Abstract. Although ticagrelor has been demonstrated to possess an anti-atherosclerosis (AS) effect, its underlying mechanism remains unclear. In the present study, it was investigated whether ticagrelor reduces oxidized low-density lipoprotein (ox-LDL)-induced endothelial cell apoptosis, an initial step for the development of AS, and alleviates AS in apolipoprotein-E-deficient (ApoE\(^{-/-}\)) mice by inhibiting the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9). The human endothelial cell line EAhy926 was treated with ox-LDL, ox-LDL + ticagrelor (40 \(\mu\)mol/l) and ox-LDL + ticagrelor (60 \(\mu\)mol/l) for 24 h. Cell apoptosis was detected using Annexin V-fluorescein isothiocyanate/propidium iodide staining. The expression levels of PCSK9, apoptosis-associated proteins and signaling pathways were determined by reverse transcription-quantitative polymerase chain reaction and western blotting. ApoE\(^{-/-}\) mice fed a high-fat diet were used to induce an AS model. After 20 weeks, ApoE\(^{-/-}\) mice were randomly assigned to receive saline or ticagrelor intragastrically for 10 days. The formation of atherosclerotic plaques was detected by hematoxylin and eosin staining. The expression of PCSK9 in the arterial tissues was measured by immunohistochemistry. The results demonstrated that treatment with ticagrelor was able to decrease ox-LDL-induced apoptosis in a concentration-dependent manner (40 \(\mu\)mol/l vs. ox-LDL, 17.58\(\pm\)2.66 vs. 27.25\(\pm\)5.54%; 60 \(\mu\)mol/l vs. ox-LDL, 12.26\(\pm\)1.54 vs. 27.25\(\pm\)5.54%). The mRNA and protein expression level of PCSK9 significantly decreased following treatment with ticagrelor, accompanied with upregulation of B-cell lymphoma (Bcl) 2 and downregulation of Bcl-2 associated X, apoptosis regulator, caspase-3, p38, phosphorylated-(p) p38, p-c-Jun N-terminal kinases (JNK), p-extracellular signal-regulated kinases and the ratio of p-JNK to JNK. Histological analysis of arterial tissues revealed ticagrelor markedly decreased the atherosclerotic plaque area and inhibited the expression of PCSK9. The present results suggested that ticagrelor may alleviate AS via downregulation of PCSK9-mediated endothelial cell apoptosis, which may be JNK-dependent.

Introduction

Atherosclerosis (AS) is one of the most prevalent diseases in elderly people. AS is characterized by the accumulation of lipids in the intima of large- and medium-sized arteries (1) (including coronary or carotid arteries); this leads to a compromised flow to target organs, resulting in hypoxic and ischemic cardiovascular events, including myocardial infarction and stroke, which are two leading causes of mortality worldwide (2,3). Therefore, the development of treatments to prevent the formation and control the progression of atherosclerotic lesions has attracted considerable attention. Accumulating evidence has demonstrated that oxidized low-density lipoprotein-(ox-LDL)-induced endothelial dysfunction is the initial step in the development of AS (4). By binding to the lectin-like endothelial ox-LDL receptor, ox-LDL promotes the production of reactive oxygen species and activates the endoplasmic reticulum stress response, which sequentially phosphorylates the mitogen-activated protein kinase (MAPK) cascade and mediates nuclear factor (NF)-xB...
activation to upregulate the expression of pro-apoptotic proteins, including caspase-3, caspase-9, caspase-12 and Bcl-2 associated X, apoptosis regulator (Bax), ultimately leading to apoptosis of endothelial cells (4-6). Endothelial cell apoptosis may destroy the integrity of endothelium and increase vascular permeability, which facilitates the infiltration and deposition of local lipids within the arterial wall and finally results in atherogenesis (7). Furthermore, disruption of the endothelial lining may additionally cause the plaque instability and rupture that triggers the coagulation cascade and platelet aggregation, eventually increasing the formation of atherothrombosis and resulting in sudden mortality (8-10). Therefore, inhibition of endothelial dysfunction may represent a promising approach for the treatment of AS (11-13).

