

Received: 2019.04.16
Accepted: 2019.07.02
Published: 2019.12.21

Beneficial Effects of Ticagrelor on Oxidized Low-Density Lipoprotein (ox-LDL)-Induced Apoptosis in Human Umbilical Vein Endothelial Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDE 1 **Meini Kang***
ABCDE 2 **Shuai Shao***
DEFG 2 **Yue Zhang**
DEF 2 **Xue Liang**
DEF 2 **Kai Zhang**
AEFG 2 **Guangping Li**

1 Department of Family Medicine, Tianjin United Family Hospital, Tianjin, P.R. China
2 Tianjin Key Laboratory of Ionic-Molecular Function of Cardiovascular Disease, Department of Cardiology, Tianjin Institute of Cardiology, The Second Hospital of Tianjin Medical University, Tianjin, P.R. China

* Meini Kang and Shuai Shao contributed equally to this work

Corresponding Author: Guangping Li, e-mail: tic_tjcardiol@126.com

Source of support: National Natural Science Foundation of China (No: 81570304); Tianjin Municipal Science and Technology Commission (13ZCDSY01400); Key Laboratory Research Foundation of Second Hospital of Tianjin Medical University (2017ZDSYS04)

Background: Ticagrelor is the first oral anti-platelet agent which has direct anti-platelet aggregation effect by combining with ADP P2Y₁₂ receptors in platelets. It has been approved to reduce the incidence of thrombus cardiovascular events in acute coronary syndrome patients. However, the effects of ticagrelor on endothelial apoptosis have not been investigated.





Material/Methods: Oxidized low-density lipoprotein (ox-LDL) was used to establish a human umbilical vein endothelial cell (HUVEC) apoptosis model. To investigate the effects of ticagrelor on endothelial apoptosis, the HUVECs were treated with different dose of ticagrelor. Apoptosis rates of HUVECs was evaluated by flow cytometry, and the expression levels of Akt, p-Akt, Bcl-2, Bax, caspase-3, endothelial nitric oxide synthase (eNOS), and nitric oxide (NO) concentration were assessed.

Results: After treatment with 50 ug/mL ox-LDL or 100 ug/mL ox-LDL, we found that the late apoptosis and necrosis rate and the expression levels of Bax and caspase-3 were significantly increased in HUVECs, whereas the expression levels of Akt, p-Akt, Bcl-2, eNOS, and NO were significantly decreased. Ticagrelor restored the apoptosis rate of ox-LDL-induced HUVECs in a dose-dependent manner. In addition, compared with ox-LDL group, ticagrelor treatment significantly increased the expression levels of Akt, p-Akt, Bcl-2, eNOS, and NO concentration, and significantly decreased the expression levels of Bax and caspase-3.

Conclusions: We found that ox-LDL induced significant apoptosis and necrosis in our model, which was dose-dependently improved by ticagrelor. These changes might be explained by alterations in apoptosis and antioxidant pathways.

MeSH Keywords: **Apoptosis Inducing Factor • Platelet Aggregation Inhibitors • Proto-Oncogene Proteins c-akt • Receptors, Oxidized LDL**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/917001>

 2024  —  4  19



Background

Vascular endothelial cells form a biological barrier to prevent the invasion of vascular exogenous substances across the vessel wall, and their apoptosis has been considered one of the important factors for the initiation and development of atherosclerosis [1]. Phosphatidylinositol 3 kinase/serine-threonine kinase (PI3K/Akt) is one of the most important pathways to regulate proliferation and maintain biological characteristics of cells. Akt activated by PI3K can regulate apoptosis of cells in pleiotropic ways. The main role of Akt is anti-apoptosis although it shows apoptosis-promoting effect in some special conditions, and it may participate in the anti-apoptosis process of endothelial cells in the early period of atherosclerosis [2,3].

Ticagrelor is the first oral anti-platelet agent which has direct anti-platelet aggregation effect by combining with adenosine diphosphate (ADP) P2Y₁₂ receptors in platelets reversibly [4]. It has been approved to reduce the incidence of thrombus cardiovascular events in acute coronary syndrome patients and improve the function of endothelium [5–7]. In previous studies on clopidogrel, another ADP receptor antagonist, the authors found that clopidogrel-treated rabbits showed a significant reduction in progression of atherosclerosis, including low expression levels of high sensitivity C-reactive protein and platelet-derived growth factor, reduction in intima thickness, and decreased ratio of bcl-2/bax in the vascular wall. These results suggest that clopidogrel can retard the progression of established lesions that is related to inhibiting inflammation, cell proliferation, and promotion of cell apoptosis [8].

