

Simvastatin alleviates epithelial-mesenchymal transition and oxidative stress of high glucose-induced lens epithelial cells *in vitro* by inhibiting RhoA/ROCK signaling

JIANMING FU¹ and XIAOJIE HU²

¹Department of Ophthalmology, The Second People's Hospital of Yueqing, Wenzhou, Zhejiang 325608;

²Department of Ophthalmology, Wenzhou People's Hospital, Wenzhou, Zhejiang 325000, P.R. China

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Abstract. Diabetic cataracts (DC) is one of the main causes of blindness among patients with diabetes mellitus. The aim of the present study was to examine the effect of simvastatin on lens epithelial cells in DC and the underlying mechanism. The viability of SRA01/04 cells treated with different concentrations of simvastatin was detected using a Cell Counting Kit-8 assay before and after high glucose (HG) treatment. The expression levels of E-cadherin, N-cadherin, Vimentin and α -smooth muscle actin (α -SMA), proteins associated with epithelial-mesenchymal transition (EMT), in addition to RhoA, Rho-associated kinases (ROCK)1 and ROCK2, proteins related to RhoA/ROCK signaling, were also measured in SRA01/04 cells treated with HG and simvastatin, with or without U46619, using western blot analysis. DCFH-DA dyes, superoxide dismutase (SOD) and glutathione (GSH)/glutathione disulfide (GSSG) kits were used to measure the levels of oxidative stress parameters in SRA01/04 cells treated with HG and simvastatin with or without U46619. The cell viability of SRA01/04 cells treated with simvastatin was found to be significantly elevated after HG treatment. The protein expression levels of E-cadherin were increased but those of N-cadherin, Vimentin and α -SMA decreased after HG and simvastatin treatment, and this was reversed by U46619. The levels of SOD and GSH-GSSG were found to be increased whereas reactive oxygen species levels were decreased, effects that were reversed by U46619. Additionally, the protein expression levels of RhoA, ROCK1 and ROCK2 were markedly decreased. These findings provided evidence that simvastatin increased HG-induced SRA01/04 cell viability and exerted inhibitory effects on EMT and oxidative stress that occurs during DC.

Introduction

The prevalence of diabetes mellitus is on the increase worldwide (1), leading to escalating global health concerns. Diabetic cataracts (DC) is a common complication of diabetes (2), which is an important cause of blindness in patients with diabetes (3). Cataracts tend to be more prevalent and deteriorate more rapidly in patients with diabetes compared with the general population (4). Human lens epithelial cells forms part of the main optical tissue that is involved in nutrition and ions transportation, metabolism and detoxification during lens development (5). In addition, these cells have been reported to have a key role in cataract formation. In particular, oxidative stress, apoptosis (6), and epithelial-mesenchymal transition (EMT) (2) in human lens epithelial cells induced by high glucose have been previously implicated in the pathogenesis of cataract formation. Cataract surgery is currently the frontline method of treatment for DC. However, cataract surgery in patients with diabetes may lead to poor visual acuity caused by posterior capsular opacification and postoperative cystoid macular edema (7). Thus, optimization of surgical and development of novel pharmacological methods may increase the efficacy for patients with diabetes (8).

Simvastatin (Fig. 1A) is a recognized drug in the statin family that is commonly used to reduce the risk of cardiovascular diseases associated with hypercholesterolemia (9,10). High glucose (HG) contributes to cardiomyocyte dysfunction and injury by promoting apoptosis and decreasing autophagy (11). Simvastatin has been shown to alleviate the injury induced by HG levels in cardiomyocytes (12) and the kidney (13). In addition, findings of a previous showed that simvastatin conferred no adverse effects on the lens (14). Although simvastatin has been reported to reduce the apoptosis of lens epithelial cells at HG levels (15), the precise mechanism underlying the effects of simvastatin on DC remains poorly understood.

Simvastatin is capable of relieving neuropathic pain by inhibiting RhoA activity (16). RhoA is a small guanosine binding protein that is normally localized to the plasma membrane (17). Results of a previous study showed that RhoA/Rho-associated protein kinase (ROCK) signaling activation exerts a key role in the occurrence and development of diabetes complications (18). Additionally, inhibition of RhoA/ROCK signaling, not only alleviated diabetic retinopathy (19), but also inhibited

Correspondence to: Dr Xiaojie Hu, Department of Ophthalmology, Wenzhou People's Hospital, 57 Canghou Lane, Wenzhou, Zhejiang 325000, P.R. China
E-mail: huaxiaojie8019@163.com

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EMT in TGF- β -induced lens epithelial cells (20). However, the effects of simvastatin on DC and the potential mechanism remain unclear. Therefore, the aim of the present study was to investigate the biological effects of simvastatin on oxidative stress and EMT in HG-induced lens epithelial SRA01/04 cells and explore whether the RhoA/ROCK signaling participates in simvastatin-regulated diabetic cataracts.

