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Original Research

SKF38393 prevents high glucose (HG)-induced endothelial dysfunction by inhibiting the effects of HG on cystathionine γ-lyase/hydrogen sulfide activity and via a RhoA/ROCK1 pathway

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Abstract

Background: Endothelial dysfunction plays a crucial role in diabetic vascular complications. A decrease in hydrogen sulfide (H_2S) levels is increasingly becoming a vital factor contributing to high glucose (HG)-induced endothelial dysfunction. Dopamine D1-like receptors (DR1) activation has important physiological functions in the cardiovascular system. H_2S decreases the dysfunction of vascular endothelial cells. However, no studies have reported whether DR1 protects the function of vascular endothelial cells by regulating H_2S levels. **Aim**: The present study aimed to determine whether DR1 regulates the levels of endogenous H_2S, which exerts protective effects against HG-induced injury of human umbilical vein endothelial cells (HUVECs) via Ras homolog gene family member A (RhoA)/Rho-associated coiled-coil containing kinase 1 (ROCK1) signalling. **Methods**: HUVECs were exposed to HG (30 mM) or normal glucose (5.5 mM) after different treatments. Cell viability, proliferation and migration were measured by Cell Counting Kit-8, EdU cell proliferation assay, transwell assay and wound healing assay, respectively. H_2S probe (7-Azido-4-Methylcoumarin) was used to detect levels of H_2S. The intracellular calcium concentration ($[Ca^{2+}]_i$) were measured using Fluo-4 AM. The protein expressions were quantified by Western blot. **Results**: We found that HG decreased the expression of DR1 and cystathionine γ-lyase (CSE) and H_2S production. The DR1 agonist SKF38393 significantly increased DR1 and CSE expression and H_2S production, whereas NaHS (a H_2S donor) only increased CSE expression and H_2S production but had no effect on DR1 expression. Meanwhile, SKF38393 further increased the $[Ca^{2+}]_i$ induced by HG. In addition, HG reduced cell viability and the expression of Cyclin D1 and proliferating cell nuclear antigen and increased the expression of $p21^{Cip/WAF-1}$, collagen I, collagen III, matrix metalloproteinase 9, osteopontin and α-smooth muscle actin and the activity of phosphorylated RhoA and ROCK1. SKF38393 and NaHS reversed these effects of HG. PPG (a CSE inhibitor) abolished the beneficial effect of SKF38393. These effects of SKF38393 were similar to those of Y-27632 (a ROCK inhibitor). **Conclusion**: Taken together, our results suggest that DR1 activation upregulates the CSE/H_2S pathway by increasing the $[Ca^{2+}]_i$, which protects endothelial cells from HG-induced injury by inhibiting the RhoA/ROCK1 pathway.

Keywords: Dopamine D1-like receptors; Endothelial cells; Hydrogen sulfide; High glucose; RhoA/ROCK1 pathway

1. Introduction

Diabetes mellitus (DM) is the most common metabolic disease, with an increasing morbidity and mortality rates worldwide and causes serious hyperglycaemia and several complications, such as nephropathy, retinopathy, neuropathy, and cardiovascular disease [1]. Vascular endothelial cell dysfunction (VECD) induced by long-term hyperglycaemia and other diabetes-associated physiological changes in individuals with DM is a critical initiating factor and most fundamental pathological change in diabetes; it is also a key factor contributing to the development of diabetic complications [2]. VECD may result in impaired vasodilation and barrier functions of endothelial cells, disturbances in proliferative capacities, impaired migratory and tube formation properties, impaired angiogenic properties, an attenuation of synthetic function, and a deterrence of white blood cell adhesion and diapedesis [3]. High glucose have been shown to trigger the shift of endothelial to mesenchymal transition (EndMT). During the EndMT, endothelial cells lose their characteristic phenotype and acquire mesenchymal features, which are characterized by the development of invasive and migratory abilities as well as the expression of extracellular matrix proteins [4]. The EndMT appears to represent the key link in the interaction between inflammation and endothelial dysfunction in diabetic complications [4]. Furthermore, transforming growth factor receptor β (TGFβ) signalling is central to the EndMT, and the Ras homolog gene family member A (RhoA)/Rho-associated coiled-coil containing kinases

(ROCKs) pathway is also involved in the EndMT [4,5]. ROCKs were shown to be downstream effectors of RhoA activation and play important roles in regulating several cellular functions including proliferation, migration, and angiogenesis [6,7]. Two similar isoforms of ROCK have been identified: ROCK1 and ROCK2. RhoA/ROCK signalling is involved in hyperglycaemia-induced injury [8]. ROCK deletion attenuates diabetes induced vascular endothelial dysfunction by preventing increased arginase activity and a reduction in NO production [9]. Human endothelial cells exposed to hyperglycaemia exhibit increased ROCK activity, and hyperglycaemia stimulates ROCK activity via protein kinase C (PKC) and oxidative stress-dependent pathways. ROCK1 plays a predominant role in hyperglycaemia-induced increases in ROCK activity [8].

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter with multiple functions in the cardiovascular system [10]. H_2S generation mainly depends on three major enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MST) [11]. CSE is primarily responsible for most of the H_2S produced within the cardiovascular system [12]. Recent studies have shown that H_2S plays a critical role in HG-induced endothelial injury, such as migration dysfunction [13], mitochondrial dysfunction [12], apoptosis [14], oxidative stress, and matrix protein accumulation [15]. Moreover, Ying *et al*. [16] indicated that H_2S protects against the endoplasmic reticulum stress-induced EndMT in subjects with cardiac fibrosis.

Dopamine receptors (DRs) are classified into D1-like receptors (DR1), including D1 and D5, which stimulate adenylyl cyclases (AC), or D2-like receptors (DR2), including D2, D3 and D4. The effect of DR2 is the opposite of DR1 [17,18]. DR1 couples to $G_{\alpha q}$ to modulate phospholipase C (PLC), thus leading to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG). This activation results in the activation of PKC by DAG and increased intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to IP_3. The increase of $[Ca^{2+}]_i$ in the cytoplasm induces the activation of calcium/calmodulin-dependent PK II (CaMKII) [17,18]. DRs are widely expressed in in the brain and in the periphery, including blood vessels, and the heart [19]. The DRs expressed at the highest levels in the blood vessels is DR1 [20]. DRs activation are involved in the occurrence and development of myocardial ischemia-reperfusion injury [21], diabetes and obesity, atherosclerosis, hypertension and other diseases [22]. Additionally, DR1 activation can inhibits the proliferation and migration of vascular smooth muscle cells, thereby exerting anti-atherosclerotic effects [23].

H_2S can protects against endothelial cell dysfunction induced by high glucose. Yang *et al*. [24] reported that an increase of $[Ca^{2+}]_i$ activates CSE, which in turn promotes the production of endogenous H_2S in endothelial cells and protects endothelial cells from damage [24]. Moreover, DR1 activation can increases $[Ca^{2+}]_i$. Researchers have not clearly determined whether DR1 activation functions by increasing $[Ca^{2+}]_i$ to promote the production of endogenous H_2S in vascular endothelial cells is unclear. Therefore, in the present study, we will explore this question and the related mechanism and signalling pathway (RhoA/ROCK1).

