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Original Article

Effects of ticagrelor on proliferation, apoptosis, and inflammatory factors of human aortic vascular smooth muscle cells through lncRNA KCNQ1OT1

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Abstract: Objective: This study was designed to determine the effects of ticagrelor on the proliferation and apoptosis of and inflammatory factors in human aortic vascular smooth muscle cells (HAVSMCs). Methods: A total of 20 patients who were first diagnosed with coronary heart disease (CHD) from August 2020 to March 2021 and 20 healthy adults were enrolled into the study. Oxidized low-density lipoprotein (ox-LDL) and different concentrations of ticagrelor were applied in the treatment of HAVSMCs, and then the cell proliferation and apoptosis and the expression of apoptosis-related proteins, inflammatory factors, and IκBα in them were determined. Results: Compared with the ox-LDL group, the OD value was significantly increased after ticagrelor treatment, and the apoptosis rate was significantly reduced ($P<0.05$); compared with the ox-LDL group, the B lymphoma-2 (Bcl-2) protein, IκB, KCNQ1OT1 expression in the ticagrelor group increased significantly, Bcl-2-associated X protein (Bax), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) expression decreased significantly ($P<0.05$); The expression of serum KCNQ1OT1 in patients with coronary heart disease was significantly higher than that in healthy individuals ($P<0.05$). Conclusion: Ticagrelor may regulate the expression of lncRNA KCNQ1OT1 and up-regulate the expression of IκBα to promote proliferation and anti-apoptosis, so as to prevent ox-LDL from oxidative damage to HAVSMCs.

Keywords: Ticagrelor, HAVSMCs, lncRNA KCNQ1OT1, IκBα, proliferation, apoptosis, inflammation

Introduction

Coronary heart disease (CHD) is a threat to the lives and health of Chinese residents. It is considered as the top cause of disability and death [1], with a crucial pathological basis of aberrant proliferation and apoptosis of human aortic vascular smooth muscle cells (HAVSMCs) [2]. Some studies have pointed out that inflammation exerts a crucial impact on CHD and other atherosclerotic diseases [3]. Nuclear factor κB (NF-κB) is a key transcription factor that controls inflammation, cell migration, proliferation, and apoptosis. Inflammation medicated by it is found to be involved in the procession of atherosclerosis in previous studies [4]. Studies have also revealed that ticagrelor, a platelet P2Y12 receptor inhibitor with a strong antiplatelet aggregation effect, can abate inflammation and oxidative stress by suppressing the NOX4/ROS/NF-κB signaling pathway, thereby alleviating myocardial ischemia-reperfusion injury [5]. In addition, strong evidence of lncRNAs that exert an important regulatory effect on the proliferation and apoptosis of vascular smooth muscle cells (VSMCs) has been detected in many studies [6]. Furthermore, reportedly, KCNQ1OT1 is up regulated in apoE-/- mice fed with a high-fat diet and THP-1 macrophages treated with oxidized low-density lipoprotein (ox-LDL) [7], and it exerts an effect on the proliferation of VSMCs and the level of inflammation by binding to IκBα [8]. IκBα can regulate NF-κB activation and transcription, as a crucial member of the NF-κB signaling pathway. In view of the above research background, this study aimed to probe into the effects of ticagrelor on the proliferation and apoptosis of HAVSMCs and the expression of inflammatory factors induced by ox-LDL through lncRNA KCNQ1OT1,

with the purpose of providing a reliable theoretical basis for clinical treatment.

Materials and methods

Materials

Source of materials: A total of 20 patients diagnosed with CHD from August 2020 to early March 2021 were enrolled in the study, and all of them signed informed consent. The inclusion criteria: (1) patients at 18-80 years old; (2) patients diagnosed with CHD for the first time; (3) patients with complete clinical data. The exclusion criteria: (1) patients with a history of infectious diseases in the past month or at the acute stage of chronic inflammation; (2) patients with other comorbid cardiovascular diseases such as severe heart failure; (3) patients with comorbid malignant tumors, autoimmune diseases, blood system diseases, severe liver, or kidney dysfunction; (4) patients who had received coronary intervention or bypass treatment. In addition, during the same period, 20 healthy adults who underwent a medical examination in the hospital were included as controls. This study was approved by the hospital ethics committee (No. 2019-04-22). For preparation, their peripheral venous blood was collected and centrifuged at 2000 r/min for 10 minutes to separate the serum, and the serum was stored at -80°C for later use. HAVSMC: fibroblast-like, adherent growth, Shanghai Huzhen Biotechnology Co., Ltd., No. HZ-H355.

