μM AMPK activator AICAR, for 0.5 h. To detect the inhibitory effect of the AMPK inhibitor compound C on sitagliptin-induced AMPKα phosphorylation, HUVECs were incubated with 1 μM sitagliptin, 10 μM compound C or 1 μM sitagliptin plus 10 μM compound C for 2 h. The cytoplasmic protein of cells was extracted with ice-cold hypotonic lysis buffer [50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 0.1% (vol/vol) Triton X-100, 100 mM NaCl and complete protease inhibitor cocktail] as previously described (15). Cell lysates were first snap frozen in liquid nitrogen and then centrifuged at 12,000 x g at 4˚C for 20 min, for collection of the supernatant. Protein concentration was measured using the BCA method. Equal amounts of protein (10 μg per sample) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis and blotted onto polyvinylidene difluoride membranes. Following incubation with no fat milk at 25˚C for 20 min, the membranes were reacted with anti-phospho-AMPKα antibody (1:1,000) and anti-AMPKα antibody (1:1,000) at 4˚C overnight, then reacted with appropriate horseradish peroxidase-conjugated secondary antibodies (1:3,000) for 2 h at 25˚C. Proteins were visualized with an enhanced chemiluminescence kit, as previously described (16). Densitometry analysis was performed for three independent experiments using the Image J Gel Analysis tool (National Institutes of Health, Bethesda, MD, USA).

Measurement of endothelial apoptosis. HUVECs ($1x10^5$) were incubated with HG (33 mM) in the presence of 1 μM sitagliptin, 100 μM AICAR or 1 μM sitagliptin plus 10 μM AMPK inhibitor compound C for 48 h. Induction of apoptosis in the treated groups was assessed by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer's protocol. Briefly, cells were incubated with 33 mM D-glucose in the presence of the aforementioned agents for 48 h and gently digested with 1 ml 0.25% trypsin (Thermo Fisher Scientific, Inc.) for 2 min. The trypsinized cells were washed once with endothelial cell medium containing 5% fetal bovine serum prior to collection by centrifugation at 1,000 x g and room temperature for 20 min. Cells were resuspended in 500 μl of 1X binding buffer, followed by incubation with 5 μl of Annexin V-FITC and 5 μl of PI (50 μg/ml) for 10 min in the dark. Binding buffer, Annexin V-FITC and PI are components of the detection kit. All procedures subsequent to cell incubation were performed at room temperature. Stained cells were monitored by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA) and analyzed with BD FACSDiva™ software (version 6.0; BD Biosciences).

Measurement of ROS generation. A ROS-specific fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes; Thermo Fisher Scientific, Inc.) was used for the measurement of cytosolic ROS production. HUVECs were incubated with 33 mM D-glucose in the presence of 1 μM sitagliptin, 100 μM AICAR or 1 μM sitagliptin plus 10 μM compound C for 48 h, then cells were stained with 10 μM H_2DCFDA fluorescent probe in serum-free endothelial cell medium at 37˚C for 30 min. The labeled cells were then washed twice with serum-free endothelial cell medium and the levels of ROS were immediately analyzed by flow cytometry (BD FACSCalibur; BD Biosciences).

Mitochondrial membrane potential (ΔΨm) assay. To measure ΔΨm, HUVECs were treated with 1 μM sitagliptin, 100 μM AICAR or 1 μM sitagliptin plus 10 μM compound C prior to exposure to 33 mM D-glucose for 48 h. Following incubation,

