Department of Cardiovascular Medicine[1], Shanxi Dayi Hospital Affiliated to Shanxi Medical University; Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology[2], Shanxi University; Department of Gerontology[3], Shanxi Dayi Hospital Affiliated to Shanxi Medical University, Shanxi, China

GLP-1 attenuates Ang II-induced proliferation and migration in rat aorta smooth muscle cells *via* inhibition of the RhoA/ROCK2 signaling pathway

Qian-Feng Xiong[1,#], Shao-Hua Fan[2,#], Xin Zhang[2], Yu Niu[1], Xue-Wen Li[1,*], Ya-Wei Shi[2,*], Li-Hui Zhang[3,*]

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**Corresponding authors: Xue-Wen Li, Department of Cardiovascular Medicine, Shanxi Dayi Hospital Affiliated to Shanxi Medical University; 99 Longcheng street, Taiyuan 030024, Shanxi, China*
Xuewenli1010@126.com
Ya-Wei Shi, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan 030006, Shanxi, China
yaweishi@sxu.edu.cn
Li-Hui Zhang, Department of Gerontology, Shanxi Dayi Hospital Affiliated to Shanxi Medical University, 99 Longcheng street, Taiyuan 030024, Shanxi, China
13485385229@163.com
 # These authors contributed equally to this work

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Glucagon-like peptide 1 (GLP-1), a neuroendocrine hormone produced by the gastrointestinal tract, plays a significant role in blood glucose regulation; drugs derived from GLP-1 are currently used for the treatment of type 2 diabetes. In addition to regulating glucose homeostasis, the protective effects of GLP-1 on the cardiovascular system are also of interest. However, the vascular protective mechanisms of GLP-1 remain unclear. The present study was designed to evaluate the role of GLP-1 in the proliferation and migration of vascular smooth muscle cells, and the underlying mechanisms. In this study, proliferation, migration, cyclin D1 expression, and phosphorylation of MLC, as well as RhoA and Rho-associated coiled-coil forming protein kinase 2 (ROCK2) expression, were increased in rat aorta smooth muscle cells (RASMCs) following incubation with angiotensin II (Ang II). These effects were significantly attenuated by GLP-1, forskolin (a cAMP activator) and Y-27632 (a ROCK2 inhibitor). However, H89 (a PKA inhibitor) inhibited the action of GLP-1, both in terms of inhibition of RASMC proliferation and migration, and RHOA/ROCK2 expression. These results indicate that GLP-1 inhibits Ang II-induced RASMC proliferation and migration via the cAMP/PKA/RhoA/ROCK2 signaling pathway. Our data suggest that GLP-1 should be considered for use in the clinical treatment of cardiovascular diseases, in addition to its current use in the treatment of diabetes mellitus.

1. Introduction

Vascular smooth muscle cell (VSMC) proliferation and migration are important pathological processes in cardiovascular diseases, such as hypertension and atherosclerosis, and are likewise key factors for restenosis after angioplasty (Orr et al. 2010). Previous studies have shown that VSMC proliferation and migration are closely related to a vast number of signaling pathways (Wang et al. 2018). Although the molecular mechanism of vascular diseases is complex and incompletely understood, there is great interest in the development of therapeutic interventions that protect blood vessels from malignant VSMC proliferation and migration.

Glucagon-like peptide 1 (GLP-1), a gut hormone synthesized by specialized enteroendocrine cells located in the small intestine, exerts multiple physiological actions (Drucker 2016). The original physiological effect described for GLP-1 was that of an incretin that stimulates insulin secretion in a glucose-dependent manner (Kreymann et al. 1987). In addition to islet cells, GLP-1 receptors (GLP-1Rs) are also expressed in the heart and blood vessels (Ban et al. 2008). The positive role of GLP-1 in the cardiovascular system is also attracting increasing attention from researchers. The LEADER study (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results – A Long Term Evaluation) found that the GLP-1R receptor agonist liraglutide effectively reduces systolic blood pressure by 1.2 mmHg while treating type 2 diabetes, and the relative risk of cardiovascular death and all-cause mortality was significantly lower in patients with type 2 diabetes and high cardiovascular risk (Marso et al. 2016). Our previous study also demonstrated that liraglutide ameliorated rat blood pressure elevated by angiotensin II (Ang II) treatment (Zhang et al. 2015). Certain reports also indicate that GLP-1 plays an important role in the pathogenesis of atherosclerosis, and animal studies have demonstrated that GLP-1 significantly delays atherosclerotic in ApoE-deficient mice (Nagashima et al. 2011; Arakawa et al. 2010; Jojima et al. 2017; Burgmaier at al. 2013). Considering the potential relationship between the abovementioned diseases and VSMC proliferation and migration, we hypothesized that the positive action of GLP-1 in vascular diseases occurs *via* inhibition of VSMC proliferation and migration, and the molecular mechanism by which GLP-1 exerts anti-proliferation and anti-migration characteristics should be clarified.

Considerable evidence suggests that Rho GTPases and downstream molecules, such as Rho-associated coiled-coil forming protein kinase 2 (ROCK2), regulate cell proliferation, migration, adhesion, and apoptosis by regulating cell shape, cytoskeletal dynamics and cell-cell contacts (David et al. 2012). ROCK1 and ROCK2 promote VSMC proliferation via ERK nuclear translocation, thus regulating the expression of PCNA and cyclin D1 protein (Zhao et al. 2013). ROCK2 also phosphorylates myosin light chain (MLC) by activating myosin light chain kinase, causing the contraction of myosin, which leads to VSMC migration. Researchers have also

found that GLP-1 inhibits the glucotoxicity-induced activation of RhoA/ROCK, which potentiates glucose-stimulated insulin secretion and disassembles stress fibers (Kong et al. 2014). GLP-1 protects cardiac microvascular endothelial cells (CMECs) against oxidative stress and apoptosis, where those effects exerted by GLP-1 are dependent on the downstream inhibition of Rho (Wang et al. 2013). However, the regulatory effect of GLP-1 in the Rho/ROCK pathway on VSMCs is still unclear.