2. Materials and methods

2.1 Materials and drugs

Sodium hydrogen sulfide (NaHS), PPG (a CSE inhibitor) and 7-Azido-4-Methylcoumarin (AzMC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SKF38393 (a DR1 agonist) was obtained from Abcam (Cambridge, MA, USA). Y-27632 (a ROCK inhibitor) was obtained from MedChemExpress (Shanghai, China). The primary antibodies for anti-CSE, Cyclin D1, proliferating cell nuclear antigen (PCNA), $p21^{Cip/WAF-1}$, collagen I (Col-1), collagen III (Col-3), matrix metalloproteinase 9 (MMP-9), osteopontin (OPN) and α-smooth muscle actin (α-SMA) were purchased from Proteintech (Wuhan, China). The anti-p-RhoA, t-RhoA, p-ROCK1, t-ROCK1 were from Affinity Biosciences (Cincinnati, OH, USA). The anti-DR1 antibody was from GeneTex (Irvine, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG antibody, the Cell Counting Kit-8 (CCK-8) and anti-β-actin were obtained from Boster Bioengineering Limited Company (Wuhan, China). The EdU Cell Proliferation Assay Kit were obtained from Ribobio (Guangzhou, China). Fluo-4 AM were obtained from Beyotime Biotechnology (Shanghai, China). Enhanced ECL Chemiluminescent Substrate Kit was obtained from Yeasen Biotechnology (Shanghai, China). All other chemicals were from Solarbio (Beijing, China) or Beyotime Biotechnology.

2.2 Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Resource Database of Chinese Academy of Sciences (China) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 at 37 °C. When the cells had reached 80% confluence, they were passaged by digestion with 0.25% trypsin-EDTA at a ratio of 1:2. Cells between the 4th to 8th passages were used in experiments.

For cell treatment, HUVECs were starved for 12 h in serum-free medium and then pretreated with NaHS (100 μM), or DR1 agonists SKF38393 (10 μM), or CSE inhibitor PPG (10 μM), or ROCK inhibitor Y-27632 (10 μM) respectively for 30 min before expose to HG (30 mM) or normal glucose (Control, 5.5 mM). Cells were then treated for 48 h. SKF38393 and Y-27632 were dissolved in DMSO with a final concentration of less than 0.1%. Osmotic control group

(5.5 mM D-glucose + 24.5 mM D-mannitol) showed that osmotic pressure did not affect parameters tested in HUVECs (data not shown).

2.3 Cell viability

Cell Counting Kit-8 (CCK-8) was used according to the manufacturer's instructions. HUVECs were seeded at a density of 1×10^3 cells/well in 96-well plates, after different treatments, the cells were washed with PBS, and incubated with 10 μL CCK-8 in culture medium at 37 °C for 1 h. The resulting absorbance was measured at 450 nm on a spectrophotometer.

2.4 EdU proliferation assay

HUVECs proliferation was detected through the EdU Cell Proliferation Assay Kit according to the manufacturer's instructions. Briefly, HUVECs were seeded in 96-well plate, after different treatments, the cells were incubated with 10 μM EdU for 12 h then fixed with 4% paraformaldehyde, after that, the cells were stained with Apollo for 30 min and Hoechst 33342 for 30 min respectively, the number of cells was counted in six random fields of each well and presented as the ratio of EdU positive cells to total cells using fluorescence microscope (Olympus, IX71, Japan).

2.5 Wound healing assays

Wound healing assays were conducted as previously described with minor modifications [25]. Briefly, the cells were seeded into 6 well plates and were cultured up to subconfluence and serum deprived for 12 h. Then, the confluent monolayer was scratched with a 200 μL sterile pipette tip and cells were washed twice with PBS and then fresh FBS-free DMEM was added. Next, the cells were given different treatments. Migration was followed by phase-contrast microscopy (Nikon Eclipse TS100-F microscope coupled to a digital sight Nikon DS-L3 camera) at different time points (0, 24 and 48 h) up to wound healing closure. The initial and final wound sizes were measured using Image J software (version: 1.53c, National Institutes of Health, Maryland, United States) [26]. Relative migration rate (%) was calculated according to area of migration.

2.6 Transwell migration assay

To assess endothelial cell migration, a transwell assay was performed as previously described [27]. Briefly, after different treatments, serum deprived HUVECs were added to the upper chambers of the transwell (8.0 μm pore size, Corning, NY, USA) and allowed to migrate for 24 h. The number of migrated cells in three random fields was counted.

2.7 Measurements of H_2S levels

The H_2S levels in the HUVECs were measured as previously described [28]. Briefly, after different treatments, cells were incubated with 50 μM 7-Azido-4-Methylcoumarin (AzMC) in PBS for 30 min, followed by washing of the cells with PBS. Visualization of the fluorescence response of AzMC to H_2S in HUVECs was carried out using fluorescence microscope (Olympus, IX71, Japan), and semi-quantitative fluorescence value were measured using Image J.

2.8 Detection of intracellular calcium concentration ($[Ca^{2+}]_i$)

The $[Ca^{2+}]_i$ was detected with the Fluo-4 AM calcium probe as described previously with minor modifications [29,30]. Briefly, after different treatments, the HUVECs were washed with Ca^{2+}-free PBS and incubated in 5 μM Fluo-4 AM for 30 min at 37 °C. Cells were then washed three times with Ca^{2+}-free PBS and incubated for 30 min. Excitation was set at 488 nm, and emission was set at 530 nm. The fluorescence intensity of Fluo-4 AM was determined using fluorescence microscope (Olympus, IX71, Japan).

2.9 Western blotting

Western blotting was performed as described previously [31]. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor PMSF. Protein concentrations were confirmed by BCA Protein Assay Reagent. Equivalent amounts of protein samples were separated by 8–12% SDS-PAGE, and the target proteins were transferred to PVDF membranes. Membranes were blocked in 5% skim milk for 1 h at room temperature, followed by incubation with primary antibodies at 4 °C overnight, and then washed with TBST three times. Subsequently, the membranes were incubated with corresponding HRP-conjugated secondary antibody. The resulting immunoreactive bands were visualized with the Enhanced ECL Chemiluminescent Substrate Kit according to the manufacturer's directions. β-actin was used as the loading control. Protein detection was performed using Image J. Adobe Photoshop (version: 22.0.0 Free Trial, Adobe Inc., San Jose, California, USA) was used to prepare image panels and annotations.

2.10 Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Data are from at least three independent experiments. Data involving more than two groups were analyzed using one-way ANOVA followed by Fisher's LSD post-hoc test (GraphPad Prism 8.02 Free Trial, GraphPad Software, San Diego, California, USA), with p values $<$ 0.05 considered statistically significant.

3. Results

3.1 DR1 activation upregulates the CSE/H_2S pathway in HG-induced HUVECs

We investigated the effects of HG on DR1 and the CSE/H_2S pathway in HUVECs by assessing the expression of DR1 and CSE and H_2S production in HG-treated HUVECs. The expression of DR1 and CSE and the endogenous H_2S production rate were reduced in the HG group compared with the control group. Compared with the HG, the DR1 agonist SKF38393 markedly increased DR1 and CSE expression and endogenous H_2S generation, whereas NaHS (a H_2S donor) only increased CSE expression and endogenous H_2S generation but did not affect DR1 expression (Fig. 1). In the present study, our data showed that SKF38393 upregulates DR1 expression, consistent with a previous study [32]. Based on these results, HG-induced injury of HUVEC injury is related to the downregulation of the DR1-CSE/H_2S pathway, and DR1 is an upstream regulatory factor of the CSE/H_2S pathway.