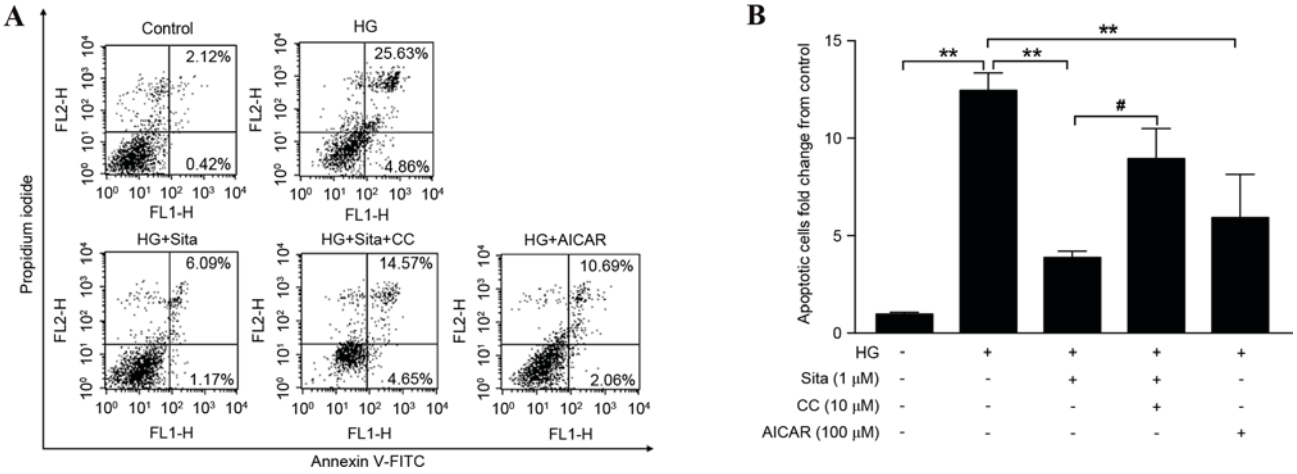

Figure 1. Sitagliptin prevents HG-induced apoptosis in vascular endothelial cells. (A) The apoptosis rate of endothelial cells in the five groups was assessed by Annexin V-FITC/PI staining and monitored with flow cytometry. The lower right quadrant: Annexin V-FITC-positive/PI-negative cells, representing early apoptotic cells. The upper right quadrant: Annexin V-FITC-positive/PI-positive cells, indicating late apoptotic cells. The values represent the percentage of the total cells in the two quadrants. (B) Quantification of apoptotic cell rate compared with control group. HG-induced cell apoptosis was prevented by sitagliptin or AMPK activator, AICAR. Compound C, an AMPK inhibitor reversed inhibition of cell apoptosis by sitagliptin. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. HG group, $^{\#}$P<0.05. Sita, sitagliptin; CC, compound C; HG, high glucose; AICAR, 5-aminoimidazole-4-carboxamide riboside; FITC, fluorescein isothiocyanate; PI, propidium iodide.

cells were collected and stained with 2 μM ΔΨm-specific fluorescent dye JC-1 (Molecular Probes; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2, for 20 min. Flow cytometry (BD FACSCalibur; BD Biosciences) was used to detect ΔΨm for each treatment group. JC-1 accumulates in mitochondria in a potential-dependent manner. In normal mitochondria with high ΔΨm, JC-1 aggregates with red fluorescence. In apoptotic cells with injured mitochondria membrane, JC-1 alters to monomers, and emits green fluorescence. ΔΨm is determined by red/green fluorescence intensity ratio.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. One-way analysis of variance was used to determine differences among the mean values of treatments. SPSS software, version 20.0 (IBM SPSS, Armonk, NY, USA) was used for the statistical data analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Sitagliptin prevents HG-induced endothelial apoptosis. The present study examined the effect of sitagliptin on HUVECs incubated with HG. Cell apoptosis of the pretreated groups was measured by Annexin V-FITC/PI double staining and monitored by flow cytometry (Fig. 1A). It was observed that HG significantly increased cell apoptosis, and this HG-induced endothelial cell apoptosis was prevented by sitagliptin or the AMPK activator, AICAR. However, compound C, an AMPK inhibitor, reversed the inhibition of apoptosis by sitagliptin (Fig. 1B). This therefore indicated that AMPK is important in the regulatory action of sitagliptin.