In this research, we studied the role of GLP-1 in the alleviation of Ang II-induced VSMC proliferation and migration. The potential mechanisms underlying these effects were also investigated.

2. Investigation and results

2.1. *GLP-1 inhibited the Ang II-induced proliferation of RASMCs*

The effect of GLP-1 at various concentrations (5, 10, and 20 nM) on RASMC proliferation in response to Ang II was determined. RASMCs treated for 24 h with Ang II showed a significant increase in cell proliferation compared to the control group, and pretreatment with GLP-1 significantly attenuated Ang II-induced proliferation in a concentration-dependent manner (Fig. 1B, $P < 0.05$). GLP-1, at a concentration of 20 nM, significantly inhibited the proliferation of RASMCs. Thus, this concentration was selected for further studies. To exclude the possible influence of GLP-1 on RASMC proliferation, GLP-1 at different concentrations was used to treat cells alone, which had no effect on the proliferation of RASMCs (Fig. 1A). The expression levels of cyclin D1 were also assessed. Stimulation with Ang II for 24 h markedly increased the expression level of cyclin D1 in RASMCs and GLP-1 suppressed this increase (Fig. 1C and D, $P < 0.05$).

2.2. *GLP-1 inhibited the Ang II-induced migration of RASMCs*

The migration capacity of RASMCs was measured by wound-healing assay. The scratch wound assay indicated that incubation with Ang II resulted in significantly greater wound healing than in the control group. Pretreatment with GLP-1 attenuated the effects of Ang II on RASMC migration (Fig. 2A and B, $P < 0.05$). In addition, Western blot analysis showed that Ang II treatment markedly increased the level of p-MLC relative to the control. With the addition of GLP-1 and the increase in concentration, the phosphorylation level of MLC gradually decreased (Fig. 2C and D, $P < 0.05$).