In addition to platelets, a previous study suggested that P2Y purinoreceptor 12(P2Y12) receptors may additionally be highly expressed in endothelial cells of culprit coronary plaques (14). The use of the P2Y12 receptor antagonists clopidogrel or ticagrelor may improve endothelial dysfunction in patients with acute (15), and stable coronary artery disease (16,17) or pig models with coronary stent restenosis (18), suggesting that P2Y12 receptor blockers may be potential drugs for the treatment of AS. This hypothesis has been preliminarily verified by Preusch et al (19), which documented that treatment with ticagrelor induces a reduction in lesion size in the necrotic core area within the aortic sinus of apolipoprotein-E-deficient (ApoE−/−) mice (an AS animal model). In vitro studies additionally demonstrated that ox-LDL uptake and induced apoptosis of RAW264.7 macrophages are decreased following incubation with ticagrelor (19). Furthermore, Ren et al (20) identified that clopidogrel inhibits the progression of AS in a rabbit model by reducing the ratio of Bcl-2/Bax in the vascular wall. However, the anti-AS effect of P2Y12 receptor blockers remains rarely investigated. In addition, whether a reduction in endothelial cell apoptosis is involved and the mechanism of action have not been examined.

The aim of the present study was to further investigate the anti-AS effects in ApoE−/− mice and analyze the anti-apoptotic effects in the endothelial cell line EAhY926 following treatment with ticagrelor, a novel P2Y12 receptor inhibitor. In a previous study (21), it was observed that increased expression of proprotein convertase subtilisin/kexin type 9 (PCSK9), encoding a neural apoptosis-regulated convertase 1, is associated with ox-LDL-induced apoptosis in EAhY926 cells. Furthermore, downregulation of PCSK9 by short hairpin (sh)RNA inhibits apoptosis of EAhY926 cells and decreases levels of apoptosis-associated proteins (Bax and caspase-3) and the mitogen-activated protein kinase (MAPK) signaling pathway (21). Accordingly, it was hypothesized that the inhibition of PCSK9 expression may be a potential mechanism for ticagrelor to exert anti-AS effects. This has not been reported previously, to the best of the authors’ knowledge, and was investigated in the present study.

Materials and methods

Cell culture and grouping. The human umbilical vein endothelial cell line EAhY926 was provided by the Pathology Laboratory of Tianjin Medical University (Tianjin, China). EAhY926 cells were maintained in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin G and 100 µg/ml streptomycin and cultured at 37°C in a humidified incubator with 5% CO₂.

EAhY926 cells were divided into four groups; control, ox-LDL, ox-LDL + 40 µmol/l ticagrelor (T40) and ox-LDL + 60 µmol/l ticagrelor (T60). Oxidized LDL (50 µg/ml; Beijing Xinyuan Jiae Biotech Co., Ltd., Beijing, China) was used to induce endothelial dysfunction of cells, as previously described (20). Cells in each group were treated for 24 h.

Apoptosis assay. The cultured cells were detached by trypsinization and stained with Annexin V labeled with fluorescein isothiocyanate (BD Pharmingen; BD Biosciences, San Jose, CA, USA) and propidium iodide (1 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in the dark for 15 min at room temperature. Cells were subsequently analyzed using a flow cytometer (FACSCalibur; BD Biosciences) with CellQuest software (Version 6.0; BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from EAhY926 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by RT into cDNA using an M-MLV RT system (Takara Biotechnology Co., Ltd., Dalian, China) with the reaction parameters of incubation at 65°C for 10 min, 42°C for 30 min and 70°C for 10 min. qPCR was performed using an ABI-7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR-Green PCR kit [Biocentury Transgene (China) Co., Ltd., Shenzhen, China]. The primers used are listed in Table I. The PCR reaction parameters were incubation at 95°C for 10 sec, then 40 cycles of 95°C for 10 sec, 58°C for 30 sec and 72 for 30 sec. The relative expression of genes, using a housekeeping gene (GAPDH) as an internal standard, was calculated by the 2-∆∆Cq method (22). All measurements were performed in triplicate.