The effect of ticagrelor on endothelial apoptosis is not completely illustrated. Hence, we established HUVECs apoptosis model by administration of ox-LDL to investigate the intervention effect and the possible molecular mechanisms of ticagrelor on the apoptosis of endothelial cells in the early period of atherosclerosis, which may provide further understanding of the molecular mechanism and new ideas for clinical treatment of atherosclerosis.

Material and Methods

Cell culture

HUVECs pools (purchased from Life Technologies) were plated on a 6-well plate and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo Fisher Scientific, Beijing). The medium consisted of D-glucose (4.5 g/L), L-glutamine (100 U/mL), sodium pyruvate (110 mg/L), streptomycin (100 mg/mL), penicillin (100 U/mL), and 10% heat-inactivated fetal calf serum (10% FBS) at 37°C with 5% CO₂.

Flow cytometry

Cells were washed 3 times with phosphate buffered solution and divided into 6 groups: negative control 24-hour (NC 24h) group, NC 48h group, 50 ug/mL ox-LDL 24h group, 50 ug/mL ox-LDL 48h group, 100 ug/mL ox-LDL 24h group, and 100 ug/mL ox-LDL 48h group. For ox-LDL groups, cells were grown in 50 ug/mL or 100 ug/mL ox-LDL for 24 hours or 48 hours. After the optimum concentration and duration to induce HUVECs apoptosis were determined, the ox-LDL-induced cells were treated with different dose of ticagrelor; ticagrelor dissolved in dimethyl sulfoxide (DMSO) was added at a final concentration of 20 µmol/L, 40 µmol/L, or 60 µmol/L. Therefore, the cells were divided into 5 groups: NC group, ox-LDL group, ox-LDL+20 µmol/L ticagrelor (ox-LDL+T20) group, ox-LDL+T40 group, and ox-LDL+T60 group. For the detection of apoptosis, HUVECs (1×10⁶) were stained with Annexin V conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI) by using AV-FITC kit according to the manufacturer's guidelines. No less than 20 000 cells were attained in a FACSVerse flow cytometer. Then the stained cells were examined with 488 nm excitation filter and a 525 nm for FITC and a 620 nm filter for PI detection. This staining allowed us to separate 3 subsets of cells, Annexin V negative/PI negative represented viable cells, Annexin V positive/PI negative indicated early apoptotic cells, and Annexin V positive/PI positive indicated late apoptotic and necrotic cells.