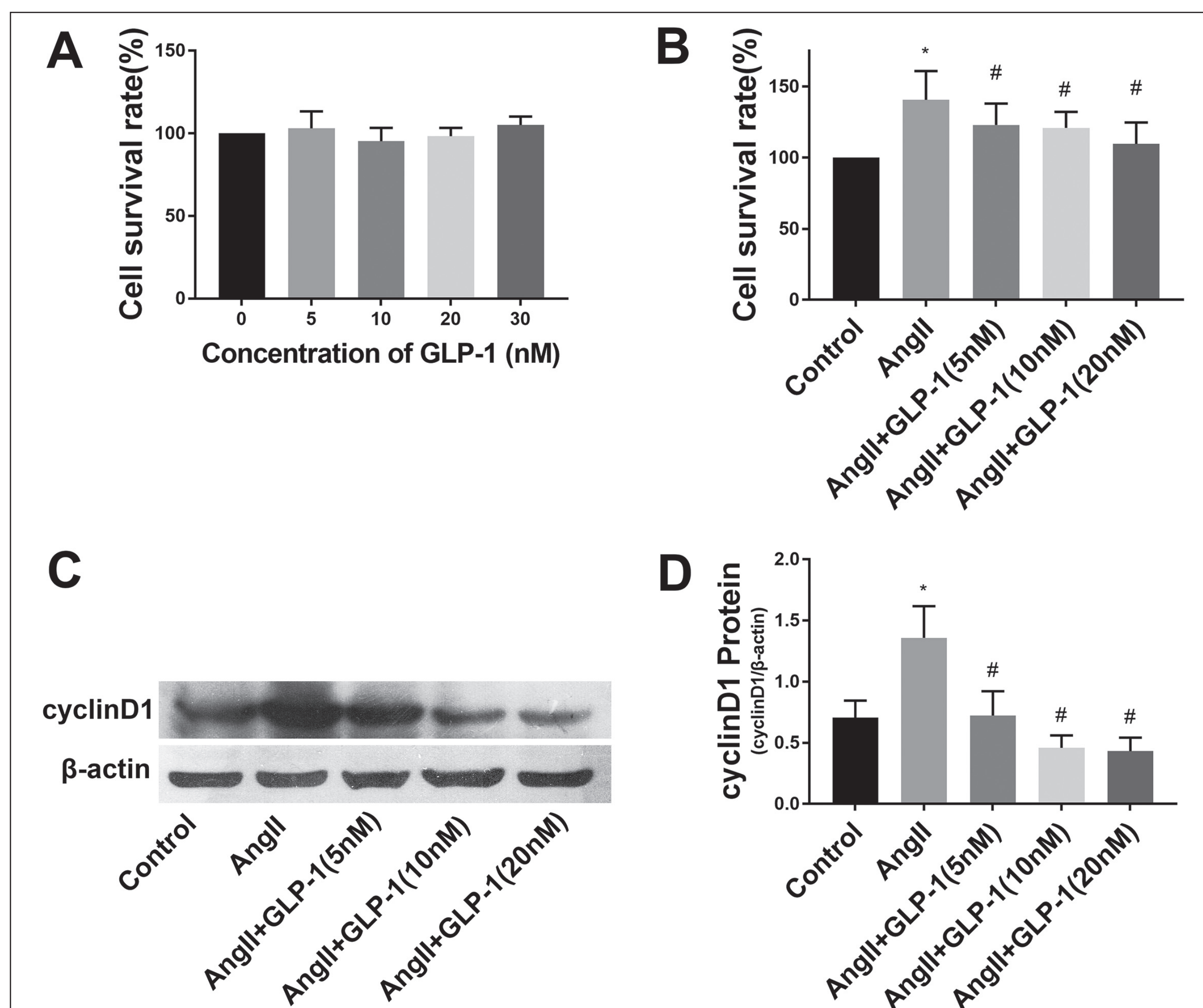

Fig. 1: Glucagon-like peptide 1 (GLP-1) inhibited angiotensin II (Ang II)-induced proliferation in rat aorta smooth muscle cells (RASMCs). (A) The effect of various concentrations of GLP-1 (5, 10, 20, and 30 nM) on RASMC proliferation. Cell proliferation was determined using the MTT assay. (B) The effect of GLP-1 (5, 10, and 20 nM) on Ang II-induced RASMC proliferation. Cell proliferation was determined using the MTT assay. (C) Cells were pretreated with different concentrations of GLP-1 (5, 10, and 20 nM) in the absence or presence of Ang II (10^{-7} M) for 24 h. The protein expression levels of cyclin D1 were evaluated by Western blot. (D) Densitometric data of cyclin D1. Data are presented as means ± SD (n = 3, *$P < 0.05$ vs. control, # $P < 0.05$ vs. Ang II).

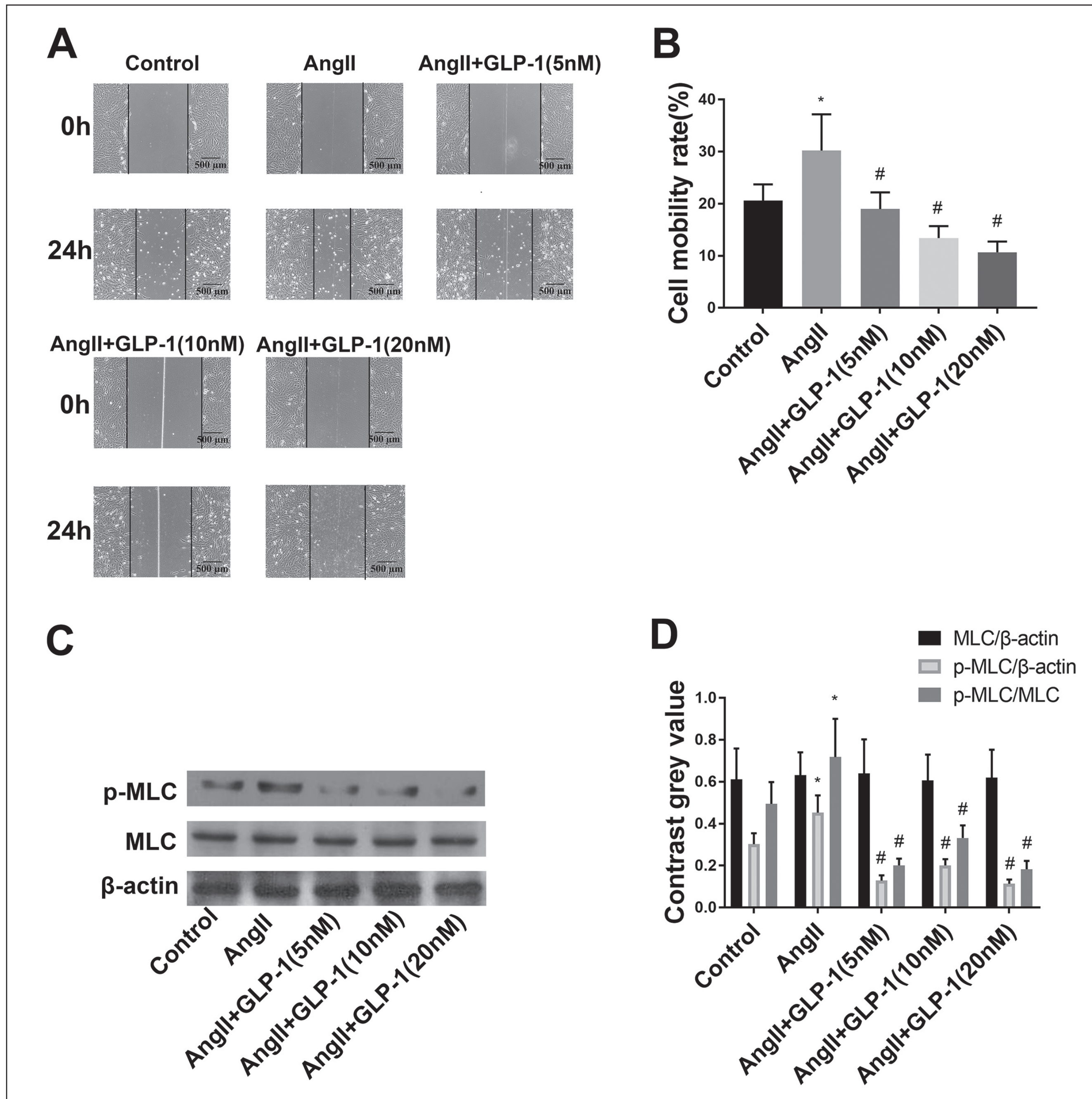

Fig. 2: GLP-1 inhibited Ang II-induced migration in RASMCs. (A) The effect of GLP-1 (5, 10, and 20 nM) on Ang II-induced RASMC migration. Cell migration was measured using the wound-healing assay. (B) Quantification of the wound area. (C) Protein expression levels of p-MLC were evaluated by Western blot. (D) Densitometric data of p-MLC. Data are presented as means ± SD (n = 3,* $P < 0.05$ vs. control, # $P < 0.05$ vs. Ang II).