Western blotting. Proteins were extracted from EAhY926 cells using a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were measured using a 2-D Quant kit (GE Healthcare Life Sciences, Little Chalfont, UK) and equal amounts of protein (30 µg) from each group were separated by SDS-PAGE on 10% gels prior to being transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% non-fat milk at room temperature. Cells were subsequently analyzed using an M-MLV RT system (Takara Biotechnology Co., Ltd., Dalian, China) with the reaction parameters of incubation at 65°C for 10 min, penicillin G and 100 µg/ml streptomycin and cultured at 37°C in a humidified incubator with 5% CO₂.

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Animal model. All animal experiments conformed with the Regulation of Animal Care Management of the Ministry of Public Health, People's Republic of China and were approved by the Ethical Committee of Second Hospital of Tianjin Medical University (Tianjin, China).

Six-week-old male weighing 20-25 g C57BL/6 mice (n=10) and homozygous ApoE<sup>-/-</sup> mice (n=10) were purchased from Beijing University (Beijing, China). The mice were housed in a specific pathogen-free facility under controlled conditions (temperature, 22±2˚C; relative humidity, 55±15%; noise, 65±15 dBA; light cycle, 12:12 h Light:Dark). The mice were housed in individually ventilated cages, and were fed with standard laboratory chow (Promit, Beijing, China) and water ad libitum. Animals were randomly distributed into the following groups (n=10 per group): group A: Vehicle; group B: Vehicle + APOE-/-; group C: LDL-R<sup>-/-</sup>; group D: LDL-R<sup>-/-</sup> + APOE-/-.

**Table I. Primers used in the present study.**

| Gene    | Primer                     | Annealing temperature, °C | Product size, bp |
|---------|----------------------------|---------------------------|-----------------|
| GAPDH   | F 5'-CACATGGCCTCAAGGAGTA-3  | 58                        | 163             |
|         | R 5'-TCCCCCTCTGCAAGGGA-3'   |                           |                 |
| PCSK9   | F 5'-TGGAACCTCACTCGTGGG-3'  | 58                        | 140             |
|         | R 5'-GAAGGATCTGAATGTGT-3'   |                           |                 |
| Bax     | F 5'-TGATCAGAACCATGCGCC-3'  | 58                        | 119             |
|         | R 5'-GGACATCAGTCCTTGGTG-3'  |                           |                 |
| Bcl-2   | F 5'-GAAGAAGGACCCTCAAGC-3'  | 58                        | 156             |
|         | R 5'-AGCAAGGACACCGGACCTC-3' |                           |                 |
| Caspase-3| F 5'-GAGGCCGACCTCTGATGC-3'  | 58                        | 106             |
|         | R 5'-GTTTCAGCATGGCAAAACG-3' |                           |                 |
| NF-κB   | F 5'-AGACAAATGGGCTACACCGA-3' | 58                      | 186             |
|         | R 5'-AAAGCTGAGTTTGCAGGAGG-3' |                         |                 |

F, forward; R, reverse; PCSK9, proprotein convertase subtilisin/kexin type 9; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X, apoptosis regulator; NF-κB, nuclear factor-κB.

**Table II. Antibody details.**

| Antibody | Supplier       | Primary antibody | Secondary antibody | Molecular weight, kDa |
|----------|----------------|------------------|--------------------|-----------------------|
| PCSK9    | Sigma-Aldrich  | SAB1302902       | Rabbit             | 72                    |
| Caspase-3| Abcam          | ab32042          | Rabbit             | 32                    |
| Bax      | Abcam          | ab32503          | Rabbit             | 21                    |
| Bcl-2    | Abcam          | ab32124          | Rabbit             | 26                    |
| p38      | Abcam          | ab27986          | Rabbit             | 38                    |
| p-p38    | Abcam          | ab4822           | Rabbit             | 38                    |
| ERK      | Abcam          | ab17942          | Rabbit             | 42-44                 |
| p-ERK    | Abcam          | ab214362         | Rabbit             | 42-44                 |
| JNK      | Abcam          | ab179461         | Rabbit             | 46                    |
| p-JNK    | Abcam          | ab124956         | Rabbit             | 46                    |
| β-actin  | Abcam          | ab8226           | Mouse              | 43                    |

Sigma-Aldrich; Merck KGaA Darmstadt, Germany and Abcam, Cambridge, UK. PCSK9, proprotein convertase subtilisin/kexin type 9; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X, apoptosis regulator; p-, phosphorylated; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases.