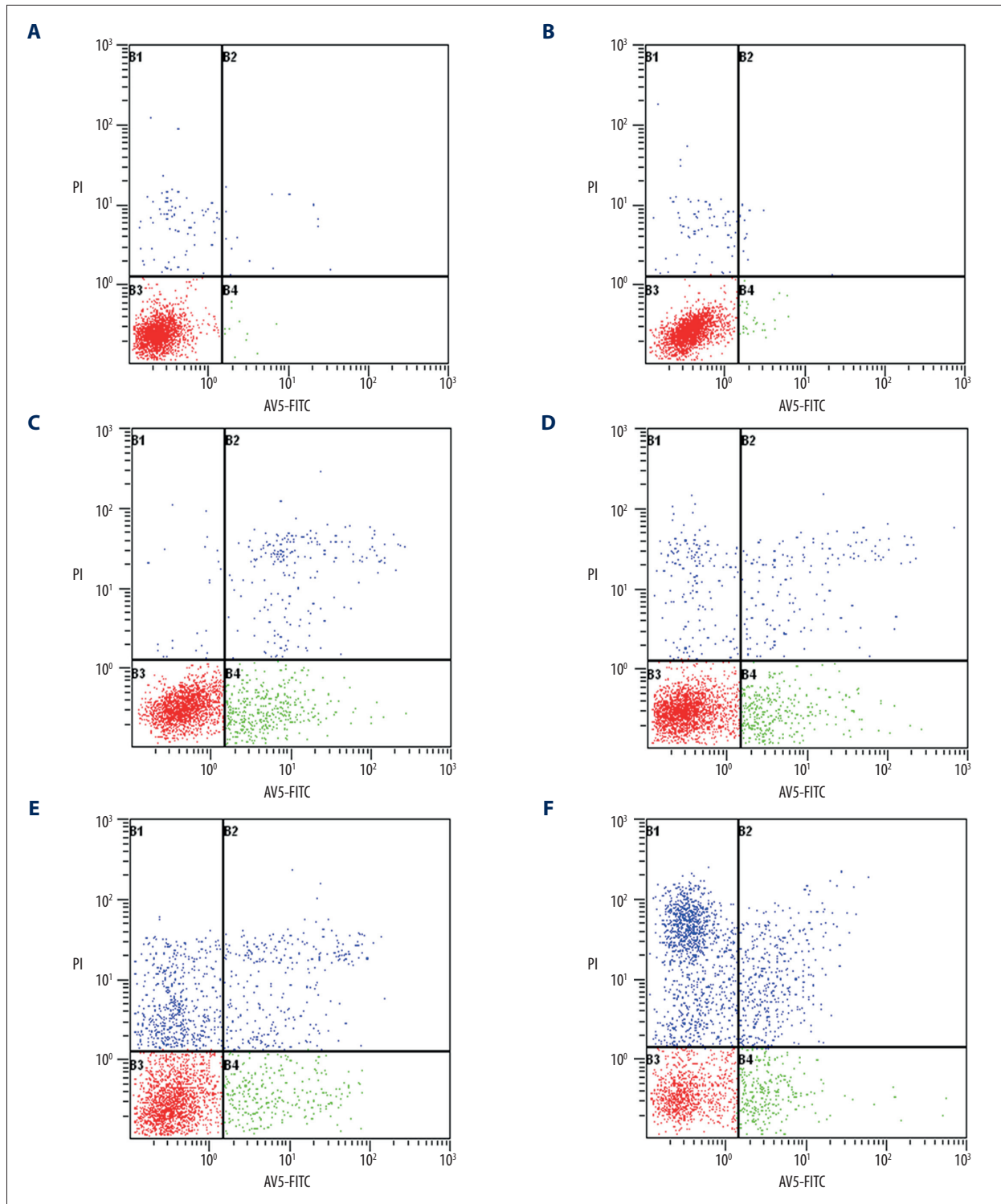
Western blot

Western blot analysis was used to determine the protein expression levels of Akt, p-Akt, Bcl-2, Bax, caspase-3, total eNOS, and β-actin. Briefly, cells were lysed in RIPA buffer (KeyGEN BioTECH, Nanjing) and phenylmethylsulfonyl fluoride (KeyGEN BioTECH), and protein concentration was quantified by BCA Protein Assay Kit (Thermo Scientific, Wilmington). The samples (about 10 µg same amount of total protein) were separated on 8% PAGE gels followed by transferring to PVDF membranes, and the membranes were incubated overnight at 4°C with the following primary antibodies: β-actin (Proteintech, Danvers, MN, USA, 1: 4000), Akt (Cell Signaling, Boston, MA, USA, 1: 1000), p-Akt (Abcam, Branford, CT, USA, 1: 7500), Bcl-2 (Abcam, Inc, Cambridge, UK, 1: 2000;), Bax (Abcam, Inc, Cambridge, UK, 1: 2000;), caspase-3 (Cell Signaling, 1: 2000), eNOS (Franklin Lakes, NJ, USA), and washed 3 times with TBS+Tween (TBST), and then the membranes were incubated with secondary peroxidase-conjugated antibodies for 1 hour at room temperature. After washed 3 times with TBST, membranes were developed using Western Lightning ECL Pro (PerkinElmer, Waltham, MA, USA). And the band intensity was assessed using ImageJ laboratory software, β-actin was used as standardization for the quantitative assessment of western blot.

Enzyme-linked immunosorbent assay (ELISA)

Concentration of NO in HUVEC was evaluated by enzyme-linked immunosorbent assay (ELISA) (Cell Signaling, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, HUVECs

were treated with different dose of ticagrelor for 24 hours. Quantitative analysis of NO was performed after 24-hour incubation. After the protein concentration was quantified by BCA kit, about 10 μg of total protein was added to each well. Data were acquired at 550 nm.