Main reagents and instruments: Eagle's minimal essential medium (EMEM) (No.: ZQ-300, Shanghai Zhongqiao Xinzhou Biotechnology Company); ox-LDL (No.: YB-002, Guangzhou Yiyuan Biological Technology Co., Ltd.); ticagrelor (NMPA No.: J20130020, 90 mg/tablet, AstraZeneca AB); MTT kit (No.: M1020, Shanghai Hengfei Biotechnology Co., Ltd.); Dulbecco's modified eagle (DMEM)/F12 complete medium containing 10% fetal bovine serum (FBS) (No.: PM150312B, Shanghai Yaji Biotechnology Co., Ltd.); Annexin V-FITC/PI apoptosis kit, BCA protein quantitative kit, chemiluminescence (ECL) kit, and cDNA first strand synthesis kit (No.: SY0471, M07270, YT061 and BTN60906, Beijing Bai Olaibo Technology Co., Ltd.); rabbit anti-Bcl-2 monoclonal antibody, rabbit anti-Bax monoclonal antibody, and rabbit anti-GAPDH polyclonal antibody (No.: ab182858, ab182733, and ab9485, ATCC company, USA); rabbit anti-IκBα monoclonal antibody (No.: YB096, Shanghai Yubo Biotechnology Co., Ltd.), monocyte chemoattractant protein-1 (MCP-1) detection kit and tumor necrosis factor-α (TNF-α) detection kit (No.: XFH10862 and XF439, Shanghai Xinfan Biotechnology Co., Ltd.); siRNA-NC and siRNA-KCNQ1OT1 (Shanghai Jima Biological Co., Ltd.); trizol reagent and SYBR Premix Ex TaqTM II PCR kit (No.: 15596 and RR820A, Beijing Zhijie Fangyuan Technology Co., Ltd.), microplate reader (SpectraMax i3x, Shanghai Meigu Molecular Instrument Co., Ltd.); flow cytometer (CytoFLEX, Beckman, USA); Gel imaging system (GelDoc Go); PCR machine (C1000, Bio-Rad, USA).

Methods

Cell culture and intervention: A cryopreservation tube containing 1 mL cell suspension was treated with a 37°C water bath for quick thawing, and added to 4 mL EMEM containing 10% fetal bovine serum. Then, it was centrifuged at 1000 r/min for 4 minutes and its supernatant was discarded, followed by addition of 1-2 mL medium and overnight incubation. Subsequently, the cells were subcultured when their confluency reached 80%-90%, and cells at the 3rd to 8th passage were collected and placed in an incubator (37°C, CO_2) for subsequent experiments. The cells were randomized into a blank control group, ox-LDL group, and low-dose, medium-dose, and high-dose ticagrelor groups, all of which were inoculated in a 6-well plate. ox-LDL (50 μg/mL) was given to the ox-LDL group, and ox-LDL (50 mg/mL) and ticagrelor (20 μmol/L) were added to the low-dose group, ox-LDL (50 mg/mL) and ticagrelor (40 μmol/L) to the medium-dose group, and ox-LDL (50 mg/mL) and ticagrelor (60 μmol/L) to the high-dose group. All groups were continuously incubated (37°C, CO_2) for 24 hours.

Cell transfection: The cells were inoculated in a six-well plate. According to the Lipofectamine 2000 reagent manual, siRNA-NC and siRNA-KCNQ1OT1 were transfected into cells in the ox-LDL group respectively, and cultured in an incubator (37°C, CO_2) when their growth confluency reached 70%-80%.

Cell proliferation determination by the MTT method: The cells were transferred to a 96-well

plate and cultured in an incubator (37°C, CO_2). After 24 hours, the cells were treated with a 4-hour incubation with 200 μL MTT solution at room temperature. The culture medium in each well was aspirated, and 150 μL DMSO solution was added and vibrated for 10 minutes. Afterwards, a microplate reader was used to measure the optical density (OD) at a wavelength of 570 nm.