2.3. GLP-1 suppressed the Ang II-induced activation of RhoA/ROCK2 in RASMCs via the cAMP/PKA pathway

As key molecules in the regulation of cardiovascular function, Rho GTPase and its downstream effector ROCK are not only involved in the regulation of cell proliferation, but also migration. The role of GLP-1 in RhoA and ROCK2 expression in RASMCs was examined. RhoA and ROCK2 expression were upregulated in RASMCs treated with Ang II, but pretreatment with GLP-1 significantly attenuated the Ang II-induced upregulation of RhoA and ROCK2 expression (Fig. 3A and B, $P < 0.05$). RASMC incubation with the PKA inhibitor H89 abrogated the effects of GLP-1 on RhoA and ROCK2 suppression (Fig. 3C and D, P < 0.05). Inhibition of RhoA and ROCK2 expression was also observed after pretreatment with exendin-4 (Ex-4) or forskolin, and the effect of Ex-4 was also abrogated by H89 (Fig. 3E and F, P < 0.05).

2.4. GLP-1 inhibited the Ang II-induced proliferation of RASMCs via the cAMP/PKA/RhoA/ROCK2 signaling pathway

To further investigate the mechanism involved in the positive effects of GLP-1 on RASMCs, a selective inhibitor of ROCK2 (Y-27632) was used. Like GLP-1, Ex-4, forskolin or Y-27632 can suppress Ang II-induced increases in the proliferation of RASMCs. The inhibitory effects of GLP-1 and Ex-4 on Ang II-induced RASMC proliferation were significantly attenuated by incubation with H89. Furthermore, the combination of GLP-1 and Y-27632 further attenuated RASMC proliferation (Fig. 4A and C, $P < 0.05$). Consistent with these above changes, the expression of cyclin D1, as assessed by Western blotting, was also suppressed by GLP-1, Ex-4, forskolin, and Y-27632, and the effects of GLP-1 and Ex-4 were eliminated by H89 (Fig. 4B and D, P < 0.05).