Sitagliptin activates AMPKα phosphorylation in vascular endothelial cells. As AMPK was observed to be involved in sitagliptin-mediated prevention of endothelial cell apoptosis, the present study aimed to determine the effect of sitagliptin on AMPK activity. HUVECs were incubated with 1 μM sitagliptin at different times ranging from 0.5-4 h. Phosphorylation of AMPKα (p-AMPKα) was determined by western blotting (Fig. 2A). Sitagliptin stimulated AMPKα (Thr^{172}) phosphorylation from 2 h, and this phosphorylation activity prevailed until 4 h. AICAR enhanced AMPK phosphorylation in endothelial cells in a similar manner to sitagliptin, following incubation with the cells for 0.5 h (Fig. 2B). The effect of compound C on sitagliptin-induced AMPKα phosphorylation was additionally examined (Fig. 2C). As presented in (Fig. 2D), sitagliptin-stimulated AMPKα activation was significantly inhibited by compound C. These findings suggested that sitagliptin induces AMPKα phosphorylation.

Sitagliptin decreases high glucose-induced ROS generation. In vascular endothelial cells, the hyperglycemia load increases generation of ROS (17), which subsequently contributes to cell apoptosis. To observe the effect of sitagliptin pretreatment on HG-induced cytosolic ROS generation, cytosolic ROS levels were detected via flow cytometry (Fig. 3A). It was observed that high glucose significantly increased ROS production, however this was suppressed with pretreatment with 1 μM sitagliptin. In addition, AICAR effectively inhibited generation of ROS, whereas compound C diminished the inhibitory effect of sitagliptin (Fig. 3B). These data suggested that sitagliptin inhibits cytosolic ROS via AMPK activation. The effect of sitagliptin on ROS-mediated mitochondrial dysfunction, under conditions of hyperglycemia were then examined.

Sitagliptin restores the loss of ΔΨm. ROS-mediated ΔΨm collapse was previously demonstrated to initiate mitochondrial-dependent apoptosis in DM (18). The present study proceeded to characterize HG-induced ΔΨm alterations and examine if sitagliptin protects against ΔΨm collapse. JC-1 staining detection by flow cytometry was performed.

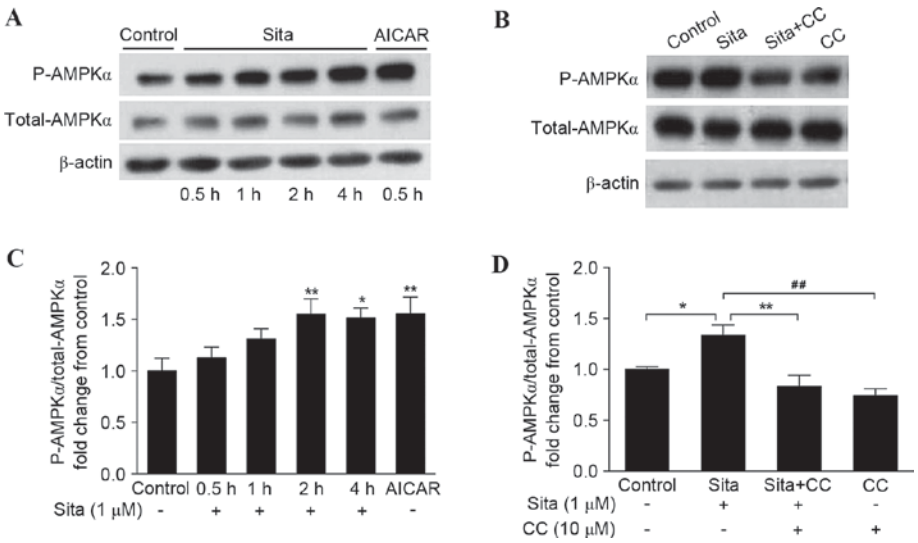

Figure 2. Sitagliptin activates AMPKα phosphorylation. (A) Phosphorylation of AMPKα was determined by western blotting. AMPKα phosphorylation was enhanced from 2-4 h. AICAR additionally activated AMPKα phosphorylation. (B) Compound C inhibited sitagliptin-induced AMPKα activation. The results were quantified and expressed as AMPKα phosphorylation normalized to total AMPKα following (C) sitagliptin plus AICAR treatment (*P<0.05, **P<0.01 vs. control), and (D) sitagliptin plus compound C, in bar graphs. Data are presented as the mean ± standard error of the mean of three independent experiments (*P<0.05, **P<0.01, $^{\#\#}$P<0.01). Sita, sitagliptin; CC, compound C; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, adenosine monophosphate-activated protein kinase; P, phosphorylated.