Cell apoptosis determination by flow cytometry: The cells were transferred to a 6-well plate and placed in an incubator (CO_2) at 37°C overnight. After the original medium was discarded, the cells were rinsed with phosphate buffer saline (PBS) solution twice, trypsinized, and incubated for 2 minutes at room temperature, and then added with complete DMEM medium to terminate the trypsinization. After 5-minute centrifugation at 1000 r/min, the supernatant was discarded, and the cells were resuspended in PBS solution, followed by addition and mixing of 5 μL Annexin V-FITC reagent and 5 μL PI reagent. The cells were kept still in the dark for 15 minutes. Finally, flow cytometry was carried out for aapoptosis rate determination.

Western blot: The cells were transferred to a 6-well plate and placed in an incubator (37°C, CO_2) overnight. The original medium was discarded, and PBS solution was used to rinse the plate twice. Then the cells were centrifuged at 12,000 r/min for 20 minutes after being lysed on ice, and the supernatant was obtained and transferred to another centrifuge tube. Afterwards, the BCA method was used for protein quantification, and the cells were transferred to a PVDF membrane by the wet method after electrophoresis in SDS-PAGE. Subsequently, the membrane was placed in membrane blocking solution and let to stand still at 4°C overnight. Then, it was rinsed 3 times with TBST solution, 10 minutes each time. Antibodies were added dropwise: B lymphocyte tumor-2 protein (Bcl-2), Bcl-2 associated X protein (Bax), and IκBα antibody with a dilution ratio of 1:800 for each. With GAPDH as an internal standard at a dilution ratio of 1:8000, the membrane was treated with overnight incubation at 4°C and a 2-hour incubation at room temperature with the HRP-labeled secondary antibody. Finally, the ECL method was used to conduct color development, gel imaging system to observe the bands, and Image J software to do quantitative analysis. The relative expression of target gene = the gray value of target gene band/the gray value of internal control gene band.

Enzyme-linked immunosorbent assay (ELISA): The cells were treated with trypsinization and 5-minute centrifugation at 1000 r/min, and the supernatant was then discarded. Subsequently, the cells were resuspended in PBS solution and disrupted by ultrasound, and the MCP-1 and TNF-α expression were determined.

Real time fluorescent quantitative polymerase chain reaction (RT-PCR) assay: Total RNA was extracted from cells and blood by the Trizol method, and then reversely transcribed into cDNA. PCR primers were designed and synthesized by Beijing Dacome Technology Co., Ltd., with KCNQ1OT1 (forward primer: 5'-CCCAGAAATCCACACCTCGG-3'; reverse primer: 5'-TCCTCAGTGAGCAGATGGAGA-3') and GAPDH (forward primer: 5'-GTACGACTCACTATAGGGA AGG-3'; reverse primer: 5'-AGGTGACACTATAGAATAGCTGT-3') as internal standards. The reaction system and conditions were set according to the SYBR Premix Ex TaqTM II PCR kit. The cycle threshold (Ct) was determined by a PCR instrument, and the $2^{-\Delta\Delta Ct}$ method was used for data analysis.

Statistical analysis

SPSS20.0 software was used to analyze the data obtained. The measured data in normal distribution were represented as ($\bar{x} \pm s$). The independent-sample t-test was adopted for inter-group comparison, the paired-sample t-test for intra-group comparison, the one-way analysis of variance for multiple-group comparison, and the Snk-q test for pairwise comparison. $P<0.05$ was considered significant.

Results

Effect of ticagrelor on the ox-LDL-induced proliferation of HAVSMCs

The results of the MTT experiment showed that compared with the blank control group, the OD value of the ox-LDL group was significantly reduced ($P<0.05$); compared with the ox-LDL group, the OD value of the ticagrelor group was significantly increased ($P<0.05$, **Figure 1**).

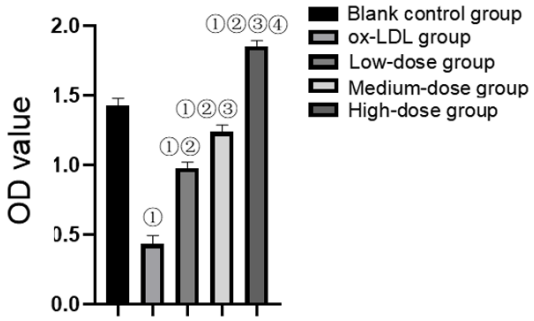

Figure 1. Effect of ticagrelor on the ox-LDL-induced proliferation of HAVSMCs. Notes: ①P<0.05 *vs.* the blank control group; ②P<0.05 *vs.* the ox-LDL group; ③P<0.05 *vs.* the low-dose group; ④P<0.05 *vs.* the medium-dose group.