Animal model. All animal experiments conformed with the Regulation of Animal Care Management of the Ministry of Public Health, People's Republic of China and were approved by the Ethical Committee of Second Hospital of Tianjin Medical University (Tianjin, China). Six-week-old male weighing 20-25 g C57BL/6 mice (n=10) and homozygous ApoE<sup>-/-</sup> mice (n=10) were purchased from Beijing University (Beijing, China). The mice were housed in a specific pathogen-free facility under controlled conditions (temperature, 22±2˚C; relative humidity, 55±15%; noise, 65±15 dBA; light cycle, 12:12 h Light:Dark). The mice were housed in individually ventilated cages, and were fed with standard laboratory chow (Promit, Beijing, China) and water ad libitum. Animals were randomly distributed into the following groups (n=10 per group): group A: Vehicle; group B: Vehicle + APOE-/-; group C: LDL-R<sup>-/-</sup>; group D: LDL-R<sup>-/-</sup> + APOE-/-.
<60 dB; light/dark cycle, 12/12 h) and were used following one week of accommodation. All animals were provided with free access to water/food. ApoE<sup>−/−</sup> mice were fed with a high-fat diet containing 0.25% cholesterol and 15% cocoa butter (Hunan Huakang Biotech Inc., Hunan, China) to induce the formation of the atherosclerotic plaques. Normal C57BL/6 mice were given standard chow throughout the experiment to serve as a control. After 20 weeks of feeding, ApoE<sup>−/−</sup> mice were randomly divided into the model group (AS, n=5) and treatment group (ticagrelor, n=5). Mice in the treatment group received ticagrelor (100 mg/kg, corresponding to concentration T60 in vivo) through intragastric administration for 10 consecutive days, while an equal amount of saline was intrastragically administered to AS and control mice in the same period.

**Biochemical analysis.** Mice in each group were anesthetized with 5% pentobarbital sodium (50 mg/kg) by intraperitoneal injection and blood was collected through direct cardiac puncture. The samples were subsequently centrifuged at 1,200 x g for 10 min at room temperature to obtain the plasma for biochemical measurements. The plasma levels of triglyceride (TG), cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined using an automatic biochemical analyzer (Olympus AU5400; Olympus Corporation, Tokyo, Japan).

**Histological analysis.** Following cardiac exsanguination, the aorta were quickly harvested, fixed in 4% paraformaldehyde at 4°C for 24 h, dehydrated in a graded series of alcohol (70, 80, 90, 95 and 100%, each 90 min), embedded in 5-µm-thick paraffin and stained with hematoxylin (5 min) & eosin (5 min) at room temperature. Images were captured using a light microscope (OLYMPUS X81; Olympus Corporation) at magnification, x10 and x40.

**Immunohistochemical staining.** The embedded tissue sections were incubated overnight at 4°C with a primary antibody against PCSK9 (1:200; Sigma-Aldrich; Merck KGaA) following a rehydration in a graded series of alcohol (100, 95, 80, 70%, each 5 min), hydrogen peroxide-induced endogenous peroxidase activity inhibition, microwave-based antigen retrieval (121°C for 10 min) and non-specific binding blocked with 10% normal goat serum (Invitrogen; Thermo Fisher Scientific, Inc.; at room temperature, 10 min). Following three washes in sterile PBS, the sections were incubated with the secondary antibody (1:500; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. The immunohistochemical reaction color was developed with diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA, USA; 2-8 min) and counterstained with hematoxylin (2 min) at room temperature. The expression of PCSK9 in the tissues was viewed under a light microscope (OLYMPUS X81; Olympus Corporation) at magnification, x10 and x40 and semi-quantitatively measured using Image Pro Plus software (Version 6.0; Media Cybernetics, Inc.).