3.2 DR1 activation upregulates the CSE/H_2S pathway by increasing the intracellular calcium concentration in HG-induced HUVECs

Compared with the control group, the intracellular calcium concentration ($[Ca^{2+}]_i$) was not changed in the control + SKF38393 group and was increased in the HG group. In addition, $[Ca^{2+}]_i$ was further increased in the HG+SKF38393 group compared with the HG group (Fig. 2).

3.3 DR1 activation increases the proliferation of HG-induced HUVECs by activating the CSE/H_2S pathway

Compared with the control group, cell viability and proliferation and the expression of PCNA and Cyclin D1 were decreased and the expression of $p21^{Cip/WAF-1}$ was increased in the HG group. Compared with the HG group, cell viability and proliferation and the expression of PCNA and Cyclin D1 were significantly increased and the expression of $p21^{Cip/WAF-1}$ was obviously decreased in the HG+SKF38393 and HG+NaHS groups. PPG blocked the effect of SKF38393 on HG-induced HUVEC proliferation. The beneficial effect of SKF38393 was similar to that of Y-27632 (a ROCK inhibitor) (Figs. 3,4). These results indicate that DR1 activation promotes HUVEC proliferation by upregulating the CSE/H_2S pathway.

3.4 DR1 activation alleviates the EndMT and promotes the migration of HG-induced HUVECs by activating the CSE/H_2S pathway

Our data showed that HG suppressed HUVEC migration at 24 h and 48 h, while the effect of HG was reversed by SKF38393 and NaHS. PPG reversed the effect of SKF38393. The beneficial effect of SKF38393 was similar to that of Y-27632 (Fig. 5).

A previous study revealed that endothelial cells undergo the EndMT under HG conditions [33]. We confirmed that the protective effects of the DR1-CSE/H_2S pathway were associated with the regulation of EndMT by measuring the levels of the EndMT markers Col-1, Col-3, MMP-9, OPN, and α-SMA using Western blotting [34]. HG increased the expression of Col-1, Col-3, MMP-9, OPN and α-SMA. However, all these changes were reversed by SKF38393 and NaHS treatments. PPG abolished the effect of SKF38393. The beneficial effect of SKF38393 was similar to that of Y-27632 (Fig. 6). These results suggest that DR1 activation inhibits the EndMT and promotes migration of HG-induced HUVECs by upregulating the CSE/H_2S pathway.

3.5 Activation of the DR1-CSE/H_2S pathway attenuates HG-induced HUVEC dysfunction by inhibiting the RhoA/ROCK1 pathway

A previous study indicated that RhoA/ROCK was activated under HG conditions [35]. In the present study, we measured the levels of t-RhoA, p-RhoA, t-ROCK1 and p-ROCK1 using Western blotting to determine the effects of the RhoA/ROCK1 pathway on HG-induced HUVECs and whether the protective effects of activation of the DR1-CSE/H_2S pathway were associated with the regulation of the RhoA/ROCK1 signalling pathway. HG increased the levels of p-RhoA/t-RhoA and p-ROCK1/t-ROCK1 compared with the control. The levels of p-RhoA/t-RhoA and p-ROCK1/t-ROCK1 were markedly decreased in the HG+SKF38393, HG+NaHS and HG+Y-27632 groups (compared with the HG group). PPG abolished the effect of SKF38393 on the RhoA/ROCK1 pathway. The total levels of the RhoA and ROCK1 proteins remained unchanged after exposure to different stimuli (Fig. 7). Therefore, activation of the DR1-CSE/H_2S pathway attenuates HG-induced HUVEC dysfunction by inhibiting the RhoA/ROCK1 pathway.

4. Discussion

Our findings provide new insights into the mechanisms of diabetes-induced vascular endothelial cell dysfunction and the protective effect of DR1 on regulating the CSE/H_2S pathway. Our results suggest that (i) DR1 expression and the activity of the CSE/H_2S pathway are decreased in HG-induced vascular endothelial cells. (ii) DR1 activation upregulates the CSE/H_2S pathway by increasing $[Ca^{2+}]_i$. (iii) DR1 activation protects endothelial cells from HG-induced injury, which is related to the regulation of the CSE/H_2S pathway and subsequent inhibition of the RhoA/ROCK1 pathway.

Hyperglycaemia causes vascular endothelial dysfunction, which is associated with diabetic vascular complications [2], and the EndMT contributes to renal fibrosis which frequently results in deleterious outcomes in patients with diabetes [4]. These factors are in turn associated with the high morbidity and mortality rates and an enormous cost to global health care. H_2S exerts proangiogenic effects, such