Materials and methods

Cell culture and HG treatment. The human lens epithelial cell line SRA01/04 was purchased from Guangzhou Jennio Biotech Co., Ltd. Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For the establishment of an *in vitro* DC model, SRA01/04 cells were grown in DMEM with HG (25 mM) for 24 h. Subsequently, HG-induced cells were treated with 1, 5 and 10 nM simvastatin for 48 h at 37°C. The cells were maintained in media containing 5 mM glucose and served as the control group.

Cell counting kit-8 (CCK-8) assay. The CCK-8 assay was performed to measure cell viability. SRA01/04 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and then incubated with different doses of simvastatin for 48 h, followed by the addition of 10 μ l CCK-8 (Dojindo Molecular Technologies, Inc.) solution into each well. The plates were then incubated for 2 h at 37°C. The optical density of each well was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). All experiments were performed three times.

Western blot analysis. Total protein was extracted from SRA01/04 cells after lysis on ice using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA kit (Beyotime Institute of Biotechnology). A total of 30 μ g protein samples per well were then transferred to PDVF membranes after resolving using 10% SDS-PAGE gels. Subsequently, the membranes were washed, blocked with 5% skimmed milk for 1 h and then incubated with the following primary antibodies at 4°C overnight: Anti-E-cadherin antibody (1:10,000; cat. no. ab40772; Abcam), anti-N-cadherin antibody (1:5,000; cat. no. ab76011; Abcam), anti-Vimentin antibody (1:1,000; cat. no. ab92547; Abcam), anti- α -smooth muscle actin (α -SMA) antibody (1:1,000; cat. no. ab265588; Abcam), anti-RhoA antibody (1:5,000; cat. no. ab187027; Abcam), anti-ROCK1 antibody (1:1,000; cat. no. ab92547; Abcam) or anti-ROCK2 antibody (1:1,000; cat. no. ab134181; Abcam). After washing with PBS, the membranes were incubated with the HRP-conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. ab6721; Abcam) for 2 h at room temperature. All antibodies utilized in the present study were purchased from Abcam. GAPDH was used as the loading control. The protein blots were visualized using enhanced chemiluminescence (ECL) reagent and densitometry analysis of the bands was performed using ImageJ (National Institutes of Health).

Measurements of reactive oxygen species (ROS), superoxide dismutase (SOD) and glutathione (GSH)/glutathione disulfide (GSSG). Detection of ROS, SOD and GSH-GSSG

was performed to assess the extent of oxidative stress in SRA01/04 cells. ROS levels were measured using the DCFH-DA measurement kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.) in the dark at 37°C. SOD activities were assessed using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.), following the manufacturer's protocols. GSH/GSSG activities were evaluated using the Glutathione (GSH) assay Kit (Abnova) in accordance with the manufacturer's protocols.

Statistical analysis. All experimental data were calculated using SPSS 17.0 (SPSS, Inc.) and are presented as the mean \pm standard deviation (SD). Results were analyzed by one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Simvastatin increases the viability of SRA01/04 cells treated with HG. To investigate the viability of SRA01/04 cells before and after HG treatment, the CCK-8 assay was performed. No changes were observed in the viability of SRA01/04 cells after treatment with simvastatin at different concentrations (Fig. 1B). By contrast, HG treatment significantly inhibited the viability of SRA01/04 cells, which was reversed by simvastatin treatment (Fig. 1C). These results suggest that simvastatin alone exerted no effect on SRA01/04 cells, but was able to restore the viability of SRA01/04 cells treated with HG.

Simvastatin inhibits EMT in SRA01/04 cells treated with HG. To further explore the effects of simvastatin on EMT in SRA01/04 cells, the protein expression levels of E-cadherin, N-cadherin, Vimentin and α -SMA were measured. Expression of the epithelial cell marker E-cadherin was markedly decreased after HG treatment, which was reversed following the addition of simvastatin (Fig. 2). Additionally, the expression levels of mesenchymal cell markers N-cadherin, Vimentin and α -SMA exhibited the opposite trend compared with that of E-cadherin, as they were increased by HG and the levels of the three proteins were significantly decreased following treatment with 10 nM of simvastatin compared with the HG-induced cells (Fig. 2). This observation provided an indicator on the EMT process and suggested that simvastatin exerted an inhibitory effect on EMT in SRA01/04 cells treated with HG.

Simvastatin alleviates oxidative stress in SRA01/04 cells treated with HG. To investigate the role of simvastatin on oxidative stress in SRA01/04 cells, the levels of ROS, SOD and GSH-GSSG were measured. ROS levels were found to be markedly increased after culturing with HG compared with those in the control group, whilst the addition of simvastatin markedly reduced the levels of ROS (Fig. 3A). In addition, HG treatment markedly reduced the activity of SOD, which recovered following the addition of simvastatin (Fig. 3B). Similarly, the levels of GSH/GSSG were reduced by HG but were reversed by simvastatin (Fig. 3C). These findings suggest that simvastatin effectively alleviated oxidative stress in SRA01/04 cells induced by HG.