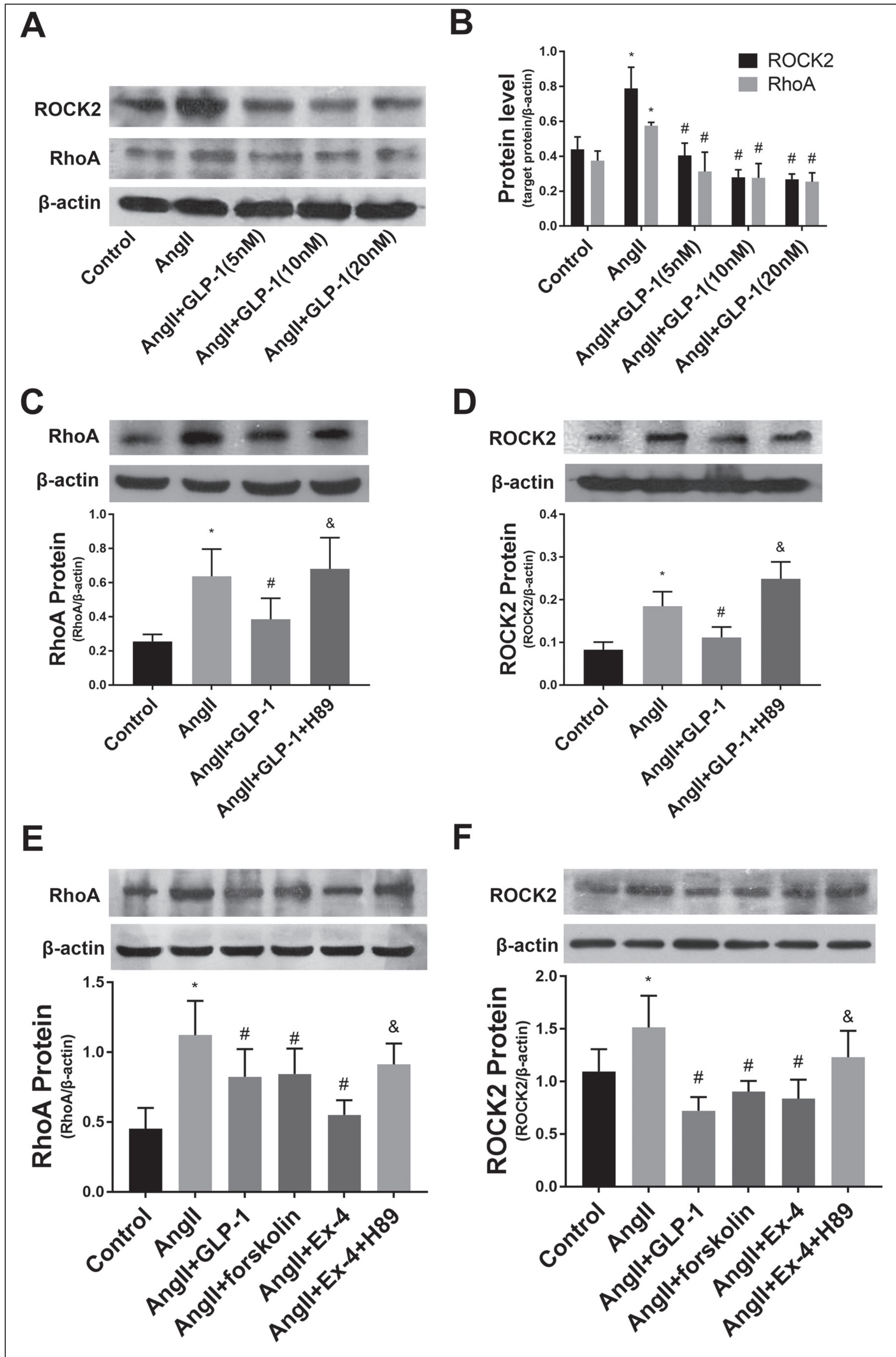

Fig. 3: GLP-1 suppressed Ang II-upregulated RhoA/Rho-associated coiled-coil forming protein kinase 2 (ROCK2) expression via the cAMP/PKA signaling pathway. (A) Protein expression levels of RhoA and ROCK2 were evaluated by Western blot. (B) Densitometric data of RhoA and ROCK2. (C) The protein expression level of RhoA was evaluated by Western blot after pretreatment with H89. (D) The protein expression level of ROCK2 was evaluated by Western blot after pretreatment with H89. (E) The protein expression level of RhoA was evaluated by Western blot after pretreatment with forskolin or exendin-4 (Ex-4). (F) The protein expression level of ROCK2 was evaluated by Western blot after pretreatment with forskolin or Ex-4. Data are presented as means ± SD (n = 3,* $P < 0.05$ vs. control, # $P < 0.05$ vs. Ang II, & $P < 0.05$ vs. Ang II+GLP-1 or Ang II+Ex-4).

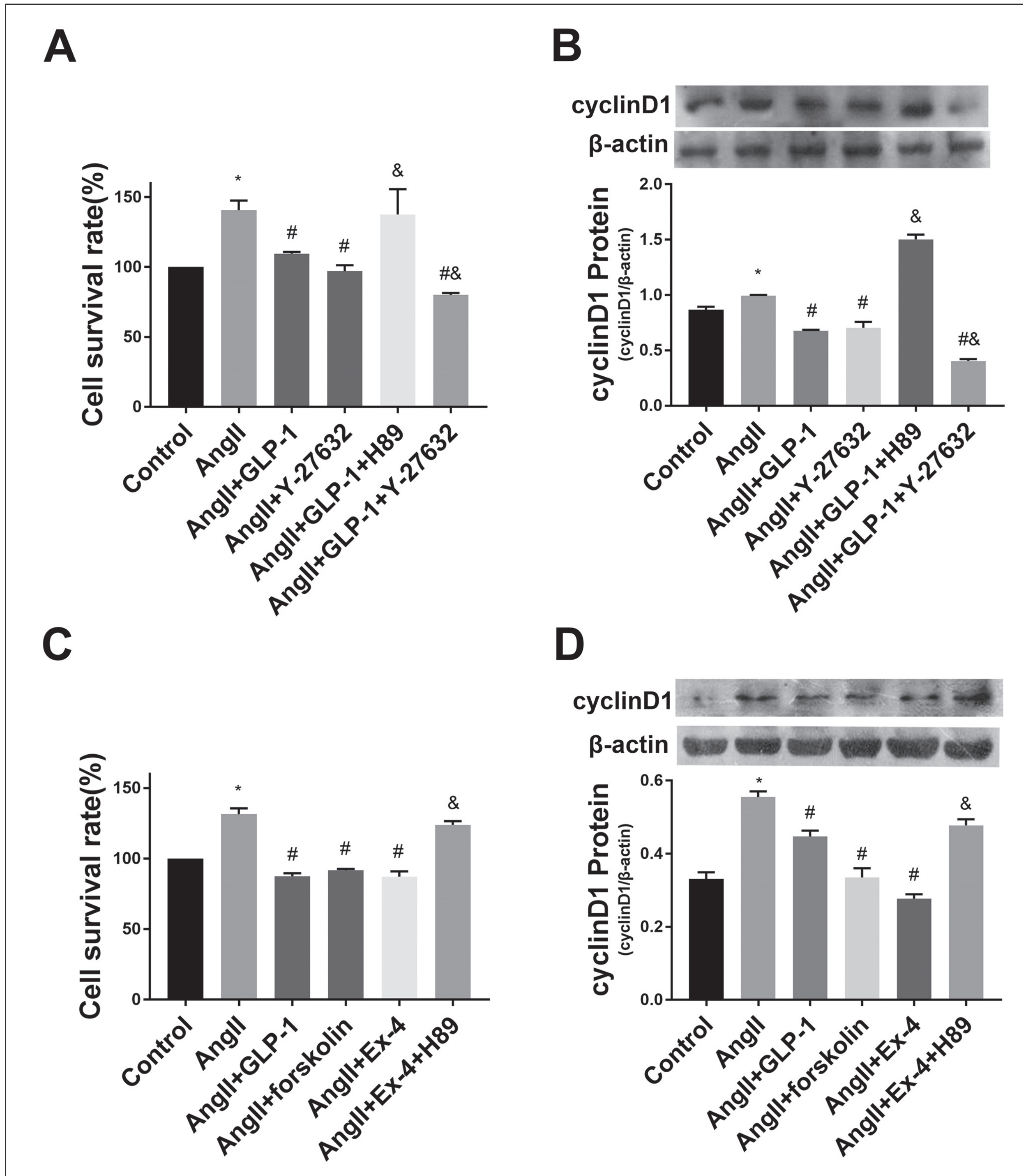

Fig. 4: Effects of GLP-1, Ex-4, forskolin, Y-27632, and H89 on Ang II-induced RASMC proliferation. (A and C) Cell survival was measured by the MTT assay. (B and D) Protein expression level of cyclin D1 was evaluated by Western blot. Data are presented as means ± SD (n = 3,* $P < 0.05$ vs. control, # $P < 0.05$ vs. Ang II, & $P < 0.05$ vs. Ang II+GLP-1 or Ang II+Ex-4).