Effect of ticagrelor on the ox-LDL-induced apoptosis of HAVSMCs

The results of flow cytometry showed that compared to the blank control group, the apoptotic rate of the ox-LDL group was significantly increased (P<0.05); compared with the ox-LDL group, the apoptotic rate of the ticagrelor group was significantly reduced (P<0.05, **Figure 2**). Western blot experiment results showed that compared with the blank control group, the relative expression of Bcl-2 protein in the ox-LDL group was significantly reduced, and the relative expression of Bax protein was significantly increased (P<0.05); compared with the ox-LDL group, the relative expression of Bcl-2 protein in ticagrelor group, increased significantly, and the relative expression of Bax protein decreased significantly, in a concentration-dependent manner (P<0.05, **Figure 3**).

Effect of ticagrelor on the expression of inflammatory factors in HAVSMCs

Results of ELISA revealed noticeably up-regulated MCP-1 and TNF-α in the group treated with only ox-LDL as compared to the group without treatment (both P<0.05), and also revealed an inhibition effect on the expression of MCP-1 and TNF-α by ticagrelor in a concentration-dependent manner (P<0.05). See **Figure 4**.

Effect of ticagrelor on the expression of IκBα in HAVSMCs

Results confirmed that ox-LDL downregulated the expression of IκBα protein, while ticagrelor exerted a stimulatory effect on it in a concentration-dependent manner (P<0.05). See **Figure 5**.

Serum KCNQ1OT1 expression in patients with CHD

Serum KCNQ1OT1 expression was significantly increased in patients with CHD compared to healthy subjects (P<0.05). See **Table 1**.

Effect of ticagrelor on the expression of KCNQ1OT1 in HAVSMCs

In comparison to the blank controls, the level of KCNQ1OT1 was significantly elevated by ox-LDL (P<0.05) but noticeably decreased after ticagrelor intervention in a concentration-dependent manner (P<0.05), as displayed in **Figure 6**.

Effect of downregulated lncRNA KCNQ1OT1 on the proliferation and apoptosis of HAVSMCs treated by ox-LDL

Compared to the siRNA-NC group, down-regulating the expression of lncRNA KCNQ1OT1 can significantly increase the proliferation of HAVSMCs induced by ox-LDL and inhibit cell apoptosis (P<0.05), see **Table 2**.

Effect of downregulated lncRNA KCNQ1OT1 on the expression of inflammatory factors in HAVSMCs

As compared to the siRNA-NC group, the expression of MCP-1 and TNF-α in the siRNA-KCNQ1OT1 group was significantly downregulated (P<0.05), as shown in **Figure 7**.

Effect of downregulated lncRNA KCNQ1OT1 on the expression of IκBα in HAVSMCs induced by ox-LDL

Results of **Figure 8** exhibited a markedly elevated relative expression of IκBα protein in the siRNA-KCNQ1OT1 group than that of the siRNA-NC group (P<0.05).

Discussion

To date, CHD in China still shows an upward trend in morbidity and mortality, with a high disability rate [9]. Drug therapy is one of the basic treatment methods for it, among which antiplatelet drugs are one of the common medications for patients. The development of vascular