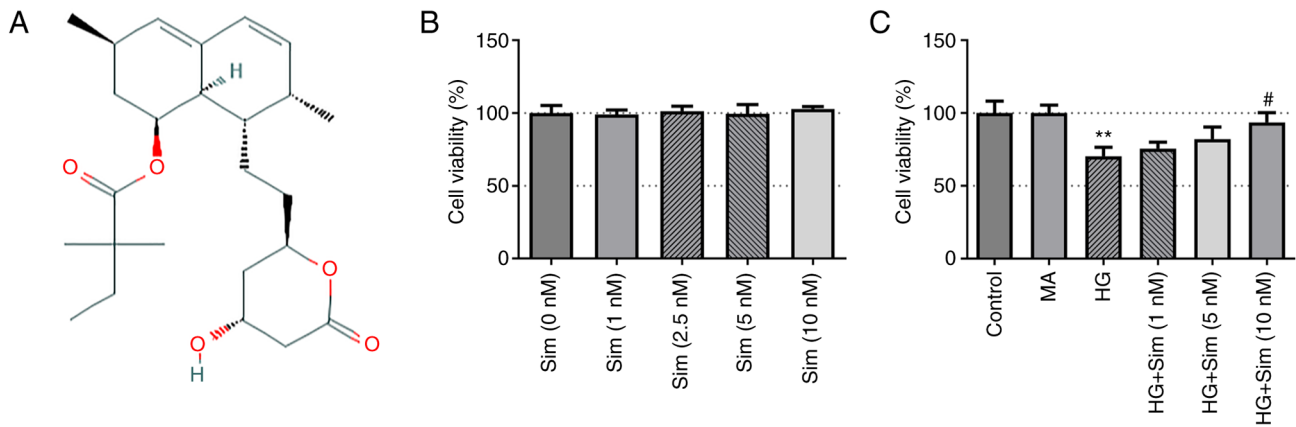


Figure 1. Simvastatin enhanced the cell viability of human lens epithelial cells SRA01/04 induced by HG. (A) The chemical formula of simvastatin. (B) The viability of SRA01/04 cells treated with different concentrations of simvastatin for 48 h was examined by CCK-8. (C) The viability of SRA01/04 cells treated with different concentrations of simvastatin for 48 h was tested by means of CCK-8 after adding different doses of HG. Data are expressed as mean \pm SD. ** $P < 0.001$ vs. Control. # $P < 0.05$ vs. HG. HG, high glucose; SD, standard deviation.

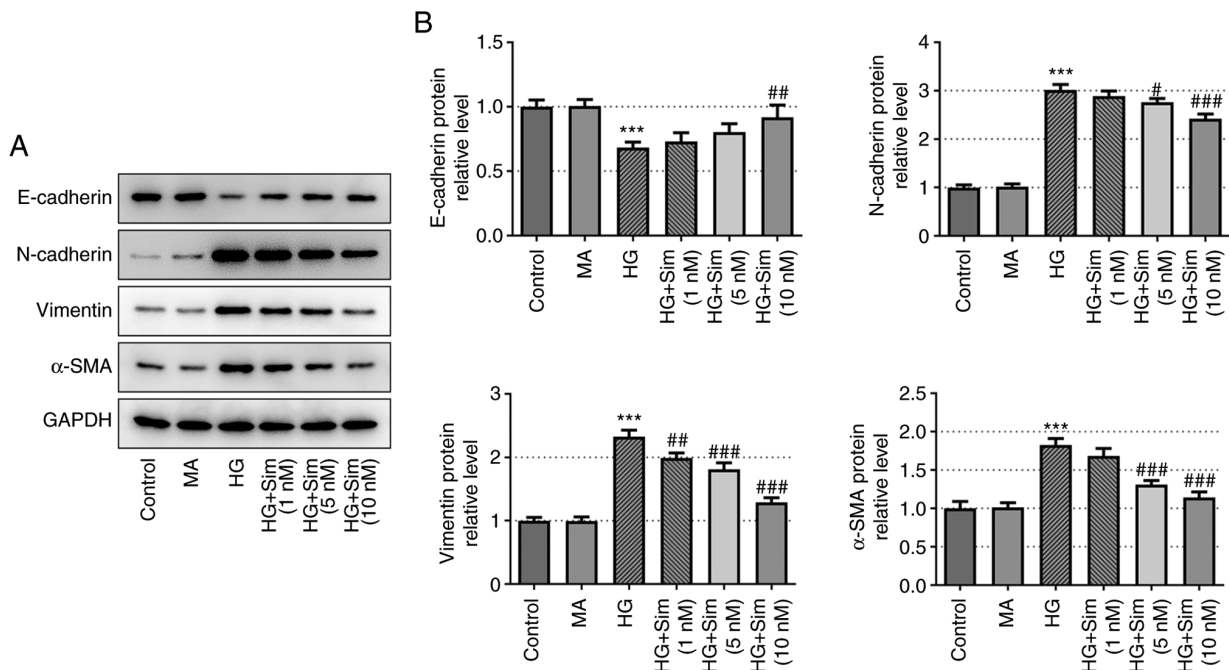


Figure 2. Simvastatin inhibited the EMT of SRA01/04 cells induced by HG. (A) Protein levels of E-cadherin, N-cadherin, Vimentin and α -SMA which were related to EMT were detected by western blot analysis. (B) Quantification of the related bands. Data are expressed as mean \pm SD. *** $P < 0.001$ vs. Control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. HG. HG, high glucose; SD, standard deviation.