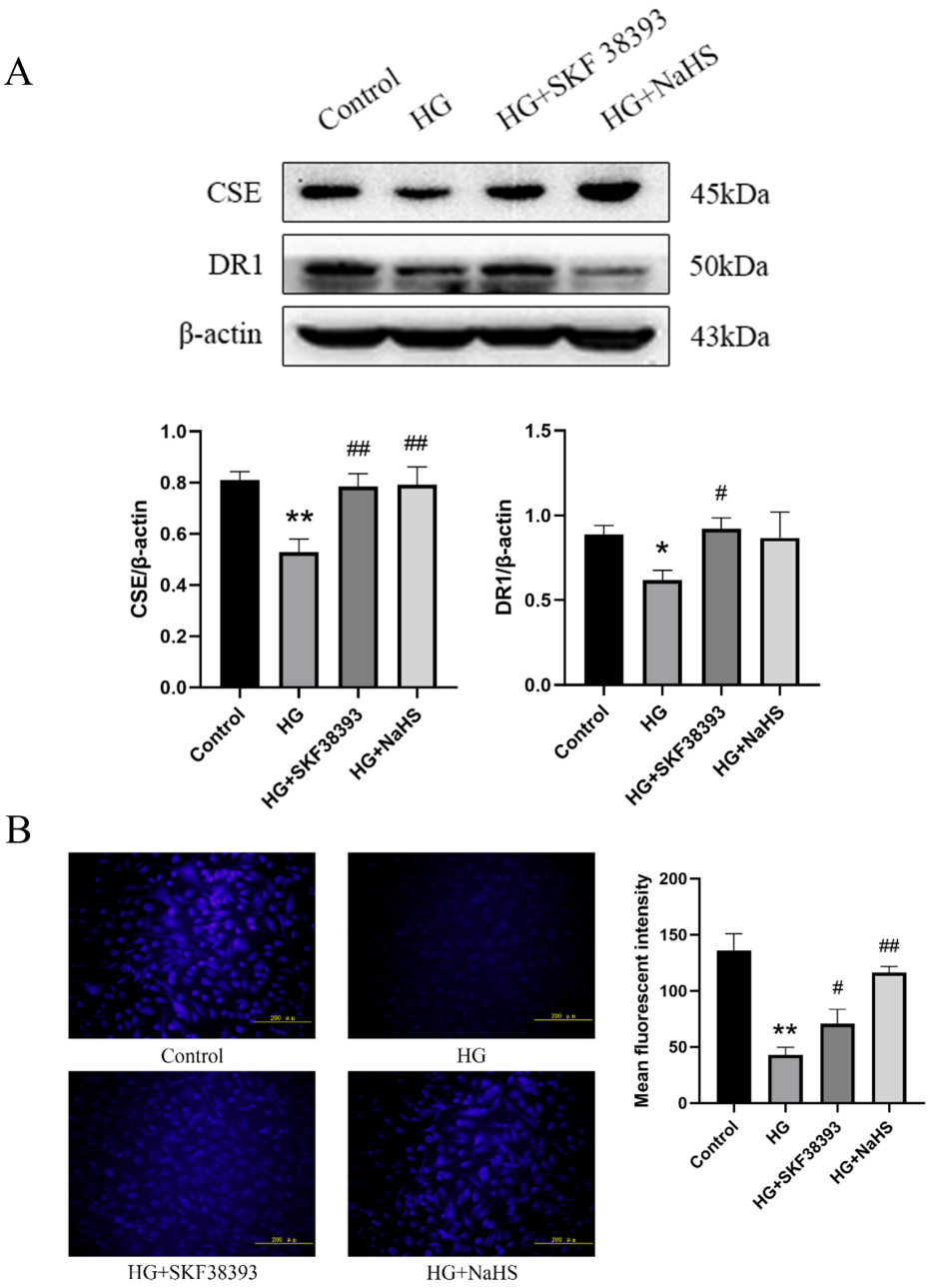

Fig. 1. DR1 activation upregulates the CSE/H_2S pathway in HG-induced HUVECs. (A) The expression DR1 and CSE was determined using western blot (n = 5). The intensity of each band was quantified by densitometry, and data was normalized to the β-actin signal. (B) Fluorescence microscopy was used to detect H_2S levels in the HUVECs (n = 4, magnification $\times$200; scale bar, 200 μm). The results were expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group.

as increased vascular endothelial cell proliferation and migration, microvessel formation and wound and ulcer healing, both *in vivo* and *in vitro* [36]. Many studies have indicated that the CSE/H_2S system is downregulated under HG conditions, including diabetic animal models and *in vitro* studies [37,38]. According to previous studies, stimulation of DR1 in dermal fibroblasts restores vascular endothelial growth factor A production, resulting in adequate angiogenesis and subsequent healing of cutaneous wounds in diabetic mice [39]. However, the function of DR1 in diabetic endothelial dysfunction has rarely been explored. The results of the present study revealed that DR1 and CSE expression and H_2S production were reduced in HG-treated HUVECs, while SKF38393 significantly increased H_2S production and DR1 and CSE expression. In addition, NaHS only increased CSE expression and H_2S production

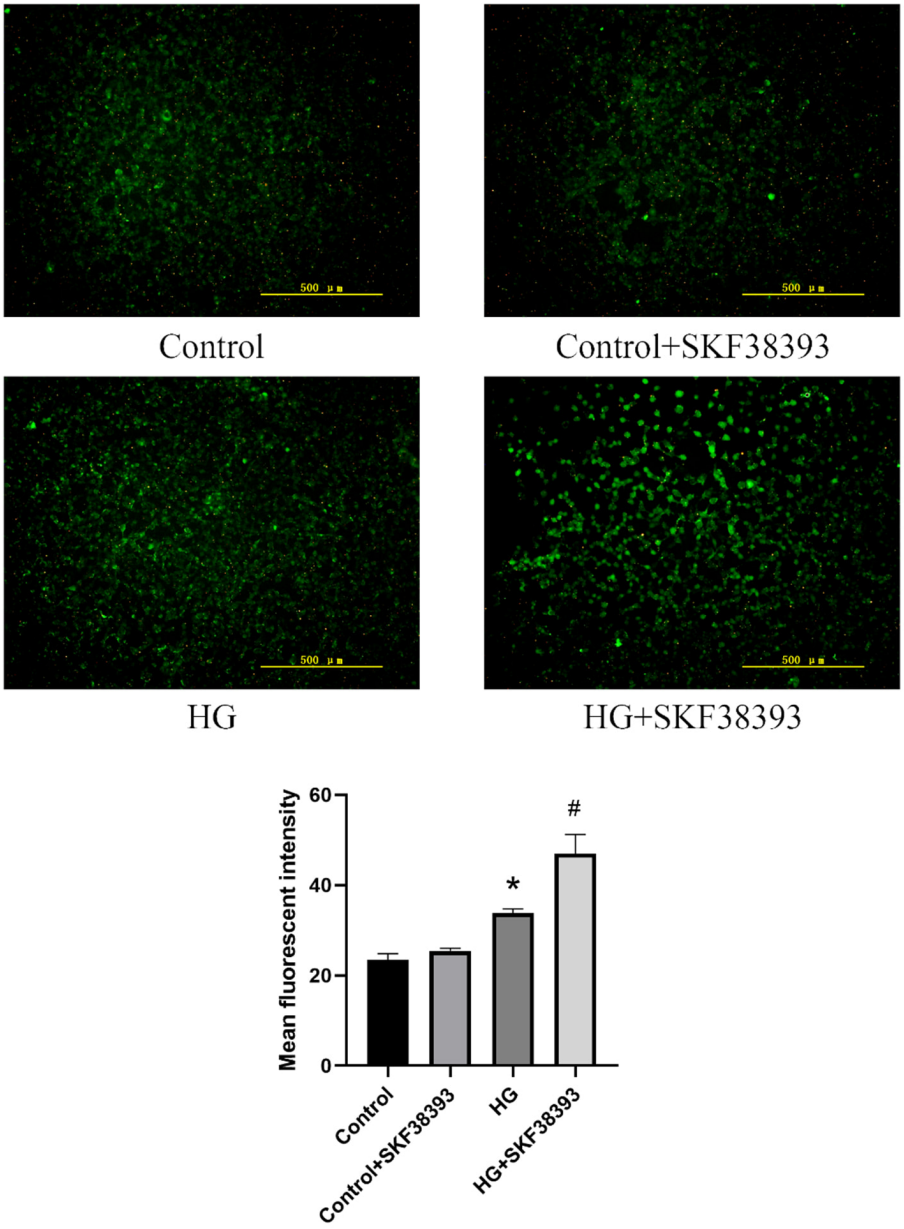

Fig. 2. DR1 activation upregulates the CSE/H_2S pathway by increasing intracellular Ca^{2+} concentration in HG-induced HUVECs. Fluorescence microscopy was used to detect intracellular Ca^{2+} concentration in the HUVECs (n = 3, magnification $\times$100; scale bar, 500 μm). The results were expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. HG group.

but had no effect on DR1 expression. Based on these results, HG-induced endothelial dysfunction is related to a decrease in the activity of the DR1-CSE/H_2S pathway. DR1 activation upregulates the CSE/H_2S pathway, and DR1 is an upstream regulatory factor of the CSE/H_2S pathway.