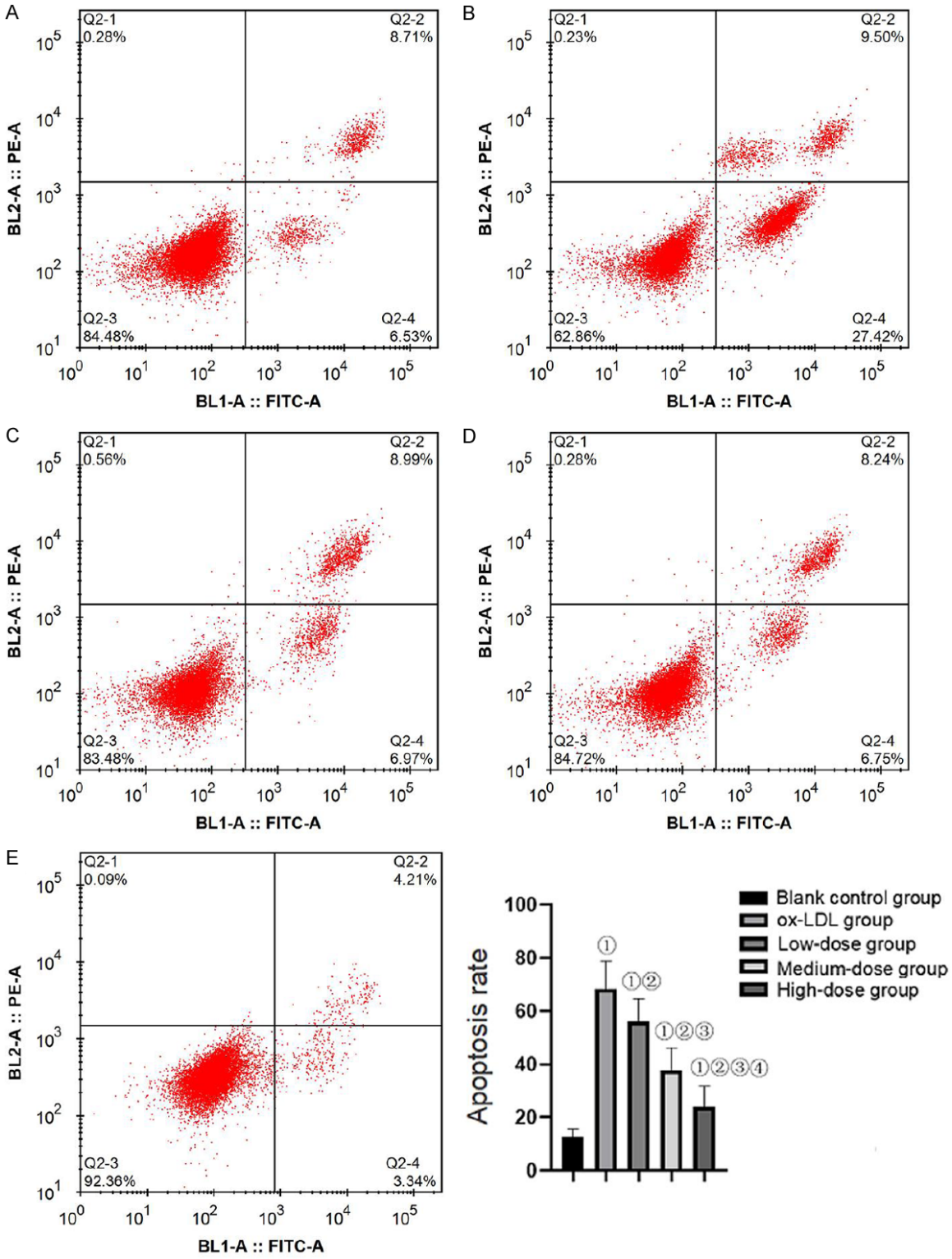

Figure 2. Effect of ticagrelor on the ox-LDL-induced apoptosis of HAVSMCs. Notes: (A) Blank control group; (B) ox-LDL group; (C) Low-dose group; (D) Medium-dose group; (E) High-dose group.

proliferative diseases was predominantly attributed to the aberrant proliferation and apoptosis of VSMCs caused by arterial injury. Specifically, the aberrant proliferation of VSMCs