Simvastatin inhibits RhoA/ROCK signaling. To assess the specific impact of simvastatin on RhoA/ROCK signaling, western blot analysis was performed to measure the expression levels of Rho family proteins RhoA, ROCK1 and ROCK2 in SRA01/04 cells treated with HG and then simvastatin. As indicated in Fig. 4, the protein expression levels of RhoA, ROCK1 and ROCK2 were found to be markedly increased by HG treatment but reversed as simvastatin was added. These observations suggest that simvastatin exerted an inhibitory effect on RhoA/ROCK signal transduction in the presence of HG.

Simvastatin suppresses EMT in SRA01/04 cells induced by HG by inhibiting RhoA/ROCK signal transduction. Simvastatin has been previously revealed to inhibit

RhoA/ROCK signal transduction (21,22). Therefore, to investigate whether simvastatin suppressed EMT in SRA01/04 cells by altering RhoA/ROCK signal transduction, the present study subsequently designated the following four groups: Control; HG; HG + 10 nM simvastatin; HG + 10 nM simvastatin + U46619. U46619 functions as an activator of RhoA/ROCK. The protein levels of E-cadherin, N-cadherin, Vimentin and α -SMA were measured using western blot analysis. As shown in Fig. 5, reduced expression of E-cadherin and increased expression of N-cadherin, Vimentin and α -SMA were observed in the HG + 10 nM simvastatin + U46619 group. These data suggest that EMT in SRA01/04 cells induced by HG was inhibited by simvastatin through the RhoA/ROCK pathway.