**Statistical analysis.** Statistical analyses were performed using SPSS software (Version 18.0; SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error. of three independent repeats. The significant difference between two experimental groups was analyzed using Student's t-test; however, statistical significance of differences among three groups were assessed using one-way analysis of variance followed by the Least Significant Difference post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Ticagrelor inhibits ox-LDL-induced apoptosis in EAhy926 cells.** Damage in the vascular endothelium has been suggested as an initial step in the pathogenesis of AS (4). To determine whether ticagrelor alleviates AS, the effect of ticagrelor on ox-LDL-induced endothelial dysfunction was evaluated. The present data demonstrated that treatment with 50 µg/ml ox-LDL resulted in a significant increase in apoptosis of EAhy926 cells compared with the control (27.25±5.54 vs. 7.62±1.76%; P<0.05; Fig. 1). However, the addition of ticagrelor was able to decrease ox-LDL-induced apoptosis, particularly at a higher concentration (T40, 17.58±2.66%; T60, 12.26±1.54%; Fig. 1). Therefore, 60 µmol/l ticagrelor was used for subsequent experimentation.

**Ticagrelor downregulates PCSK9 expression and downstream apoptosis pathways in EAhy926 cells.** To elucidate the mechanisms by which ticagrelor inhibits endothelial apoptosis, the expression of PCSK9 and downstream apoptosis pathway genes were additionally evaluated. As expected, the mRNA expression level of PCSK9 determined by RT-qPCR was significantly decreased following treatment with ticagrelor (P<0.05; Fig. 2). The use of ticagrelor additionally upregulated mRNA expression of the anti-apoptotic factor Bcl-2 and significantly downregulated the expression of the pro-apoptotic factors Bax and caspase-3 (Fig. 2; P<0.05). This result was similarly observed in the analysis of protein expression levels by western blotting (Fig. 3). Furthermore, the expression levels of apoptosis pathway proteins, including p-p38, p-JNK, JNK and p-ERK, were significantly suppressed following treatment with ticagrelor; the JNK pathway may be of particular importance, as difference between the p-JNK/JNK ratios was statistically significant (P<0.05; Fig. 3). These results suggested that ticagrelor reduced ox-LDL-induced endothelial apoptosis by downregulating PCSK9 and downstream apoptosis pathways.

**Intragastric administration of ticagrelor reduces AS development in ApoE<sup>−/−</sup> mice.** To further confirm the anti-atherosclerotic
role and mechanisms of action of ticagrelor in vivo, an AS animal model, of ApoE<sup>−/−</sup> mice fed a high-fat diet, was constructed. The health condition of the majority of mice was excellent during the modeling, with no mortality observed in
any group. Neither treatment affected food intake, and body weight increased in all groups after 20 weeks, particularly the AS and ticagrelor groups due to the high-fat diet provided (Table III). Unexpectedly, there was no significant difference in body weight between the AS and ticagrelor groups (Table III). Likewise, plasma concentrations of TC, LDL and HDL were equivalent in ApoE-/- mice irrespective of ticagrelor exposure (Table IV). These results suggested that ticagrelor had no effect on lipid metabolism. Histological analysis in the control group revealed that the vascular endothelium of the aorta was complete, smooth and arranged in order, without an increase in aortic arch inner membrane thickness and formation of plaques in the arteries (Fig. 4). However, the vascular endothelium was not intact and was even partly falling off in the AS model group. Additionally, abundant plaque formation and lipid deposition were observed in the lumen, accompanied by infiltration of numerous inflammatory cells (Fig. 4). Ticagrelor markedly decreased the atherosclerotic plaque area and increased the lumen area (Fig. 4), demonstrating the anti-AS effect of this drug.

The aortas of the mice were additionally collected to analyze the expression of PCSK9 by immunohistochemistry to determine the association between PCSK9 and AS and the mechanism of action of ticagrelor. As anticipated, there were
no brown-yellow particles (representing PCSK9 expression) around the blue nuclei in the control group. In contrast, PCSK9 was abundantly expressed in the aortic plaque of the AS group. Compared with the AS group, the expression level of PCSK9 was significantly decreased following treatment with ticagrelor ($P<0.05$; Fig. 5). These results further illustrated that downregulation of PCSK9 may be an important mechanism for ticagrelor to alleviate the formation and development of AS.