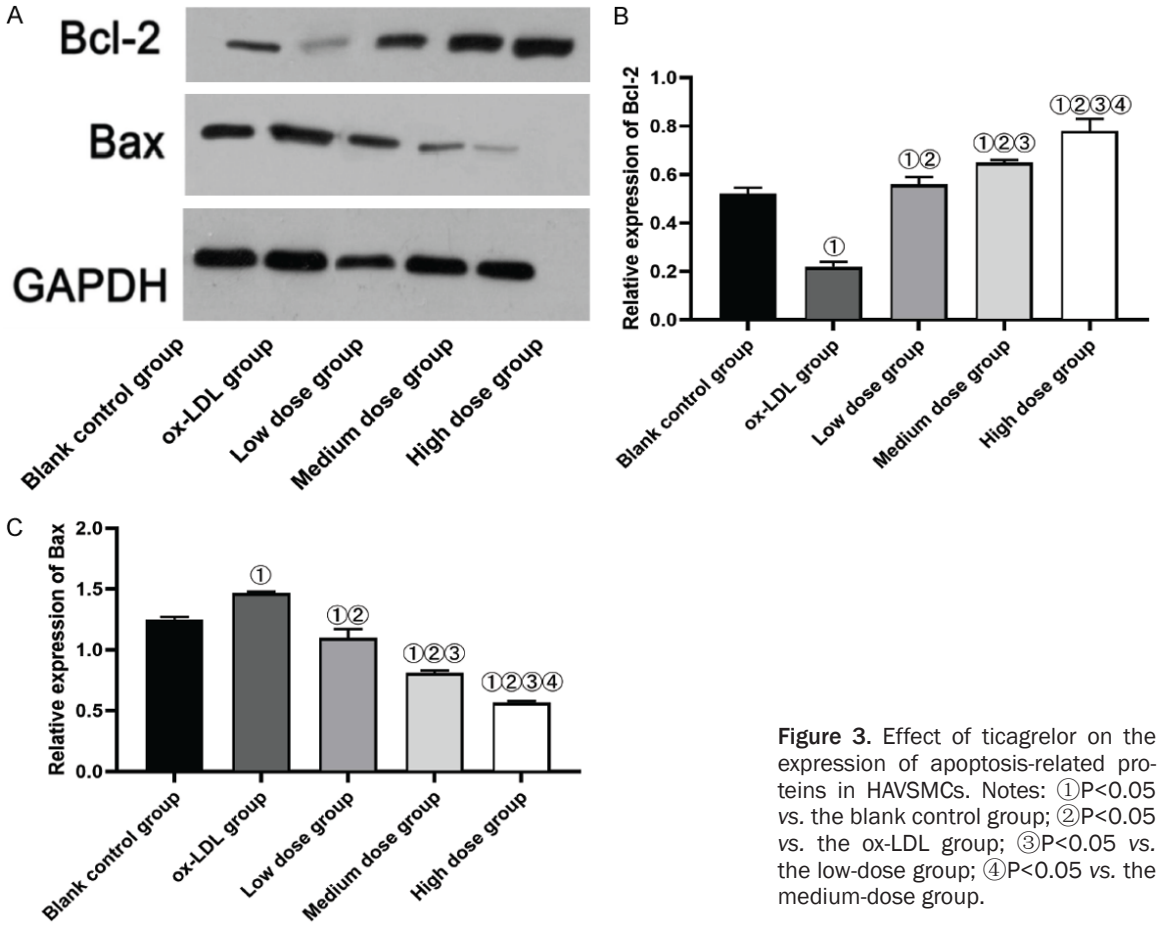

Figure 3. Effect of ticagrelor on the expression of apoptosis-related proteins in HAVSMCs. Notes: ①P<0.05 *vs.* the blank control group; ②P<0.05 *vs.* the ox-LDL group; ③P<0.05 *vs.* the low-dose group; ④P<0.05 *vs.* the medium-dose group.

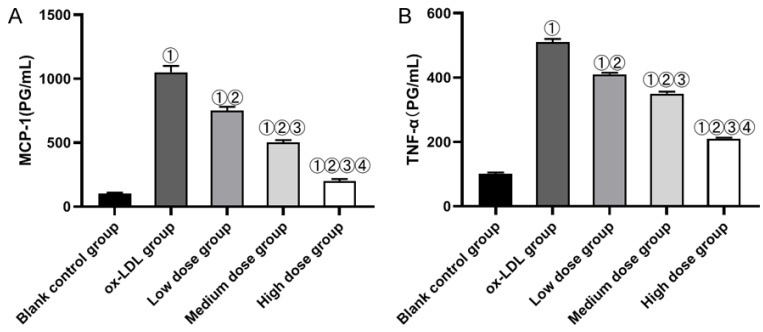

Figure 4. Effect of ticagrelor on the expression of inflammatory factors in HAVSMCs. Notes: ①P<0.05 *vs.* the blank control group; ②P<0.05 *vs.* the ox-LDL group; ③P<0.05 *vs.* the low-dose group; ④P<0.05 *vs.* the medium-dose group.