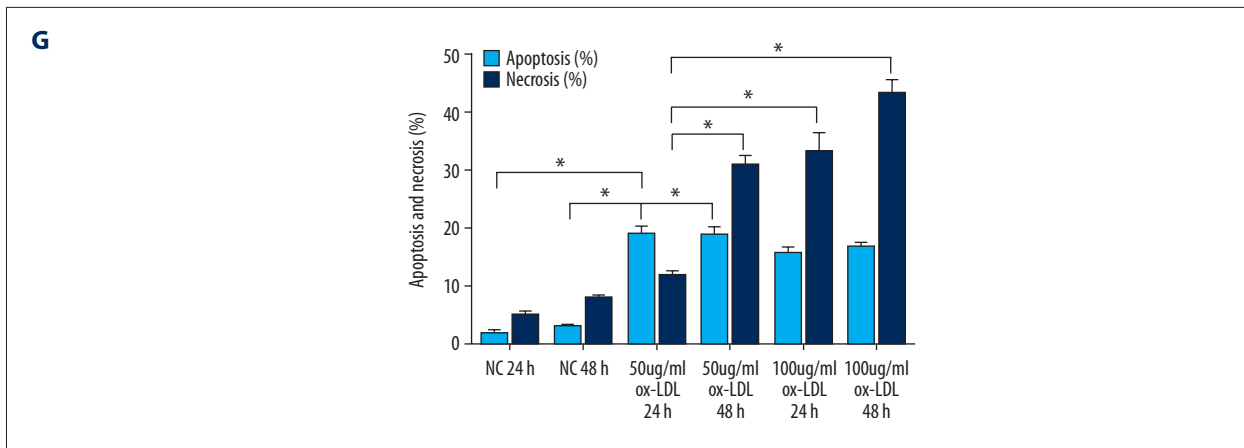


Figure 1. Representative flow cytometric data from HUVECs. The lower left quadrant represents living cells, the lower right quadrant represents early apoptosis cells, the upper left quadrant and the upper right quadrant represent late apoptosis and necrosis cells. (A) NC 24 h; (B) NC 48 h; (C) 50 ug/mL ox-LDL 24 h; (D) 50 ug/mL ox-LDL 48 h; (E) 100 ug/mL ox-LDL 24 h; (F) 100 ug/mL ox-LDL 48 h. (G) Comparison of apoptosis and necrosis rates in each group. * Indicates significant difference between the 2 groups at $P<0.05$.

Statistical analysis

All data are expressed as mean±standard error and compared with one-way ANOVA followed by Bonferroni correction, 2-tailed unpaired Student's *t*-test was used for comparison between the 2 groups. GraphPad Prism 6.0 was used for data statistical analysis. *P* value <0.05 was considered as statistically significant.

Results

Ox-LDL induced significant apoptosis in HUVECs

After induced via 50 ug/mL and 100 ug/mL ox-LDL for 24 hours and 48 hours, our results showed that the apoptosis rate in HUVECs was increased in a time-and dose-dependent manner. After stimulated with 50 ug/mL ox-LDL, the late apoptosis and necrosis rates were $19.06\pm1.65\%$ and $11.86\pm0.43\%$ at 24 hours, and $18.95\pm1.98\%$ and $30.83\pm1.95\%$ at 48 hours, respectively. And stimulated with 100 ug/mL ox-LDL, the late apoptosis and necrosis rates were $16.96\pm1.05\%$ and $33.06\pm3.03\%$ at 24 hours, and $17.37\pm0.67\%$ and $43.12\pm2.55\%$ at 48 hours, respectively (Figure 1). Figure 1 shows that comparison with other groups: 50 ug/mL ox-LDL and 24 hours induced relatively higher apoptosis rate and relatively necrosis rate. Therefore, the optimum concentration and duration to induce HUVECs apoptosis by ox-LDL might be 50 ug/mL and 24 hours.

Ticagrelor dose-dependently reduced ox-LDL-induced apoptosis in HUVECs

Compared with ox-LDL group, the apoptosis rate was significantly decreased after the intervention of ticagrelor in 50 ug/mL ox-LDL-induced HUVECs model at 24 hour, and a dose-dependent improvement in ticagrelor groups was observed. The late apoptosis rate in NC, ox-LDL, ticagrelor₂₀, ticagrelor₄₀, ticagrelor₆₀ groups were $1.06\pm0.15\%$, $15.34\pm1.47\%$, $6.51\pm1.49\%$, $4.63\pm0.39\%$, and $3.42\pm0.09\%$ respectively (Figure 2).

Ticagrelor attenuated ox-LDL-induced HUVECs apoptosis through the Akt pathway

Compared with NC group, the expression levels of anti-apoptosis related factors Akt, p-Akt, and Bcl-2 were significantly decreased, the expression levels of pro-apoptosis factors Bax and caspase-3 were significantly increased, and the expression level of antioxidant factor eNOS was also significantly decreased in the ox-LDL group. In addition, p-Akt/Akt and Bcl-2/Bax were significantly decreased in the ox-LDL group compared to the NC group. These changes were significantly improved in the ticagrelor group (Figure 3). As for NO concentration, NO concentration was significantly decreased in ox-LDL group compared with the NC group, while NO concentration in the ticagrelor groups was significantly increased (Figure 4).