Discussion

Increasing evidence demonstrates that P2Y12 receptor antagonists may improve endothelial dysfunction in patients with acute (15), stable coronary artery disease (16,17) or pig models with coronary stent restenosis (18); however, the endothelial function in these studies was determined by measuring the vasomotor responses. The effects of P2Y12 receptor antagonists on endothelial cell apoptosis, a key event in the pathogenesis of AS, have not been investigated. In the present study, for the first time to the best of the authors’ knowledge, the influence of ticagrelor, a relatively novel P2Y12 receptor inhibitor (23), on ox-LDL-induced apoptosis of the endothelial cell line EAhy926, was examined. As expected, the present results demonstrated that treatment with ticagrelor was able to decrease ox-LDL-induced apoptosis, particularly at higher doses (40 $\mu$mol/l vs. ox-LDL, 17.58±2.66 vs. 27.25±5.54%; 60 $\mu$mol/l vs. ox-LDL, 12.26±1.54 vs. 27.25±5.54%). This is in agreement with a previous study that demonstrated that clopidogrel reduces palmitic acid-induced apoptosis of human vascular endothelial cells (24). However, this finding may be attributed to the P2Y12 receptor expressed on endothelial...
cells (14) and to the interaction between the endothelium and platelets (25), as the P2Y12 receptor is primarily expressed in blood platelets (23). Ticagrelor may promote the degradation of adenine nucleotides released from platelets to adenosine (26) and thus inhibit ADP/ATP binding to endothelial P2Y receptors (13), blocking the production of pro-inflammatory factors (including nitric oxide and PGI2) (27,28) and the downregulation of nucleolin (29), which eventually suppresses cell cycle arrest in S phase and cell apoptosis and stimulates cell proliferation (29,30).

PCSK9 is a protein that was initially identified to be upregulated in apoptotic neural cells, and for this reason it was additionally termed neural apoptosis regulated conver-
tase 1 (31,32). However, previous studies documented that PCSK9 is additionally involved in endothelial cell apoptosis. Knock-out of PCSK9 by small interfering RNA or shRNA inhibits ox-LDL-induced endothelial cell apoptosis and alleviates the formation of atherosclerotic plaques (21,33). Therefore, it was speculated that downregulation of PCSK9 may be an underlying mechanism for ticagrelor to inhibit endothelial cell apoptosis. This was demonstrated, for the first time to the best of the authors' knowledge, by the present results at the mRNA and protein expression level. Although the way in which ticagrelor regulates PCSK9 expression remains to be further elucidated, it is hypothesized it may serve as a link between PCSK9 and platelets (34). It has been demonstrated that increased PCSK9 serum levels are positively associated with the platelet count (r=0.218), plateletcrit (r=0.250) (35) and platelet reactivity (r=0.30) in patients with acute coronary syndrome (36). Therefore, the anti-platelet agent ticagrelor may influence PCSK9 expression by reducing platelets.

Caspase-3 and Bax are common genes that serve important roles in the process of apoptosis; whereas Bcl-2 exerts an anti-apoptotic effect via a mitochondria-dependent caspase-9 pathway. MAPKs are a group of associated serine-threonine protein kinases, which participate in the mediation of cell growth, apoptosis, proliferation and function. Therefore, these genes were predicted to be involved in ox-LDL-induced endothelial cell apoptosis and AS. This postulation has been confirmed by numerous studies. Chen et al (37) demonstrated that ox-LDL is able to induce endothelial cell apoptosis via a Bax-mitochondria-caspase protease pathway. Furthermore, Yu et al (38) demonstrated that ox-LDL activates the cell MAPKs, particularly JNK and p38. Therefore, inhibition of the expression of caspase-3, Bax and blocking of MAPK activation may protect endothelial cells against apoptosis. In our previous study, it was observed that shRNA-PCSK9 markedly inhibits the expression of pro-apoptotic proteins and promotes anti-apoptotic proteins accompanied with alteration in the phosphorylation of p38 and c-Jun N-terminal kinases (21). This was additionally concluded in the study conducted by Wu et al (33). Accordingly, it was hypothesized that suppression of these PCSK9-mediated apoptosis signaling pathways may be the inhibitory mechanism of ticagrelor for endothelial cell apoptosis and AS. Therefore, the expression levels of caspase-3, Bcl-2, Bax and MAPK were additionally analyzed following treatment with ticagrelor. In agreement with previous studies on anti-apoptosis drugs (6,38,39), the present results demonstrated that ticagrelor suppressed ox-LDL-induced p38MAPK, JNK, ERK activation (particularly JNK), caspase-3 and Bcl-2; however, upregulated Bax in EAh926 cells.