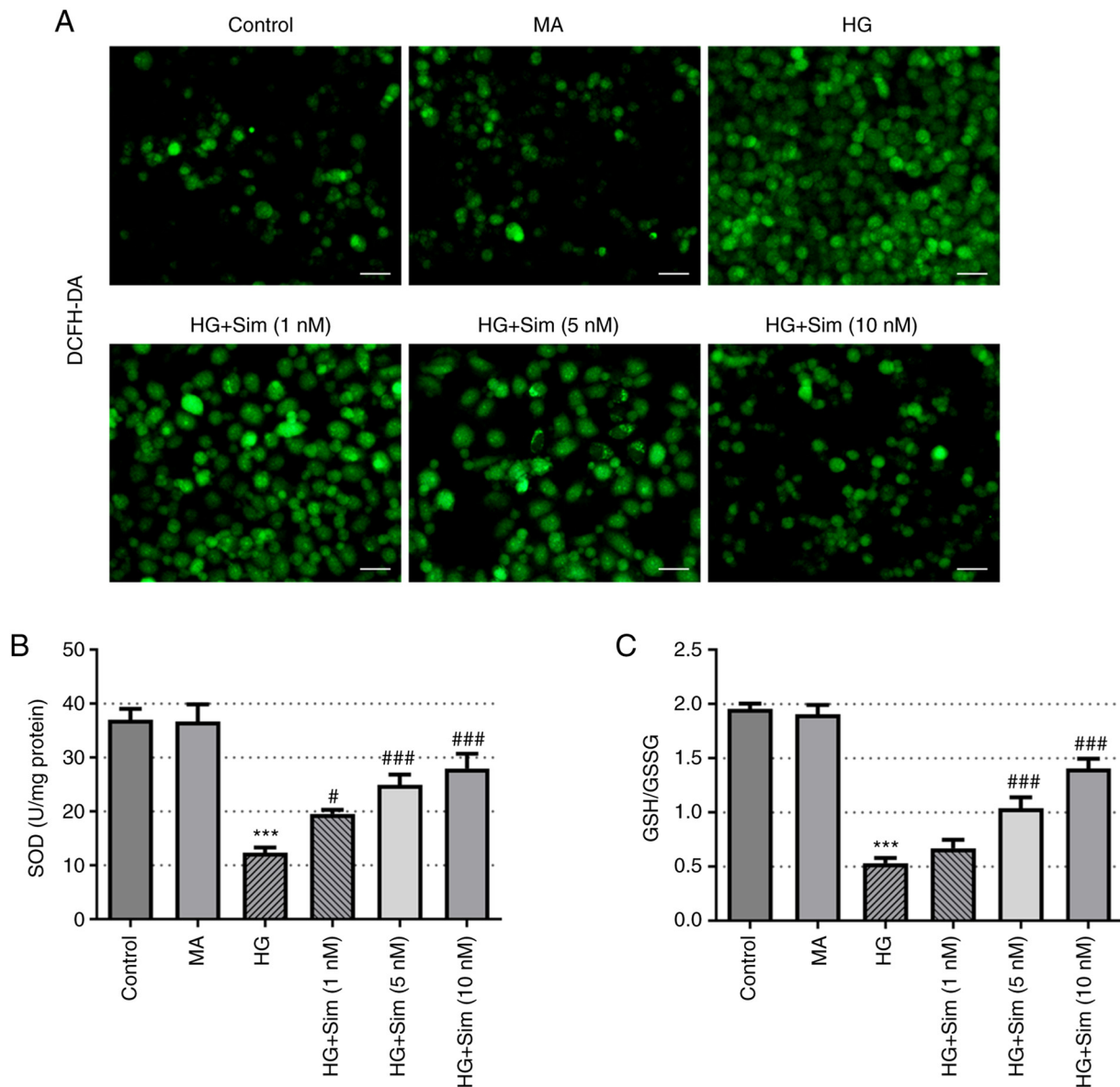


Figure 3. Simvastatin alleviated the oxidative stress of SRA01/04 cells induced by HG. (A) Level of ROS was measured by means of DCFH-DA kit after adding 25 nM HG and different doses of simvastatin. Green-stained cells are positive cells. Scale bar: 50 μ m. (B) The level of SOD was detected with the corresponding SOD kit after adding 25 nM HG and different doses of simvastatin. (C) The GSH-GSSG level was examined with GSH-GSSG kit after adding 25 nM HG and different doses of simvastatin. Data are expressed as mean \pm SD. *** P <0.001 vs. Control. # P <0.05, ### P <0.001 vs. HG. HG, high glucose; SD, standard deviation.

Simvastatin alleviates oxidative stress in SRA01/04 cells induced by HG through inhibition of RhoA/ROCK signaling. As shown in Fig. 6, the levels of ROS, SOD and GSH-GSSG in SRA01/04 cells after the addition of the RhoA activator U46619 were investigated using DCFH-DA, SOD and GSH/GSSG kits. In the HG + 10 nM simvastatin + U46619 group, the levels of ROS were increased, whereas the levels of SOD and GSH-GSSG were reduced compared with those in the HG + simvastatin group. These results highlight the role of simvastatin in attenuating oxidative stress in SRA01/04 cells induced by HG through inhibiting the RhoA/ROCK pathway.

Discussion

As the incidence of DC increases, so does the importance of developing an effective therapeutic strategy for treating this

condition. Although agents are available for treating DC, including aldose reductase inhibitors (23) and aspirin (24), their therapeutic effects remain unsatisfactory. Simvastatin is a type of statin that is widely applied for controlling hypercholesterolemia (25). Emerging evidence has indicated that simvastatin is involved in the development of diabetic complications. Simvastatin has been documented to reverse the increase in apoptosis, while also increasing autophagy after treatment with HG (12). The aim of the present study was to investigate the mechanism underlying the effects of simvastatin on DC. Specifically, the focus was on the potential effects of simvastatin on EMT and oxidative stress in the human lens epithelial cell line SRA01/04 induced by HG.

Lens epithelial cell damage in patients with diabetes is mainly caused by oxidative stress, EMT and cell apoptosis induced by HG. Previous findings showed that simvastatin