2.5. GLP-1 inhibited the Ang II-induced migration of RASMCs via the cAMP/PKA/RhoA/ROCK2 signaling pathway

Regarding cell migration, Y-27632, forskolin, Ex-4, and GLP-1 all inhibited Ang II-induced migration, and the inhibitory effect of GLP-1 or Ex-4 on migration was abolished by H89 (Fig. 5A and E, $P < 0.05$). In addition, Western blot analysis showed that Y-27632 and GLP-1 significantly suppressed the elevated level of p-MLC induced by Ang II treatment, and H89 abrogated the GLP-1-induced suppression of Ang II-induced phosphorylation of MLC (Fig. 5C and G, P < 0.05).

3. Discussion

GLP-1, one of the most promising hormones with hypoglycemic properties, exhibits potential protective effects on the cardiovascular system (Drucker 2016). However, it is unclear whether

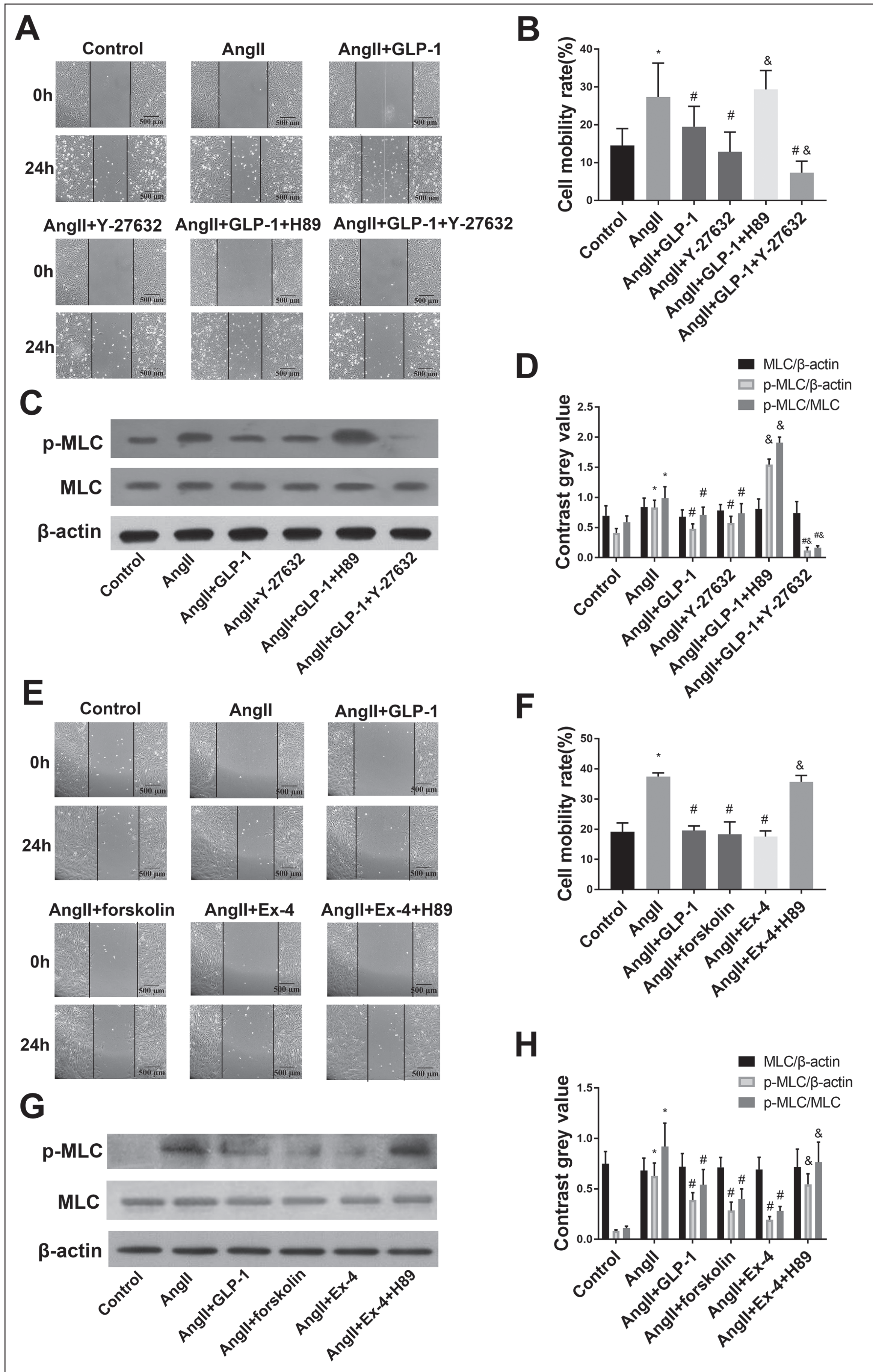

Fig. 5: Effects of GLP-1, Ex-4, forskolin, Y-27632, and H89 on Ang II-induced RASMC migration. (A and E) Cell migration was measured by the wound-healing assay. (C and G) Protein expression level of p-MLC was evaluated by Western blot. Data are presented as means ± SD (n = 3,* $P < 0.05$ vs. control, # $P < 0.05$ vs. Ang II, & $P < 0.05$ vs. Ang II+GLP-1 or Ang II+Ex-4).