In addition to in vitro experiments, an in vivo study was conducted. In accordance with previous studies, the present results demonstrated that the use of ticagrelor inhibited the progression of AS (20) and reduced the lesion size (19). Compared with the study of Preusch et al (19) who additionally used ApoE−/− mice, the present results may be of increased use to researchers as a high-fat diet was fed to the mice.

There are some limitations in the present study. First, further studies with quantitative assessment are still required to confirm the therapeutic effects of ticagrelor on the atherosclerotic plaque. Second, the MAPK agonists should be used to further validate the mechanisms of ticagrelor in AS.

In conclusion, the present results preliminarily demonstrated that ticagrelor may suppress ox-LDL-induced endothelial cell apoptosis and alleviate AS by downregulating PCSK9 and the downstream apoptotic MAPK signaling pathway. These results may provide a theoretical basis for the use of ticagrelor to treat patients with AS in a clinical setting.

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Availability of data and materials

The datasets from current study are available from the corresponding author on reasonable request.

Authors’ contributions

XX, XL and GL designed the study. XX and JL performed the experiments. SZ and JYL were involved in the statistical analyses. TL and MA participated in the interpretation of the data. XX, XL and GL wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments conformed with the Regulation of Animal Care Management of the Ministry of Public Health, People’s Republic of China and were approved by the Ethical Committee of Second Hospital of Tianjin Medical University (Tianjin, China).

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

References

1. Clarke R, Du H, Kurmi O, Parish S, Yang M, Arnold M, Guo Y, Bian Z, Wang L, Chen Y, et al: Burden of carotid artery atherosclerosis in Chinese adults: Implications for future risk of cardiovascular diseases. Eur J Prev Cardiol 24: 647-656, 2017.

2. Barquera S, Pedroza-Tobías A, Medina C and, Hernández-Barrera L, Bibbins-Domingo K, Lozano R and Moran A: An overview of the epidemiology of atherosclerotic cardiovascular disease. Arch Med Res 46: 328-338, 2015.

3. Mathers CD and Conner D: Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 3: e442, 2006.

4. Chen, M. Asasaki T and Sawamura T: LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: Implications in endothelial dysfunction and atherosclerosis. Pharmacol Ther 95: 89-100, 2002.

5. Hong D, Bai YP, Gao HC, Wang X, Li LF, Zhang GG and Hu CP: Ox-LDL induces endothelial cell apoptosis via the LOX-1-dependent endoplasmic reticulum stress pathway. Atherosclerosis 235: 310-317, 2014.

6. Bao MH, Zhang YW and Zhou HH: Paeonol suppresses oxidized low-density lipoprotein induced endothelial cell apoptosis via activation of LOX-1/p38MAPK/NF-kB pathway. J Ethnopharmacol 143: 353-361, 2013.

7. Xu F, Fan Y, Sun Y, Sun Y, Li R, Liu C, Zhang C, Wang R and Zhang Y: Endothelial cell apoptosis is responsible for the formation of coronary thrombotic atherosclerotic plaques. Tohoku J Exp Med 218: 25–33, 2009.

8. Durand E, Scouazec A, Lafont A, Bodduert J, Al Hajzen A, Addad F, Mirshahi M, Desnos M, Tedgui A and Mallat Z: In vivo induction of endothelial apoptosis leads to vessel thrombosis and endothelial denudation a clue to the understanding of the mechanisms of thrombotic plaque erosion. Circulation 109: 2503-2506, 2004.

9. Badimon L and Vilahur G: Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med 276: 618-632, 2015.

10. Raskob GE, Anghiauskisri P, Blanco AN, Böller H, Gallus A, Hunt BJ, Hylek EM, Kakkar TL, Konstantinides SV, Mathers CD and Loncar D: Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 3: e442, 2006.