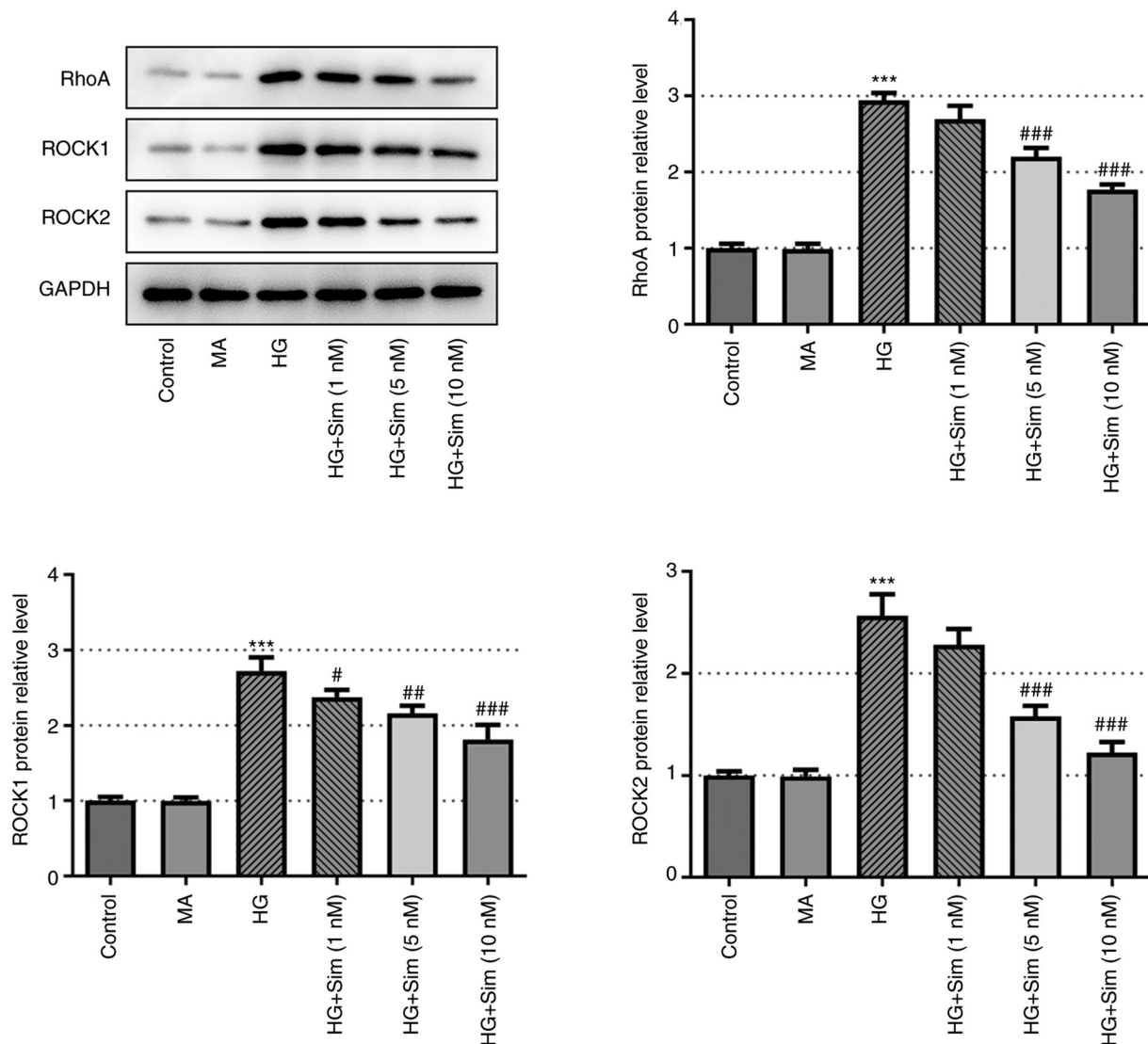


Figure 4. Simvastatin suppressed RhoA/ROCK signaling transduction. The protein levels of RhoA, ROCK1 and ROCK2 were measured by western blot analysis after treatment with HG and different doses of simvastatin. Data are expressed as mean \pm SD. *** $P < 0.001$ vs. Control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. HG. HG, high glucose; SD, standard deviation.

does not result in any deleterious effects on the lens (26). In the present study, 25 mM HG was used to stimulate SRA01/04 cells in order to simulate a HG condition and detect the effects of HG on cell viability, EMT and oxidative stress. The results showed that 25 mM HG significantly inhibited cell viability, promoted the EMT process and oxidative stress, accompanied with higher ROS, lower levels of SOD and GSH/GSSG, which indicated that a DC model was established successfully. In addition, the viability of SRA01/04 cells did not exhibit any significant differences after 48 h of treatment with different doses of simvastatin. Additionally, the viability of SRA01/04 cells was markedly decreased by HG, mimicking the physiological condition that occurs in the lens of patients with diabetes (27). In the present study, to assess cell viability, SRA01/04 cells were treated with 25 mM HG to establish an *in vitro* model of DC before simvastatin treatment. The results showed that after treatment with simvastatin, cell viability was markedly restored, suggesting that simvastatin can increase lens epithelial cell viability. If SRA01/04 cells were cultured for 7 days without continuous passage culture,

the cells would be too old to maintain normal cell morphology and function. Thus, treatment of simvastatin for one week is inappropriate. Therefore, the action time of simvastatin was selected according to another study (15).

RhoA/ROCK signaling serves key roles in a variety of cellular processes (28). Simvastatin was previously shown to inhibit the TGF- β 1-induced RhoA/ROCK signaling pathway by blocking Rho geranylation (29). Another study reported significant and specific decreases in RhoA/ROCK activity following treatment with simvastatin (30). In addition, simvastatin reduced RhoA activity by suppressing the levels of isoprenoid intermediates (31). In the present study, HG increased RhoA, ROCK1 and ROCK2 protein expression, all of which was reversed by simvastatin. This suggests that simvastatin exerted inhibitory effects on RhoA/ROCK signaling.