How does DR1 regulate CSE/H_2S pathway? DR1 couples the PLC signalling pathway that triggers intracellular calcium release [17,18,40]. A previous study confirmed that DR1 activation promotes hypoxia/reoxygenation injury of cardiomyocytes by increasing $[Ca^{2+}]_i$ [21]. Moreover, according to Yang *et al.* [24], the increase in $[Ca^{2+}]_i$ and the activation of the Ca^{2+}-CaM complex activates CSE, which in turn stimulates endogenous H_2S production in endothelial cells. Therefore, we detected the $[Ca^{2+}]_i$ to further investigate whether DR1 exerts a protective effect on HG-induced endothelial cell dysfunction by regulating $[Ca^{2+}]_i$, and the results showed that HG induced $[Ca^{2+}]_i$ overload in HUVECs, consistent with a previous study [41]. However, the CSE/H_2S pathway was inhibited in HG. Furthermore, SKF38393 increased $[Ca^{2+}]_i$ and activated the CSE/H_2S pathway under HG conditions. Thus, DR1 activation upregulates CSE/H_2S by increasing $[Ca^{2+}]_i$ to a certain level.

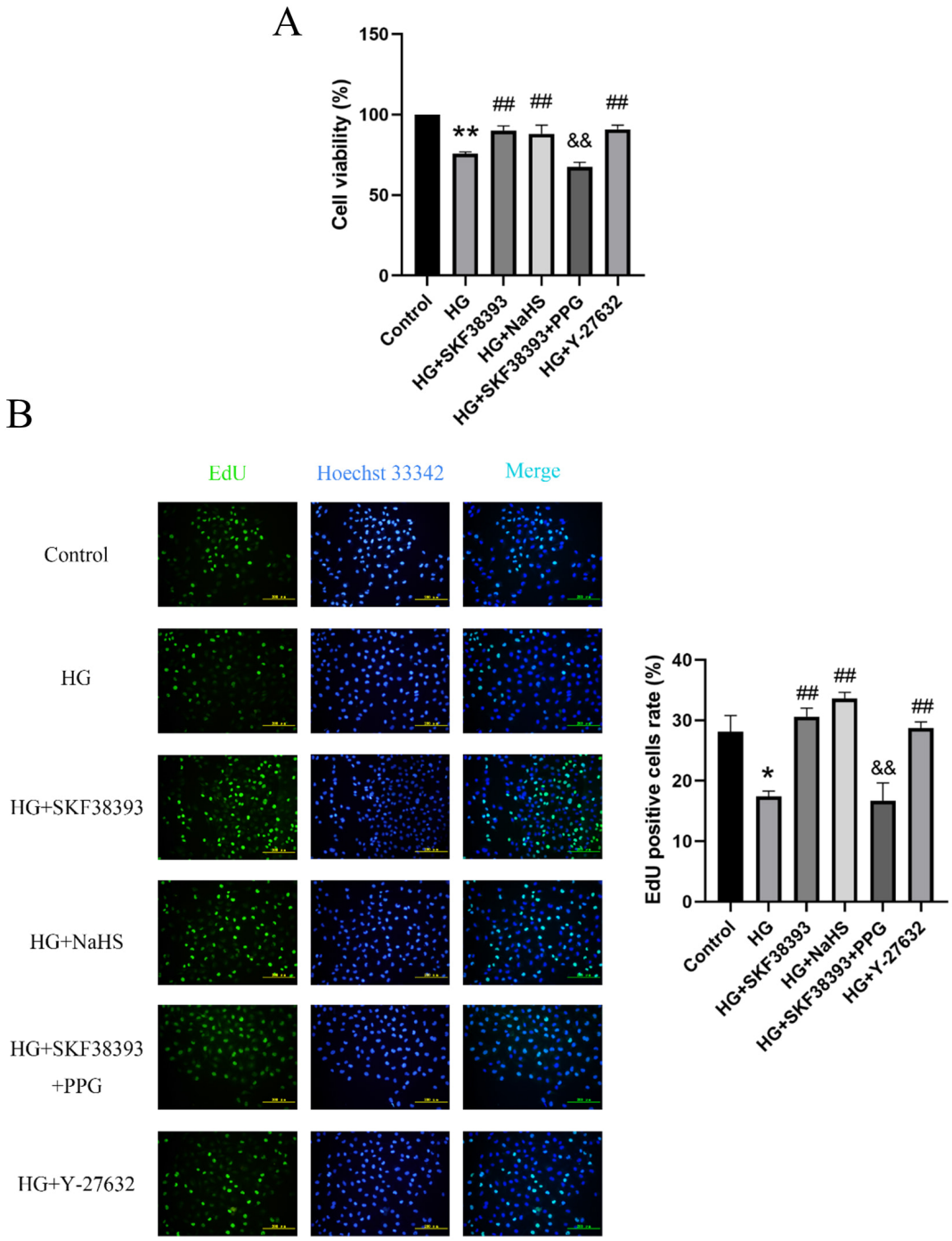

Fig. 3. DR1 activation increases the proliferation of HG-induced HUVECs by activating the CSE/H_2S pathway. (A) Cell viability was detected by CCK-8 kit assay (n = 5). (B) Cell proliferation was detected by EdU proliferation assay (n = 8, magnification $\times$200; scale bar, 200 μm). The results were expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group; && $p < 0.01$ vs. HG+SKF38393 group.

Endothelial cell proliferation and migration are required to promote angiogenesis, which are impaired under HG conditions [13]. The endothelial to mesenchymal transition (EndMT) has been identified as playing a vital role in the pathologic process of diabetic fibrosis. Moreover, HG conditions have been shown to trigger the shift of the endothelium towards the mesenchymal phenotype [4]. In this study, cyclin D1 and PCNA protein expression levels were decreased, while $p21^{Cip/WAF-1}$ protein levels were increased under HG conditions. In addition, cell migration was inhibited under HG conditions. In addition, in the present study, the increased protein levels of