11. Nakamura T, Shishido K, Fujii S, Ueno S, Tanaka T, Kadowaki T, Hara T, Yokosuka O, Tamaoki N, Tanaka K et al: P2Y12 receptor is a major contributor to global cardiovascular disease. Arch Med Res 46: 328-338, 2015.

12. Kim HK, Jeong MH, Lim KS, Kim JM, Lim HC, Kim MC, Hong YJ, Kim SS, Park KH and Chang KS: Effects of ticagrelor on neointimal hyperplasia and endothelial function, compared with clopidogrel and prasugrel, in a porcine coronary stent restenosis model. Int J Cardiol 240: 326-331, 2017.

13. Preusch MR, Rusnak J, Staudacher K, Mogler C, Uhlmann L, Sievers P, Bea F, Katus HA, Blessing E and Staudacher I: Ticagrelor promotes atherosclerotic plaque stability in a mouse model of advanced atherosclerosis. Drug Des Devel Ther 10: 151-158, 2016.

14. Ren H, Li ML, Jiang J, Zhang Y, Zhang Y and Zhu X: Effects of clopidogrel on vascular proliferation and apoptosis in an atherosclerotic rabbit model. J Cardiovasc Pharmacol 55: 617-624, 2010.

15. Li J, Jiang X, Wang Y, Xu Z and Li G: Investigation of highly expressed PCSK9 in atherosclerotic plaques and ox-LDL-induced endothelial cell apoptosis. Mol Med Rep 16: 1817-1825, 2017.

16. Livak JK and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

17. Capodanno D, Drarmashankar K and Angiolillo DJ: Mechanism of action and clinical development of ticagrelor, a novel platelet ADP P2Y12 receptor antagonist. Expert Rev Cardiovasc Ther 8: 151-158, 2010.

18. Wang J, Chen L, Li H, Yang J, Gong Z, Wang B and Zhao X: Clopidogrel reduces apoptosis and promotes proliferation of human vascular endothelial cells induced by palmitic acid via suppression of the long non-coding RNA HIF1-alpha in vitro. Mol Cell Biochem 404: 203-210, 2015.

19. Mathers CD and Loncar D: Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 3: e442, 2006.

20. Courtois MR, Rusnak J, Staudacher K, Mogler C, Uhlmann L, Sievers P, Bea F, Katus HA, Blessing E and Staudacher I: Ticagrelor promotes atherosclerotic plaque stability in a mouse model of advanced atherosclerosis. Drug Des Devel Ther 10: 151-158, 2016.

21. Wang J, Chen L, Li H, Yang J, Gong Z, Wang B and Zhao X: Clopidogrel reduces apoptosis and promotes proliferation of human vascular endothelial cells induced by palmitic acid via suppression of the long non-coding RNA HIF1-alpha in vitro. Mol Cell Biochem 404: 203-210, 2015.

22. Capodanno D, Drarmashankar K and Angiolillo DJ: Mechanism of action and clinical development of ticagrelor, a novel platelet ADP P2Y12 receptor antagonist. Expert Rev Cardiovasc Ther 8: 151-158, 2010.
37. Chen TG, Chen TL, Chang HC, Tai YT, Cherng YG, Chang YT and Chen RM: Oxidized low-density lipoprotein induces apoptotic insults to mouse cerebral endothelial cells via a Bax-mitochondria-caspase protease pathway. Toxicol Appl Pharmacol 219: 42-53, 2007.

38. Yu W, Ying H, Tong F, Zhang C, Quan Y and Zhang Y: Protective effect of the silkworm protein 30Kc6 on human vascular endothelial cells damaged by oxidized low density lipoprotein (Ox-LDL). PLoS One 8: e68746, 2013.

39. Zhang L, Jia YH, Zhao XS, Zhou FH, Pan YY, Wan Q, Cui XB, Sun XG, Chen YY, Zhang Y and Cheng SB: Trichosanatine alleviates oxidized low-density lipoprotein induced endothelial cells injury via inhibiting the LOX-1/p38 MAPK pathway. Am J Transl Res 8: 5455-5464, 2016.