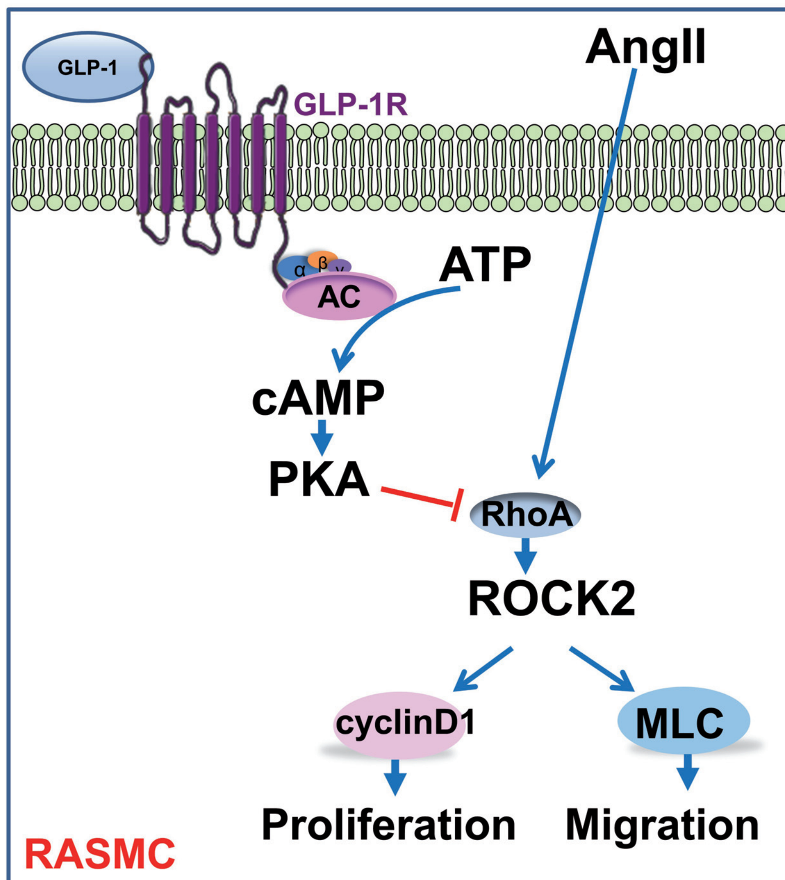

Fig. 6: Proposed mechanism of the protective effect of GLP-1 on Ang II-induced RASMC proliferation and migration. Ang II directly promotes RhoA/ROCK2 activation, triggering cell proliferation and migration by regulating cyclin D1 and MLC phosphorylation, respectively. GLP-1 pretreatment promotes the activation of cAMP/PKA, leading to a decrease in RhoA/ROCK2 expression, and inhibiting the proliferation and migration of RASMCs induced by Ang II.

GLP-1 has a direct effect on Ang II-induced RASMC proliferation and migration, and the mechanism of any such effect has not yet been determined.

VSMC proliferation and migration are important causes of vascular remodeling, which is a pathological change seen in the development of cardiovascular diseases such as hypertension, atherosclerosis and restenosis after angioplasty (Orr et al. 2010). RASMCs, embryonic rat-thoracic-derived cells line have similar properties to primary VSMCs, have often been used for in vitro studies. Ang II is well-known for causing many cardiovascular diseases through its type 1 receptor, which can effectively induce proliferation and migration in RASMCs (Zahradka et al. 2002). In this study, an Ang II-induced intervention model was used to evaluate the potential impact of GLP-1 on RASMCs.

A decrease in systolic blood pressure of 1.2 mm Hg with the GLP-1 receptor agonist liraglutide was reported in the LEADER trial, a large-scale randomized, placebo-controlled cardiovascular outcomes trial (Marso et al. 2016). Our previous study demonstrated that preservation of GLP-1 using liraglutide or linagliptin is effective at ameliorating the blood pressure increase and cardiac fibrosis induced by Ang II infusion using osmotic minipumps in vivo (Zhang et al. 2015). Additionally, GLP-1 attenuates the development of atherosclerosis in ApoE-knockout mouse models (Jojima et al. 2017). These positive effects of GLP-1 against vascular diseases suggest that GLP-1 is involved in regulating the proliferation and migration of VSMCs. In the present study, Ang II-induced proliferation and migration were dramatically inhibited by the addition of GLP-1 or the GLP-1 analogue Ex-4 in RASMCs.

Our results further demonstrate that GLP-1 had a protective role in RASMC incubation with Ang II, possibly by inhibiting the RhoA/ROCK signaling pathway. Accumulating evidence has demonstrated that the Rho/ROCK signaling pathway is involved in the process of RASMC proliferation and migration (Shimokawa and Rashid 2007). Recent studies have shown that GLP-1 enhanced glucose-stimulated insulin secretion in beta cells (Kong et al. 2014) and protected endothelial cells against hyperglycemia-induced oxidative stress (Wang et al. 2013) by inhibiting activation of the RhoA/ROCK pathway. However, few studies have explored the role of GLP-1 in Rho/ROCK pathway regulation in VSMCs. The present study revealed that treatment of RASMCs with Ang II promoted the expression of RhoA and its downstream molecule ROCK2, one of the two isoforms of ROCK protein that is mainly expressed in smooth muscle cells, both of which were inhibited by pretreatment with GLP-1 in a concentration-dependent manner. The above data demonstrate that the positive effects of GLP-1 on cell proliferation and migration may be regulated via the RhoA/ROCK2 signaling pathway in RASMCs.