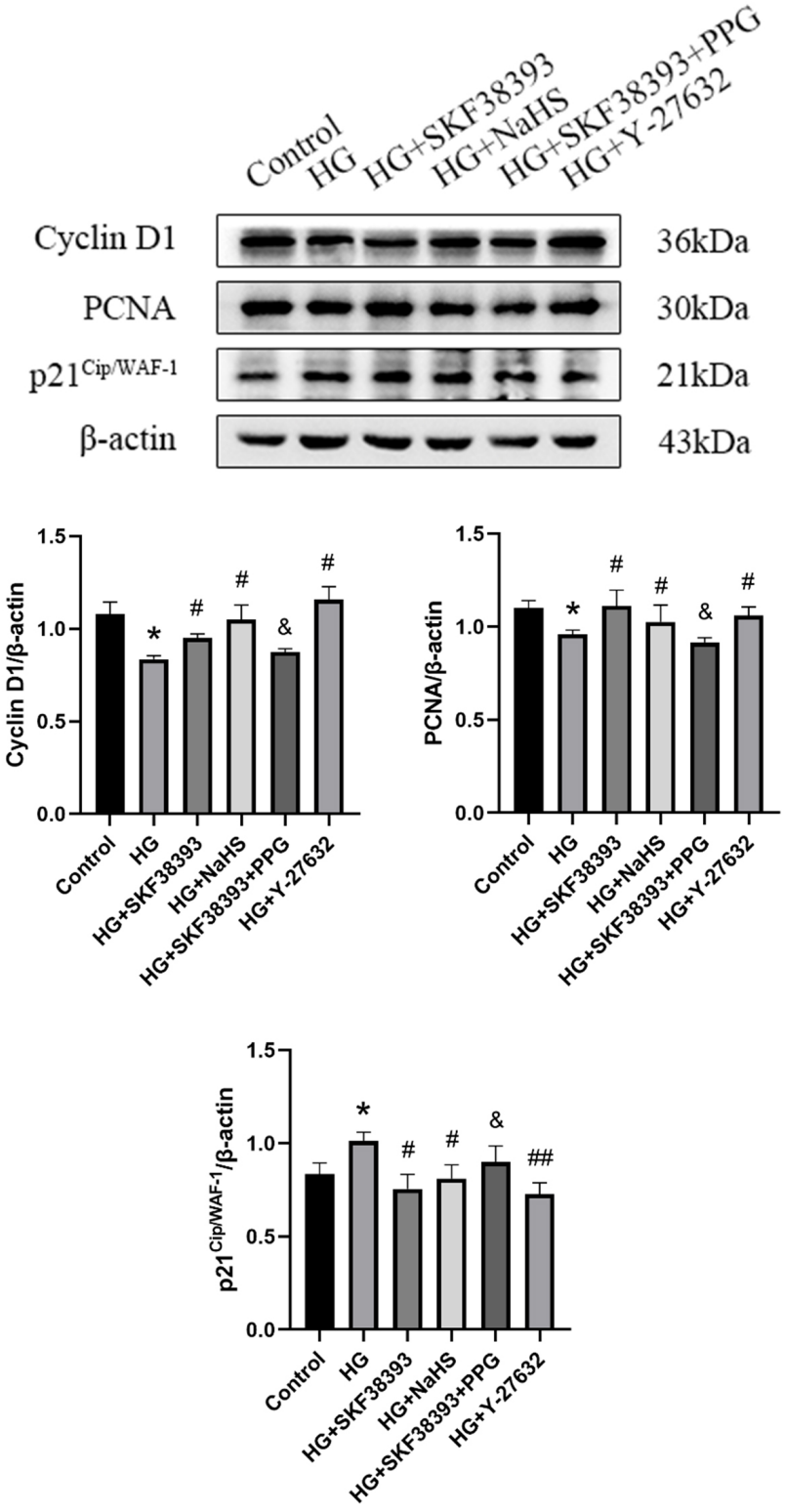

Fig. 4. Effects of DR1 activation on cell proliferation associated proteins by activating the CSE/H_2S pathway in HG-induced HUVECs. Detection of Cyclin D1 (n = 4), PCNA (n = 3) and $p21^{Cip/WAF-1}$ (n = 3) expression levels using western blot. The intensity of each band was quantified by densitometry, and data was normalized to the β-actin signal. The results were expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group; & $p < 0.05$ vs. HG+SKF38393 group.

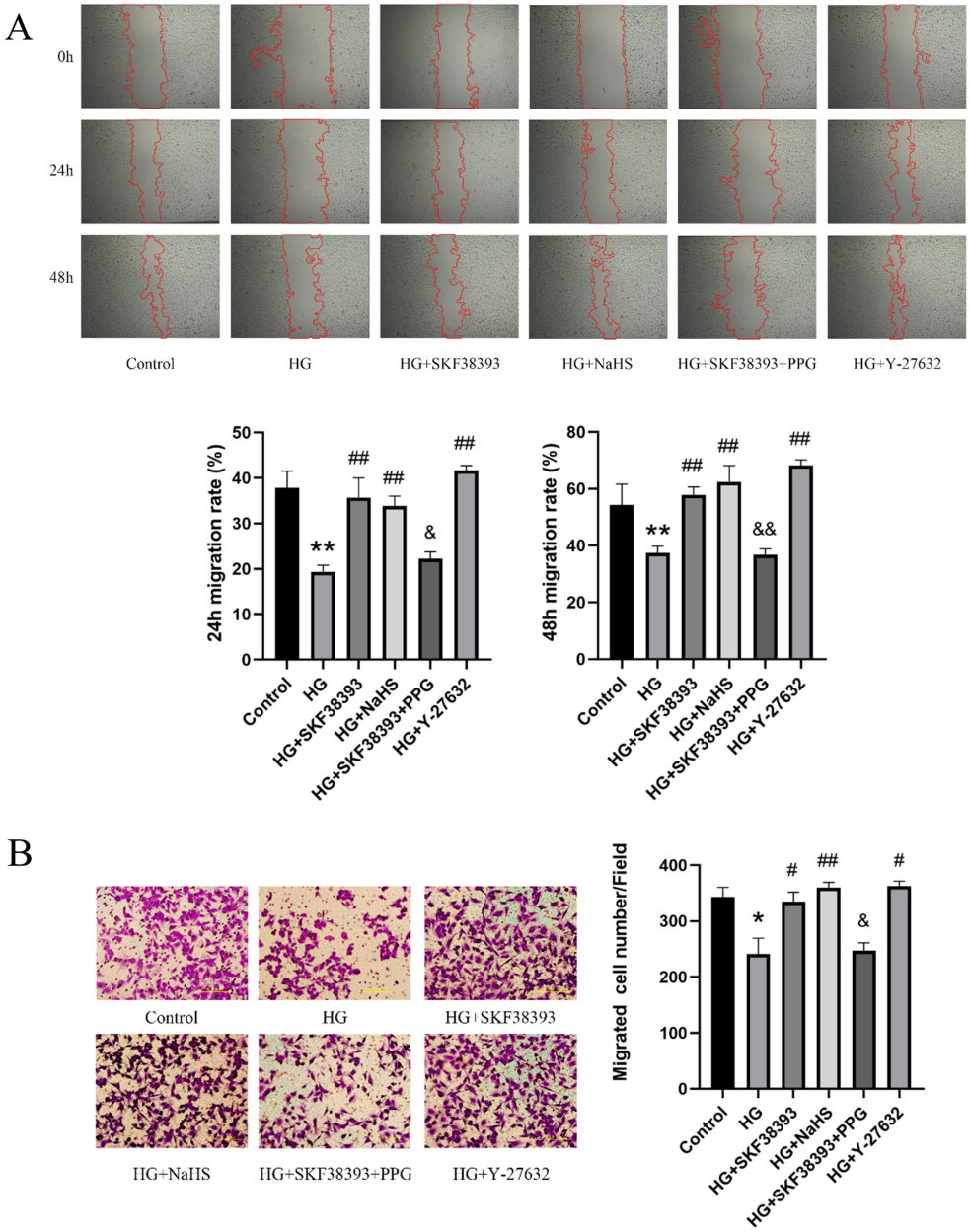

Fig. 5. DR1 activation promotes migration by activating the CSE/H_2S pathway in HG-induced HUVECs. (A) Cell migration was measured by wound healing assays (n = 6). (B) Cell migration was tested via Transwell assay (n = 3, magnification ×200; scale bar, 200 μm). The results were expressed as the mean ± SEM. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group; & $p < 0.05$ vs. HG+SKF38393 group; && $p < 0.01$ vs. HG+SKF38393 group.

mesenchymal markers Col-1, Col-3, MMP-9, α-SMA, and OPN indicated that HG participated in the occurrence of EndMT, consistent with a previous study [33]. Furthermore, HUVEC proliferation and migration were increased after SKF38393 and NaHS treatments. Moreover, DR1 and CSE/H_2S activation mitigate mesenchymal marker expression, thus alleviating the EndMT induced by HG. Our findings suggest that DR1 activation attenuates HG-induced proliferation and migration dysfunction and the EndMT by upregulating the CSE/H_2S pathway in HUVECs.