promotes the formation of atherosclerotic plaques [10], and their aberrant apoptosis is closely related to the development of CHD-related inflammatory reactions [11]. Ticagrelor, an effective reversible adenosine diphosphate (ADP) receptor antagonist, is frequently used in combination with aspirin to significantly optimize the antiplatelet efficacy, which is mostly applied in the treatment of acute myocardial infarction [12]. In-depth studies have also discovered efficient anti-inflammatory and vascular endothelial protective effects of the drug [13]. lncRNAs are a series of RNAs unable to encode proteins, with a length of more than 200 nt and an ability to regulate gene expression transcriptionally. It has been confirmed by many studies that lncRNAs exert an strong regulatory effect on the proliferation and apoptosis of VSMCs [14]. KCNQ1OT1 is known to be involved in a variety of cardiovascular diseases. According to some studies, KCNQ1OT1 is overexpressed in cases with diabetic cardiomyopathy, and inhibiting its expression can reduce cell apoptosis and mitigate abnormal cytoskel-

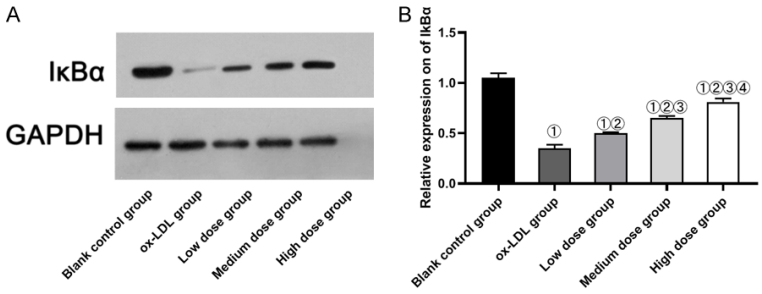

Figure 5. Effect of ticagrelor on the expression of IκBα in HAVSMCs. Notes: ①P<0.05 *vs.* the blank control group; ②P<0.05 *vs.* the ox-LDL group; ③P<0.05 *vs.* the low-dose group; ④P<0.05 *vs.* the medium-dose group.

Table 1. Expression of serum lncRNA KCNQ1OT1 ($\bar{x}$±s)

Groups	Cases	lncRNA KCNQ1OT1
Healthy subjects	20	0.41±0.12
Coronary Heart Disease	20	0.92±0.15
t		11.873
P value		<0.01

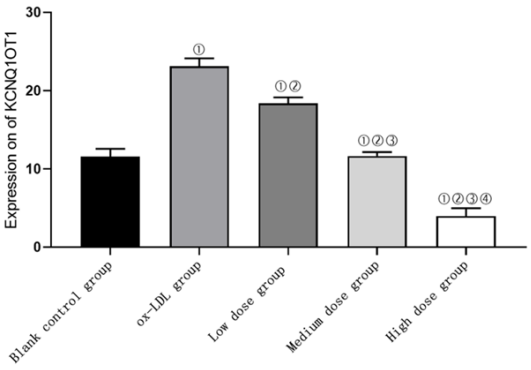

Figure 6. Effect of ticagrelor on the expression of lncRNA KCNQ1OT1 in HAVSMCs. Notes: ①P<0.05 *vs.* the blank control group; ②P<0.05 *vs.* the ox-LDL group; ③P<0.05 *vs.* the low-dose group; ④P<0.05 *vs.* the medium-dose group.

etal structure and calcium overload *in vitro*, and thus ameliorate cardiac function and morphology [15]. One prior study has pointed out that the level of KCNQ1OT1 in peripheral blood mononuclear cells of patients with CHD was significantly up-regulated [16], which indicates a correlation between the abnormal expression of KCNQ1OT1 and the onset of atherosclerosis and CHD.