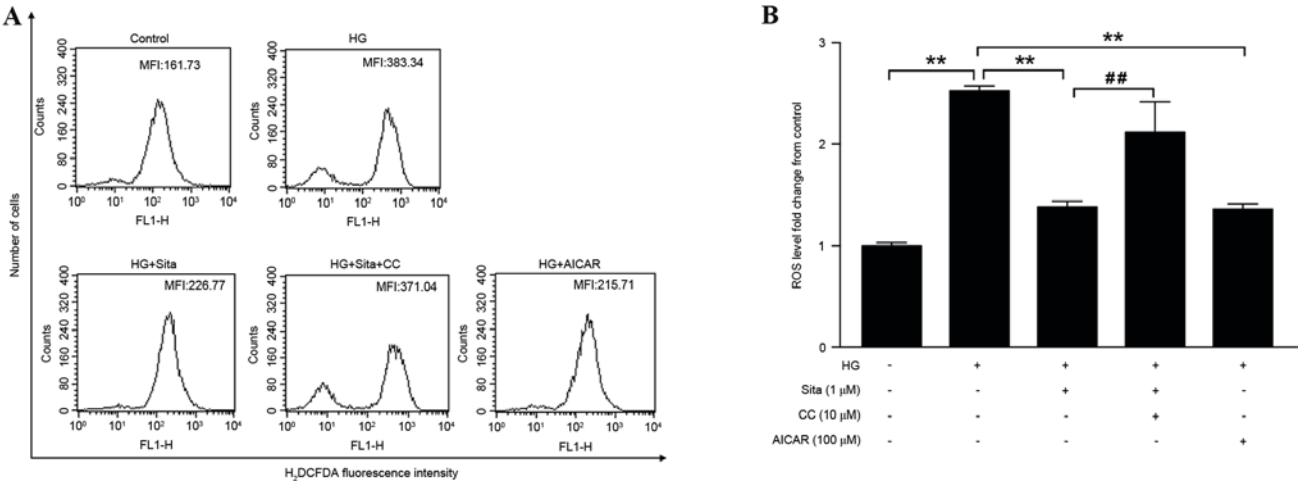

Figure 3. Sitagliptin decreases HG-induced cytosolic ROS generation. (A) The MFI of the cells was monitored using flow cytometry. (B) ROS level was quantified as MFI of each group compared with control group. Sitagliptin decreased HG-induced ROS generation, which was blocked by compound C. AICAR similarly inhibited hyperglycemia-induced intracellular ROS. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01, $^{\#\#}$P<0.01. Sita, sitagliptin; CC, compound C; HG, high glucose; ROS, reactive oxygen species; AICAR, 5-aminoimidazole-4-carboxamide riboside; MFI, mean fluorescence intensity; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate.

Mitochondrial depolarization is determined by a decrease in aggregate/monomer fluorescence ratio (Fig. 4A). In a similar manner to that exhibited by AICAR, 1 μM sitagliptin restored HG-induced ΔΨm collapse, and this effect was blocked by compound C (Fig. 4B). These results suggested that AMPK is important in the regulatory actions of sitagliptin in HG-induced endothelial apoptosis.

Discussion

The present study demonstrated that the DPP-4 inhibitor, sitagliptin, functions as a regulator of endothelial cell apoptosis. In HUVECs, sitagliptin effectively prevented HG-induced apoptosis. The underlying mechanisms may involve inhibition of ROS and the downstream ΔΨm collapsing pathway, induced via AMPKα activation, as summarized in Fig. 5. Overall, the present study has reported novel findings, suggesting DPP-4 inhibitor-mediated AMPK activation as a therapeutic target for vascular endothelial apoptosis.