The phosphorylation of RhoA and ROCK1 is necessary for activation of the RhoA/ROCK1 signalling pathway. RhoA/ROCK1 are involved in multiple important cellular processes including proliferation, migration and angiogenesis [6,42], and its dysregulation is involved in cardiovascular diseases [42]. Moreover, previous reports have shown that RhoA/ROCK1 is activated in HG-induced HUVECs, including angiogenic functions [43] and the EndMT [35]. Furthermore, H_2S can protects cerebral endothelial cells from oxygen-glucose deprivation/reoxygenation-

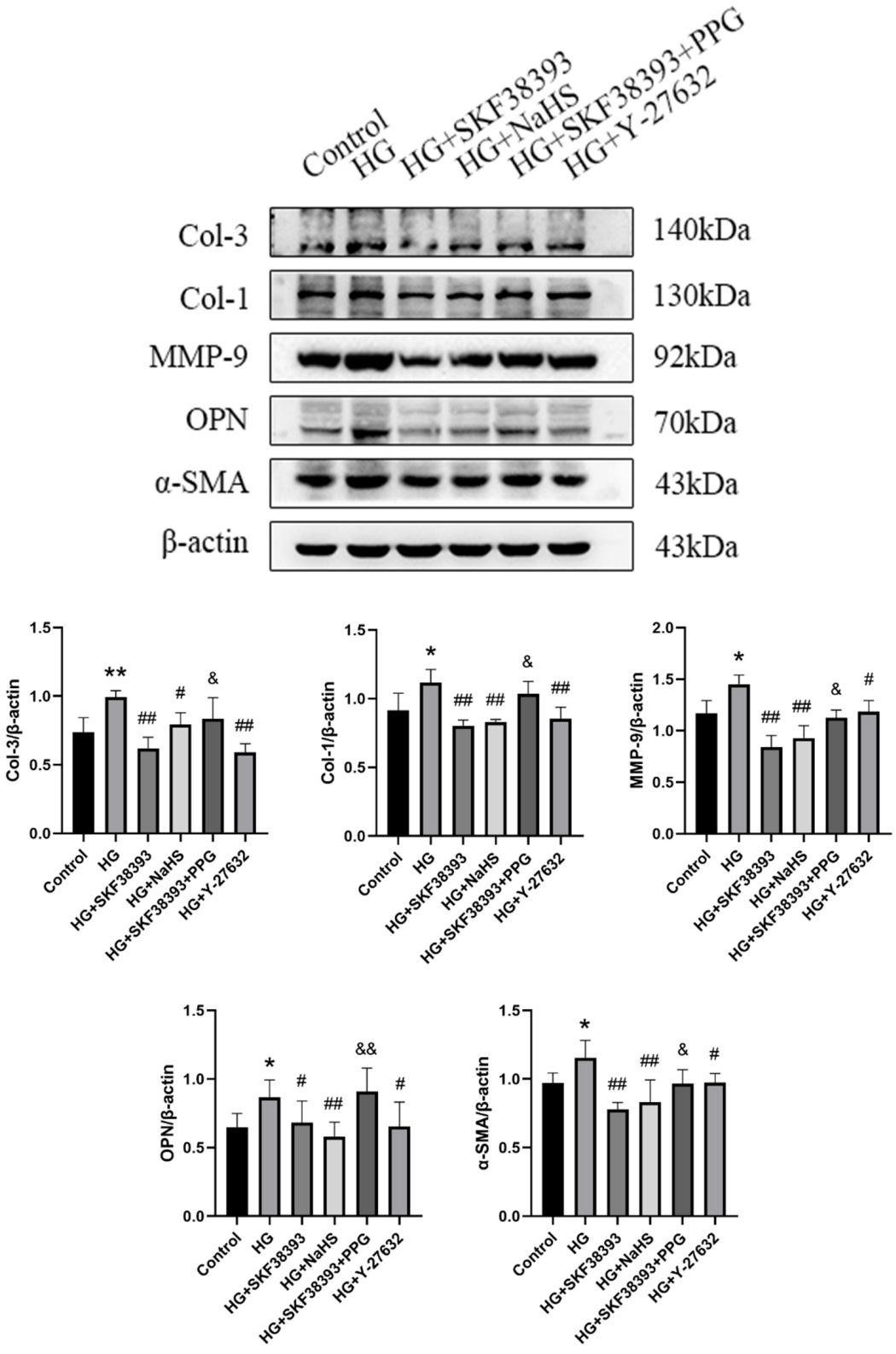

Fig. 6. DR1 activation alleviates the EndMT of HG-induced HUVECs by activating the CSE/H_2S pathway. Detection of Col-3 (n = 4), Col-1 (n = 4), MMP-9 (n = 3), OPN (n = 4) and α-SMA (n = 3) expression levels using western blot. The intensity of each band was quantified by densitometry, and data was normalized to the β-actin signal. The results were expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group; & $p < 0.05$ vs. HG+SKF38393 group; && $p < 0.01$ vs. HG+SKF38393 group.