Ox-LDL, as one of the key factors involved in the development of atherosclerosis, can induce the proliferation of VSMCs [17], promote the secretion of MCP-1 and TNF-α, and mediate inflammation [18]. In this study, HAVSMCs were treated with ox-LDL, and the results showed that such treatment significantly inhibited cell proliferation, promoted apoptosis, and increased expression levels of MCP-1 and TNF-α, thus mediating inflammation, which is in line with the results of previous studies [18-20]. As the first reversibly combined oral P2Y12R antagonist, ticagrelor has been widely used in the treatment of platelet aggregation in patients with CHD, with an anti-inflammatory effect confirmed by increasing evidences [19]. In this study, HAVSMCs induced by ox-LDL were treated with different concentrations of ticagrelor. The results show that ticagrelor can significantly suppress the inhibitory effect of ox-LDL on the proliferation of HAVSMCs, inhibit cell apoptosis, and reduce MCP-1 and TNF-α level, further confirming the usefulness of ticagrelor in the prevention and treatment of coronary heart disease. According to prior studies, ticagrelor can up-regulate IκBα by inhibiting the NF-κB pathway, an essential target of ticagrelor, thereby abating the inflammation and oxidative stress and optimizing myocardial ischemia-reperfusion injury [5]. In this study, it was shown that ticagrelor might inhibit the NF-κB pathway and up-regulate IκBα whose expression in HAVSMCs has been lowered by stimulation of ox-LDL, and thus alleviate ox-LDL-induced inflammation. KCNQ1OT1 has been confirmed to be involved in the pathophysiological mechanism of various vascular diseases such as acute myocardial injury [20]. In this study, the detection of KCNQ1OT1 expression in cells and human serum revealed an apparently increased expression of KCNQ1OT1 in the serum of patients with CHD and HAVSMCs treated with ox-LDL, suggesting an involvement of the high KCNQ1OT1 expression in the procession of atherosclerosis and CHD. However, the downregulation results of KCNQ1OT1 expression by ticagrelor indicated that the vascular protection and anti-inflammatory effects of ticagrelor might be achieved by the regulation of lncRNA KCNQ1OT1. Further research found that after

Table 2. The effect of down-regulating the expression of lncRNA KCNQ1OT1 on the proliferation and apoptosis of HAVSMCs induced by ox-LDL ($\bar{x}\pm s$)

groups	OD value	Apoptosis rate (%)
siRNA-NC group	42.96±0.33	53.79±2.85
siRNA-KCNQ1OT1 group	75.67±0.29⑤	38.32±3.67⑤

Note: ⑤P<0.05 *vs.* the siRNA-NC group.

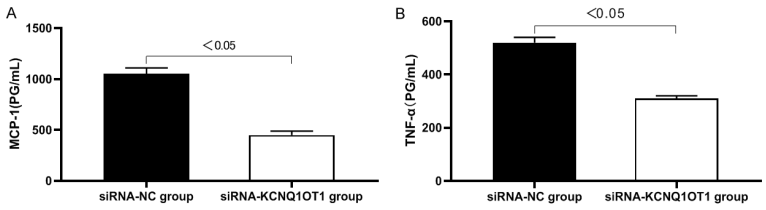

Figure 7. Effect of upregulated lncRNA KCNQ1OT1 on the expression of inflammatory factors in HAVSMCs.

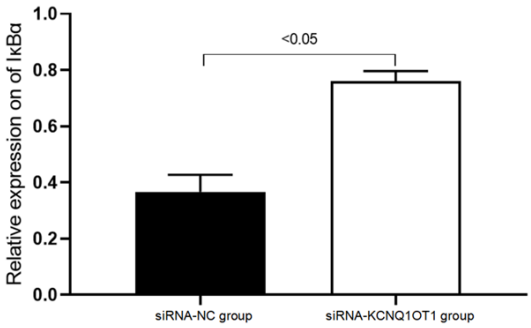

Figure 8. Effect of up-regulated lncRNA KCNQ1OT1 on the expression of IκBα in HAVSMCs induced by ox-LDL.

transfection of siRNA-KCNQ1OT1, the effects of ox-LDL on inhibiting the proliferation of HAVSMCs and inducing their apoptosis were significantly reduced, and the expression levels of MCP-1 and TNF-α were downregulated, while the relative expression of IκBα protein was notably upregulated. The results indicate that down-regulating the expression of lncRNA KCNQ1OT1 can inhibit the proliferation of HAVSMCs induced by ox-LDL and inflammatory response, and promote cell apoptosis by regulating IκBα.

Conclusions

In summary, ticagrelor may regulate the expression of lncRNA KCNQ1OT1 and up-regulate the expression of IκBα to promote proliferation and anti-apoptosis, so as to prevent ox-LDL from oxidative damage to HAVSMCs. However, this study is limited by the absence of animal tests; this will be explored in future studies.

Disclosure of conflict of interest

None.

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