Diabetes is a major risk factor for cardiovascular disease. The risk of the development of cardiovascular complications in diabetes suggests a need for further therapeutic treatments, which may modulate disease-specific mechanisms, including endothelial apoptosis. DPP-4 inhibitors, including sitagliptin, alogliptin and vildagliptin are safe, well-tolerated hypoglycemic agents that have exhibited beneficial therapeutic effects in diabetes. Previous studies have demonstrated that DPP-4 inhibitors have substantial implications in the

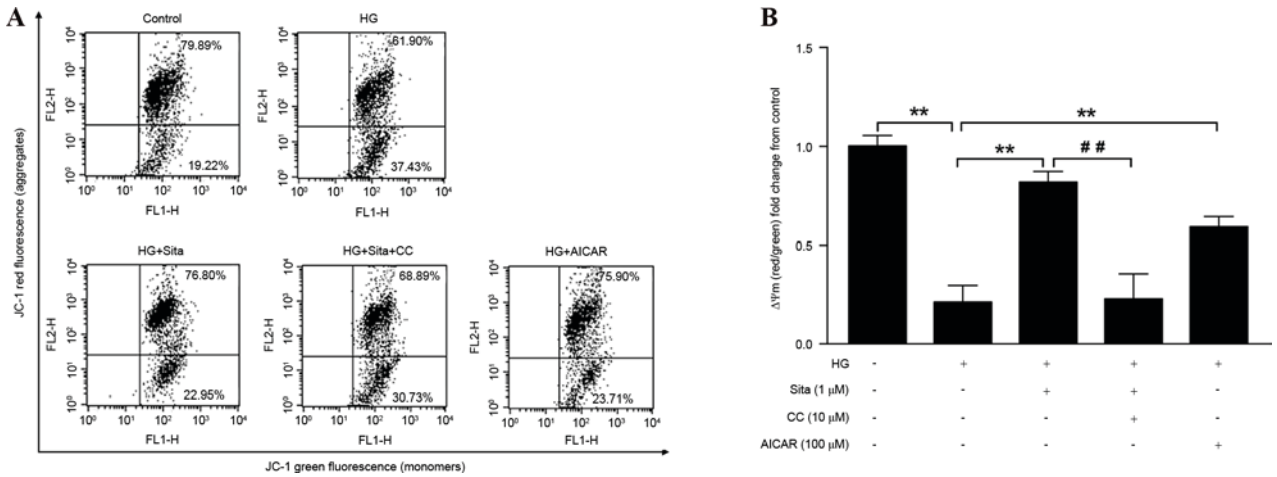

Figure 4. Sitagliptin restored ΔΨm collapse in endothelial cells. (A) Representative flow cytometry graph. The mitochondria membrane depolarization is indicated by a fluorescence emission shift from red to green. Values are refer to the percentage of total cells in each quadrant. Data from three independent experiments are expressed as the mean ± standard error of the mean. (B) Representation of red/green fluorescence intensity ratio. Sitagliptin restored HG-induced ΔΨm collapse, in a similar manner to that exhibited by AICAR. Compound C reversed prevention of ΔΨm collapse by sitagliptin. **P<0.01, $^{\#\#}$P<0.01. ΔΨm, mitochondrial membrane potential; Sita, sitagliptin; CC, compound C; HG, high glucose; AICAR, 5-aminoimidazole-4-carboxamide riboside.

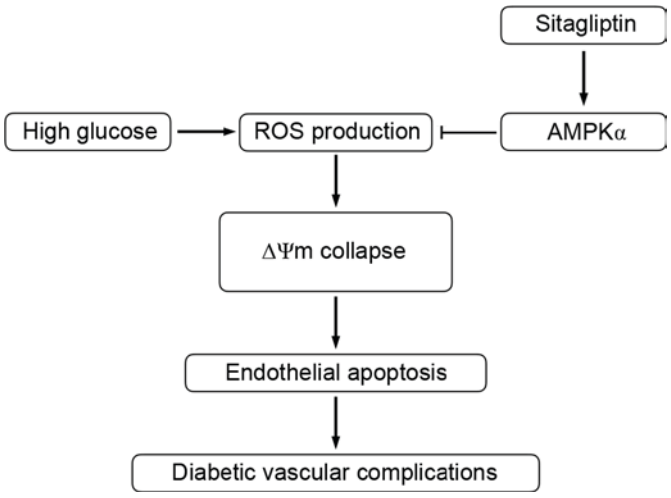

Figure 5. The proposed action pathway for sitagliptin in the prevention of diabetic vascular complications. In endothelial cells, high glucose exposure induces ROS production and the associated downstream ΔΨm collapse, leading to mitochondria-dependent apoptosis. Sitagliptin inhibits high glucose-induced ROS production by activating AMPKs and effectively inhibits ΔΨm collapse. Sitagliptin results in cell survival against high glucose exposure, and thereby prevents diabetic vascular complications under conditions of hyperglycemia. AMPK, adenosine monophosphate-activated protein kinase; ROS, reactive oxygen species.