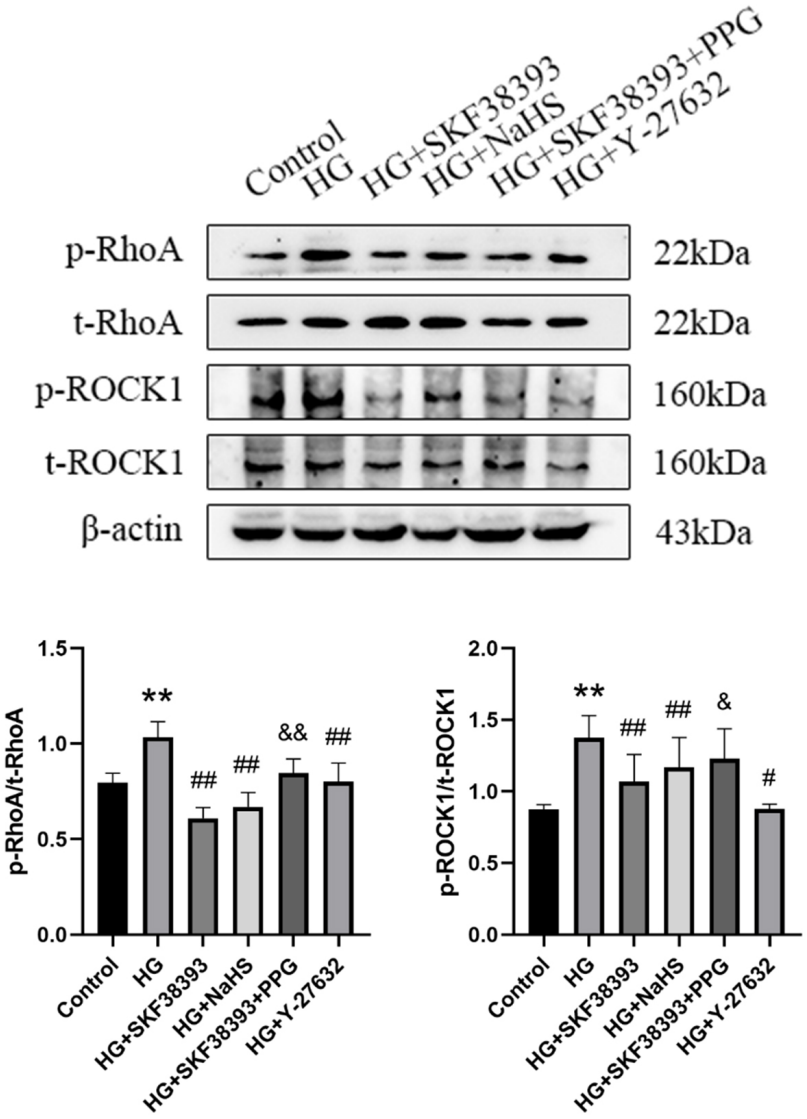

Fig. 7. Activation of the DR1-CSE/H_2S pathway attenuates HG-induced HUVEC dysfunction by inhibiting the RhoA/ROCK1 pathway. Analysis of p-RhoA, RhoA, p-ROCK1 and ROCK1 levels using western blot (n = 4). The intensity of each phosphorylated band was quantified by densitometry, and data was normalized to the corresponding total band signal. The results were expressed as the mean ± SEM. ** $p < 0.01$ vs. control group; # $p < 0.05$ vs. HG group; ## $p < 0.01$ vs. HG group; & $p < 0.05$ vs. HG+SKF38393 group; && $p < 0.01$ vs. HG+SKF38393 group.

induced injury [44], inhibits colonic smooth muscle contraction [45], inhibits reactive astrocytes proliferation and promotes neural functional recovery in cerebral ischaemia/reperfusion injury [46], and improves erectile dysfunction in bilateral cavernous nerve injury [47] by inhibiting the RhoA/ROCK pathway. However, researchers have not yet determined whether DR1 regulates CSE/H_2S and attenuates HG-induced endothelial injury by targeting the RhoA/ROCK pathway. In the present study, SKF38393 and NaHS inhibited the phosphorylation of RhoA and ROCK1 and reversed the HG-mediated EndMT and alterations in proliferation and migration. The beneficial effect of SKF38393 was similar to that of Y-27632 (a ROCK inhibitor). These results suggest that DR1-CSE/H_2S activation inhibits the HG-induced EndMT and alterations in proliferation and migration by attenuating the RhoA/ROCK pathway in HUVECs.

This study has some limitations. First, HUVECs were used to establish an *in vitro* model, which may have resulted in some unexpected outcomes, and other primary endothelial cells and diabetic animal models deserve additional research. Second, overexpression and knockdown of DR1, CSE, RhoA and ROCK1 were not performed to further determine the effects of DR1 activation and H_2S on HG-induced HUVEC injury. Third, the precise molecular targets of H_2S that inhibit RhoA and ROCK1 activity are

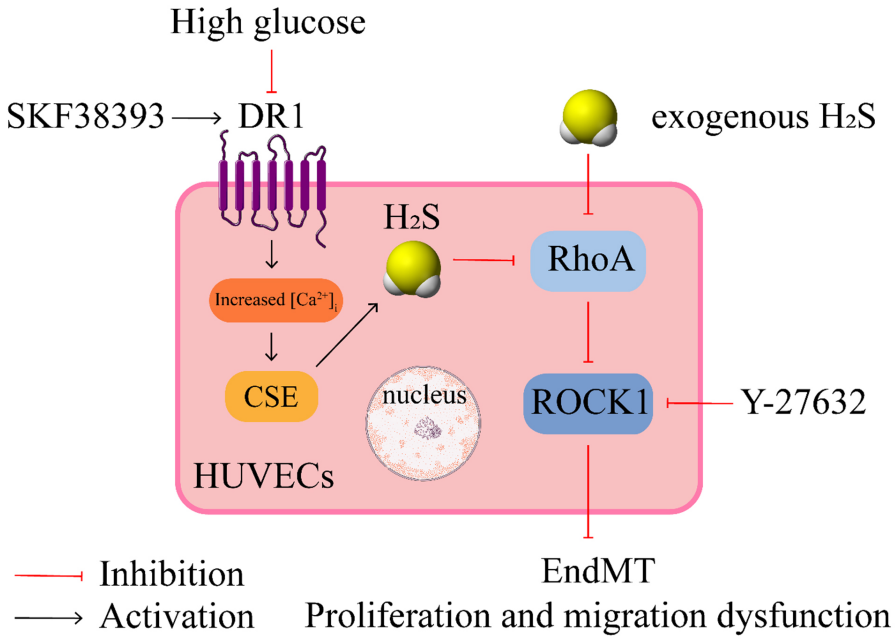

Fig. 8. DR1 up-regulates CSE/H_2S pathway by increasing $[Ca^{2+}]_i$, which inhibits HG-induced endothelial dysfunction through down-regulating RhoA/ROCK1pathway. $[Ca^{2+}]_i$, intracellular calcium concentration; EndMT, endothelial to mesenchymal transition; SKF38393, a dopamine D1-like receptor agonist; Y-27632, a ROCK inhibitor.

not clear. Nalli *et al.* [45] reported that H_2S inhibits smooth muscle contraction via *S*-sulfhydration of RhoA, resulting in inhibition of RhoA and ROCK activities. However, additional investigation is required to unveil the precise molecular mechanism of the interaction between RhoA/ROCK1 and H_2S in HG-induced HUVECs.

5. Conclusions

In summary (Fig. 8), this study showed that DR1 and CSE/H_2S were downregulated under HG conditions, DR1 activation upregulated the CSE/H_2S pathway by increasing $[Ca^{2+}]_i$, and DR1 activation attenuated the HG-induced EndMT and alterations in proliferation and migration by activating the CSE/H_2S pathway, which inhibited the RhoA/ROCK1 pathway. These findings help elucidate the role of DR1 as a significant regulator under HG conditions, and DR1 may be a beneficial target to improve vascular function in patients with diabetes mellitus.

Abbreviations

α-SMA, alpha-smooth muscle actin; AC, adenylyl cyclases; AzMC, 7-Azido-4-Methylcoumarin; CaMKII, calcium/calmodulin-dependent PK II; CBS, cystathionine β-synthase; Col-1, collagen I; Col-3, collagen III; CSE, cystathionine γ-lyase; DAG, diacylglycerol; DM, diabetes mellitus; DR1, dopamine D1-like receptor; DRs, dopamine receptors; EndMT, endothelial-mesenchymal transition; H_2S, hydrogen sulfide; HG, high glucose; HUVECs, human umbilical vein endothelial cells; IP_3, inositol trisphosphate; MMP-9, matrix metalloproteinase 9; MST, 3-mercaptopyruvate sulfurtransferase; OPN, osteopontin; PCNA, proliferating cell nuclear antigen; PLC, phospholipase C; PKC, protein kinase C; PPG, DL-propagylglycine; RhoA, Ras homolog gene family member A; ROCK, Rho-associated coiled-coil containing kinase; TGFβ, transforming growth factor receptor β; VECD, vascular endothelial cell dysfunction.

Author contributions

HZL and SZB conceived the study, designed experiments. GQC, FQS, RW performed the experiments and analyzed the data. GQC, XW, YXX, JHH and AZ prepared the figures and performed statistical analysis. GQC wrote the first draft of the manuscript. CW, AZ and HZL revised the entire manuscript. All authors read and approved the submitted version.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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