The RhoA/ROCK pathway can also regulate HG-induced EMT (32). EMT has been previously reported to serve a key role in cataract formation. It was found that simvastatin can block EMT in human alveolar epithelial cells (33) and in human prostate cancer cells (34) induced by TGF- β 1. In

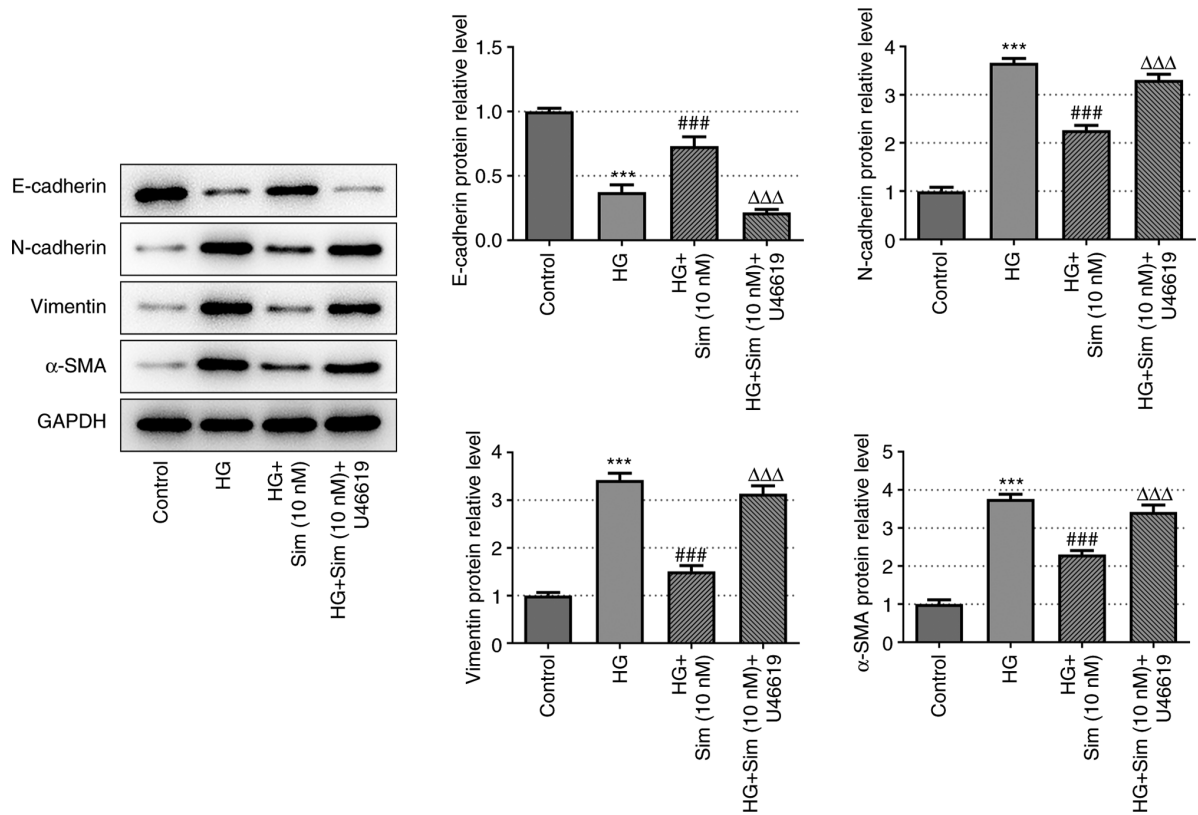


Figure 5. Simvastatin inhibited the EMT of SRA01/04 cells induced by HG by suppressing RhoA/ROCK signaling transduction. Protein levels of E-cadherin, N-cadherin, Vimentin and α -SMA were detected by western blot analysis after the treatment of HG, 10 nM simvastatin and the activator of RhoA/ROCK pathway U46619. Data are expressed as mean \pm SD. *** P <0.001 vs. Control. ### P <0.001 vs. HG. $\Delta\Delta\Delta P$ <0.001 vs. HG + Sim (10 nM). HG, high glucose; Sim, simvastatin; SD, standard deviation.