The mechanism by which GLP-1 regulates the RhoA/ROCK pathway in RASMCs remains unclear. It is well-known that GLP-1 stimulates cAMP/PKA by activating GLP-1R, resulting in insulin synthesis and release in pancreatic beta cells (Flamez et al. 1999). Reactive oxygen species-induced senescence in human umbilical vein endothelial cells was attenuated by GLP-1, which involved GLP-1R/PKA signaling (Oeseburg et al. 2010). cAMP/PKA may be a negative regulator of Rho in Nf1+/− central nervous system neurons and CMECs (Brown et al. 2012). GLP-1 protects CMECs against oxidative stress and apoptosis, where those effects, as exerted by GLP-1, depend on the downstream inhibition of Rho (Wang et al. 2013). H89, a PKA inhibitor, significantly abrogated the effects of GLP-1 and Ex-4 on cyclin D1, p-MLC, and RhoA/ROCK2 suppression. Conversely, the cAMP activator forskolin acts similarly to GLP-1, inhibiting RASMC proliferation and migration and the expression of related proteins. These findings suggest that cAMP/PKA may be involved in the regulation of GLP-1-mediated inhibition of Ang II-upregulated RhoA/ROCK2 expression in RASMCs. Furthermore, Y-27632, a selective ROCK2 inhibitor, also inhibited Ang II-induced proliferation and migration in RASMCs, and greater suppression was observed when Y-27632 was incubated together with GLP-1. Collectively, these findings establish that cAMP/PKA/RhoA/ROCK2 signaling is a possible pathway underlying GLP-1-induced protective effects.

In conclusion, this study clearly demonstrated that GLP-1 inhibited Ang II-induced RASMC proliferation and migration via the cAMP/PKA/RhoA/ROCK2 signaling pathway, which may be the main mechanism of the hypotensive and anti-atherosclerosis effects of GLP-1. Further in vivo studies are needed to confirm the positive effects of GLP-1 on angiopathy and the underlying mechanism of action; this can be accomplished by using an Ang II-induced hypertensive rat model or spontaneously hypertensive rats. Our present study provides a theoretical basis by which GLP-1 can serve as a promising agent to protect against Ang II-induced cardiovascular diseases, in addition to exerting a hypoglycemic effect.

4. Experimental

4.1. Cell culture and treatment

RASMCs, originally derived from embryonic rat aortas, were purchased from the Shanghai Institute of Cell Biology, China. Cells were cultured in Dulbecco's modified Eagle's medium (Boster, Wuhan, China) supplemented with fetal bovine serum (CellMax, Beijing, China) to a final concentration of 10%, and antibiotics (Boster) at 37 °C with 5 % CO_2.

4.2. Drug/chemical treatment

All cultures were incubated with serum-free media for 24 h prior to treatment. Cultures of RASMCs were divided into the following groups: control (phosphate-buffered saline [PBS]); Ang II (10^{-7} M) (synthesized by Sangon Biotech, Shanghai, China); and Ang II+GLP-1 (10^{-7} M Ang II and different concentrations of GLP-1 [synthesized by Sangon Biotech]: 5 nM, 10 nM, and 20 nM); the remaining groups were 20 nM GLP-1, 10 nM Ex-4, 10 μM forskolin, 10 μM Y-27632, and 10 μM H89; the latter four were purchased from MedChemExpress (Monmouth Junction, NJ, USA). All cell cultures were pretreated with the indicated drugs 1 h prior to Ang II treatment.

4.3. MTT assay

Cell proliferation was measured by the MTT assay, which was performed according to published literature (Liu et al. 2014). Briefly, cells were seeded into 96-well plates (10^4 cells/well), serum-starved and treated as described above for 24 h. Next, 20 μL of 5 mg/mL MTT (Solarbio, Beijing, China) was added to each well and incubated for 4 h. The medium was removed, and 150 μL dimethyl sulfoxide (Tianli, Tianjing, China) was added to each well. The plates were incubated on a shaker at room temperature for 10 min, and the optical density (OD) was assayed at 570 nm on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The OD value was recorded, and the relative cell survival rates were calculated.

4.4. Wound-healing assay

The migration capacity of RASMCs was also characterized using a well-established *in vitro* scratch wound model (Akiyama et al. 2008). VSMCs were grown to confluence and then a 200 μL sterile pipette tip was used to scratch the monolayer after serum starvation for 24 h. The scratch wound was allowed to heal for 24 h in the presence or absence of the indicated drug/chemical. Micrographs were captured for each sample at 0 and 24 h, and the capacity of VSMC migration was evaluated by measuring the width of the scratch wound at both time points using NIS-Elements software (Nikon, Tokyo, Japan).

4.5. Western blot analysis

RASMCs from each group were harvested after washing with ice-cold PBS and lysed using RIPA lysis buffer. Total proteins were loaded onto SDS-PAGE gels and transferred electrophoretically to PVDF membranes (Boster). Membranes were blocked with 5% skim milk for 2 h and incubated with primary antibodies overnight at 4 °C. The primary antibodies used were as follows: p-MLC (AF5443, Affinity, Beijing, China) and MLC (AF5423; Affinity) were diluted in 5 % skim milk at a dilution of 1:500; cyclin D1 (BA0770; Boster) was diluted in Tris-buffered saline containing Tween-20 (TBST) at a dilution of 1:1,000; RhoA (BM4573; Boster) and ROCK2 (BM5257; Boster) were diluted in TBST at 1:200; β-actin (AP0060; Bioworld, Nanjing, China) was diluted in TBST at 1:1,000. The membranes were washed and further incubated with a secondary antibody marked by horseradish peroxidase at room temperature for 2 h. Immunoreactive bands were visualized using enhanced chemiluminescence reagents. The level of each target protein was quantified and normalized to that of β-actin.

4.6. Statistical analysis

All group values are expressed as means ± standard deviation (SD). GraphPad Prism 7 software (GraphPad Inc., San Diego, CA, USA) was used to determine statistical significance. Differences between groups were analyzed by Student's t test when two groups were compared, or by one-way analysis of variance when more than two groups were compared. Test results were considered significant at $P < 0.05$.

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Conflicts of interest: None declared.

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