cardiovascular system. Alogliptin relaxes reconstructed aortic segments (19), and incubation of HUVECs with vildagliptin has been demonstrated to result in phosphorylation of eNOS and serine/threonine kinase 1, increasing nitric oxide synthesis (20). Various animal model studies have demonstrated beneficial effects of DPP-4 inhibitors in improving blood pressure and endothelial dysfunction (7,21). The present study revealed the novel mechanism of DPP-4 inhibitor-mediated apoptosis prevention. The results demonstrated that sitagliptin protected against HG-induced apoptosis in HUVECs, an effect additionally exhibited by the AMPK activator AICAR. Compound C, an AMPK inhibitor, diminished the inhibitory effect of the sitagliptin pretreatment. The potential link between sitagliptin and AMPK was then determined. The data demonstrated that AMPKα phosphorylation was activated by sitagliptin and compound C inhibited sitagliptin-induced AMPKα activation. Therefore, it was demonstrated that AMPK activation is important in sitagliptin-mediated protection against HG-induced vascular endothelial apoptosis.

AMPK is composed of catalytic α-subunit and regulatory β- and γ-subunits (9). The activation of AMPK occurs via α-subunit phosphorylation at Thr^{172} (22). AMPK is an important regulator of metabolic homeostasis, and is considered a therapeutic target for the prevention of diabetic complications (23). Various reports have demonstrated that the AMPK signaling pathway exhibits a protective effect against endothelial dysfunction (24) and prevents apoptosis of HUVECs (11) consistent with the findings of the present study. A previous study reported that AMPKα acts as a physiological suppressor of NAD(P)H oxidase and ROS generation in endothelial cells (12), whereas a further study conversely indicated that AMPK is activated by ROS (25). Hyperglycemia-induced intracellular ROS production and associated downstream mitochondrial fission, stimulated ΔΨm collapse, which resulted in mitochondrial-dependent apoptosis (26). The present study observed a significant increase in cytosolic ROS generation and ΔΨm collapse upon incubation with HG in HUVECs. The ROS production and ΔΨm collapse were suppressed by pretreatment with sitagliptin or AICAR. Compound C reversed the effect of sitagliptin. Therefore, there may be a negative feedback loop between AMPK and ROS, in which ROS generation potentiates AMPK activation, resulting in a further inhibition in intracellular ROS production.

Sitagliptin was demonstrated to prevent endothelial apoptosis via AMPKα activation, therefore it is necessary to elucidate the mechanism by which sitagliptin activates AMPKα. The Ca^{2+}/calmodulin-activated protein kinase kinases (CaMKK), particularly CaMKKβ (27) and the liver kinase B1 (LKB1)-STRAD-MO25 complex (28) are major upstream kinases of AMPK in mammals, and the upstream

kinase LKB1 is important for the activation of AMPK by AICAR (29). Sitagliptin mimicked the preventive effect of AICAR on HG-induced ROS production, ΔΨm collapse, and endothelial cell apoptosis, indicating that sitagliptin-mediated AMPK HG-induced AMPKα activation may involve LKB1. It was additionally reported that sitagliptin treatment improved endothelial function *in vivo* via sequential activation of the LKB1/AMPKα/eNOS axis (30).

In conclusion, the results of the present study indicated that the DPP-4 inhibitor sitagliptin effectively prevented HG-induced cytosolic ROS generation, ΔΨm collapse and apoptosis via activation of AMPKα in endothelial cells. These results suggested sitagliptin may act as a potential novel therapeutic agent to treat vascular complications in diabetes.

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