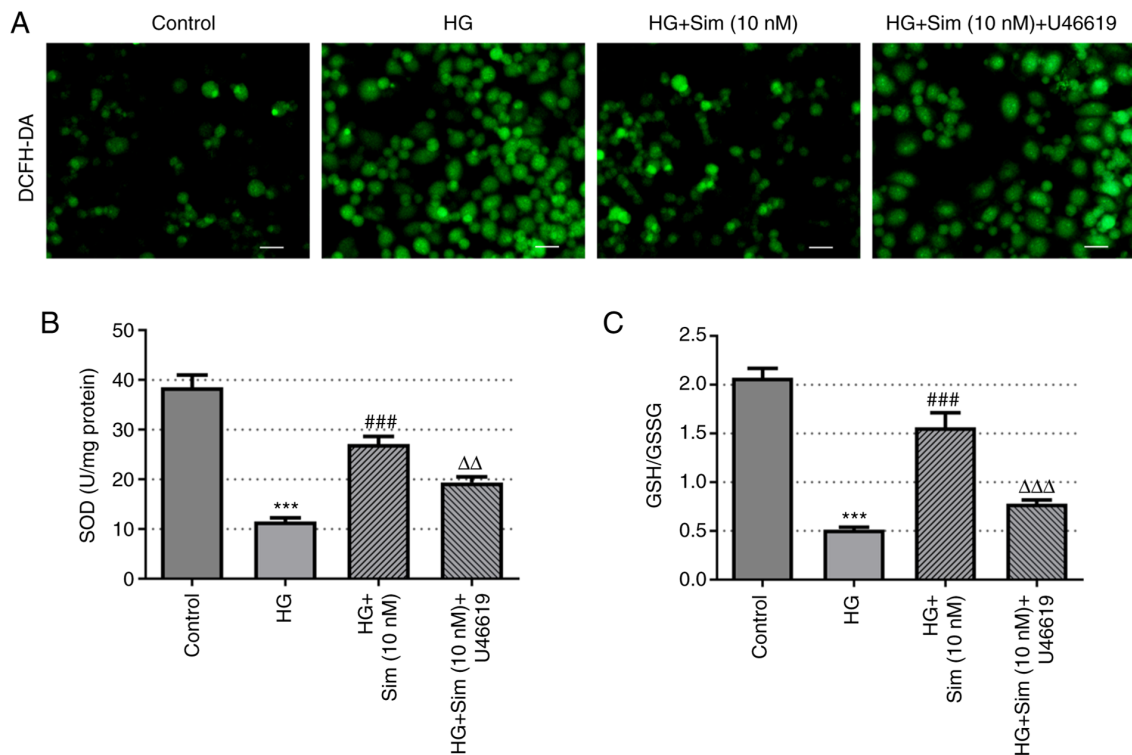


Figure 6. Simvastatin alleviated the oxidative stress of SRA01/04 cells induced by high glucose via suppressing RhoA/ROCK signaling transduction. (A) The level of ROS was measured by means of DCFH-DA kit after adding 10 nM U46619. Green-stained cells are positive cells. Scale bar: 50 μ m. (B) The level of SOD was detected with the corresponding SOD kit after adding 10 nM U46619. (C) The GSH-GSSG level was examined with GSH-GSSG kit after adding 10 nM U46619. Data are expressed as mean \pm SD. *** P <0.001 vs. Control. ### P <0.001 vs. HG. $\Delta\Delta P$ <0.01, $\Delta\Delta\Delta P$ <0.001 vs. HG + Sim (10 nM). HG, high glucose; Sim, simvastatin; SD, standard deviation.

addition, Fan *et al* (35) reported that atorvastatin partially suppressed the EMT process in A549 cells induced by TGF- β 1. The present study showed that under both HG and simvastatin presence, the protein expression levels of E-cadherin were elevated whereas the expression levels of N-cadherin, Vimentin and α -SMA were decreased. Downregulation of E-cadherin and upregulation of N-cadherin, vimentin and α -SMA are closely associated with EMT injury (36), which was contrary to the results and further suggested the inhibitory effects of simvastatin on EMT in SRA01/04 cells induced by HG. However, after U46619, the activator of RhoA/ROCK, was added, and the opposite trend in the expression of these EMT protein markers was observed, suggesting that RhoA/ROCK is important for mediating EMT in this cell type. Together, these results suggest that simvastatin can inhibit EMT in SRA01/04 cells, at least partially by suppressing RhoA/ROCK signaling.

Oxidative stress is caused by the imbalance between ROS production and activity in the anti-oxidant defense system in the body (37). Oxidative stress and subsequent oxidative damage to lens proteins is a frequently reported causative factor in cataract formation (38). A previous study has found that simvastatin can prevent cardiac hypertrophy in diabetic rats by attenuating oxidative stress and inflammation caused by the calpain-1-mediated activation of NF- κ B (39). Statins have been found to alleviate inflammatory and oxidative stress damage (40). In the present study, the levels of representative oxidative stress markers ROS, SOD and GSH-GSSG were measured after treatment with HG and simvastatin. Significantly decreased levels of ROS and elevated levels of SOD and GSH-GSSG were observed after simvastatin treatment, suggesting that simvastatin inhibited oxidative stress in SRA01/04 cells. By contrast, the addition of U46619 reversed the effects of simvastatin on SRA01/04 cells, suggesting that RhoA/ROCK signaling also exerts an inhibitory role in oxidative stress injury. These observations suggest that simvastatin alleviates oxidative stress in SRA01/04 cells induced by HG through inhibition of RhoA/ROCK. In addition, *in vivo* experiments are far more complex than cell experiments; thus it is not equivalent to treatment in humans. The dose of simvastatin used in this study was selected according to that of a previous study (15). However, the appropriate doses of simvastatin used in clinical trials need to be further investigated.

To conclude, simvastatin exerted a concentration-dependent therapeutic effect on the human lens epithelial cell line SRA01/04 induced by HG. In addition, reversal experiments using the RhoA/ROCK activator revealed that simvastatin could reduce EMT and oxidative stress by inhibiting RhoA/ROCK signaling. Therefore, simvastatin has the potential for application as a therapeutic agent for treating DC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JF and XH designed the study, performed the experiment, drafted and revised the manuscript. JF analyzed the data and searched the literature. All authors read and approved the final manuscript. JF and XH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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