Discussion

In this study, we found that ox-LDL induced significant apoptosis and necrosis in our model, and the optimum concentration and duration to induce HUVECs apoptosis by ox-LDL

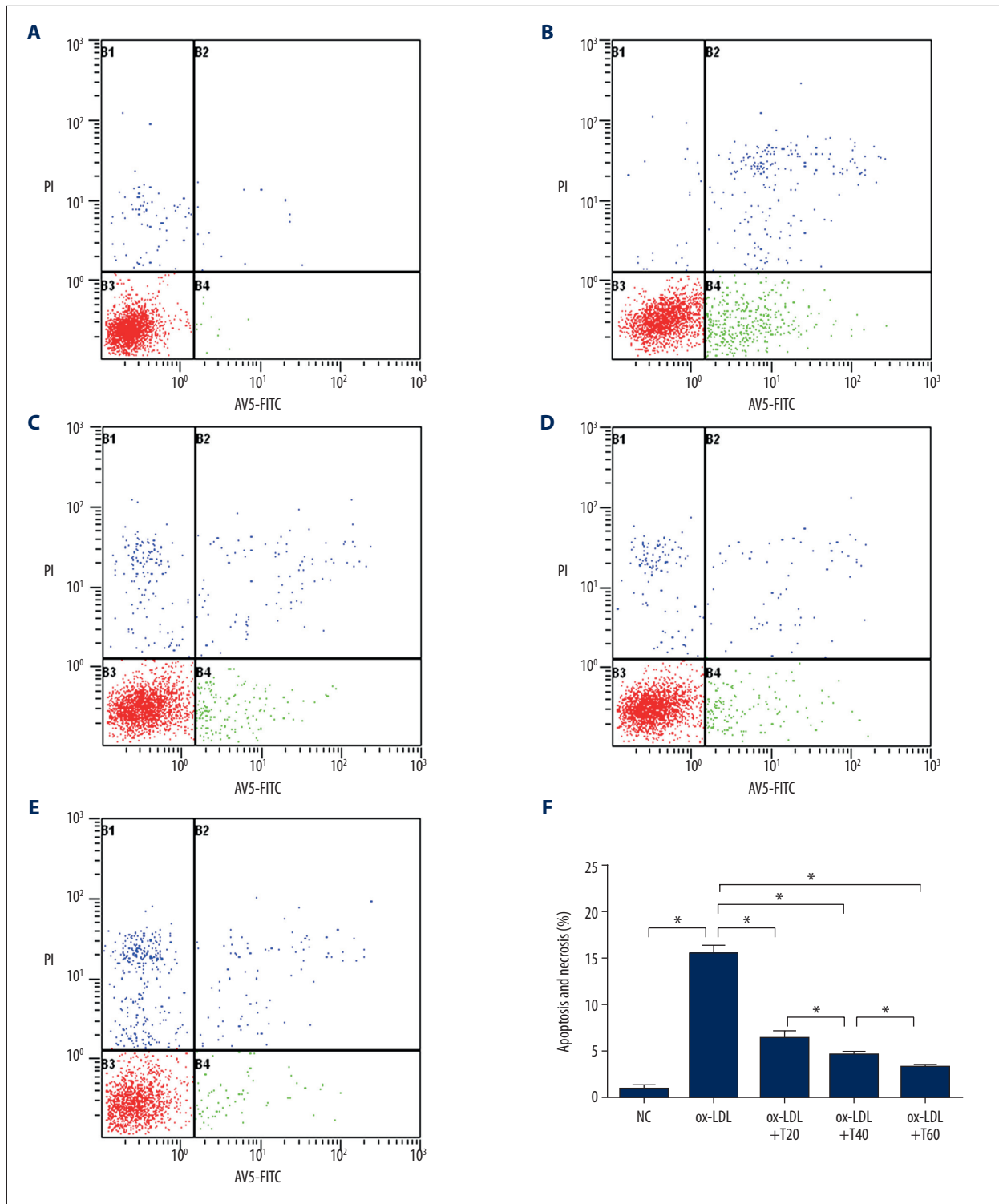
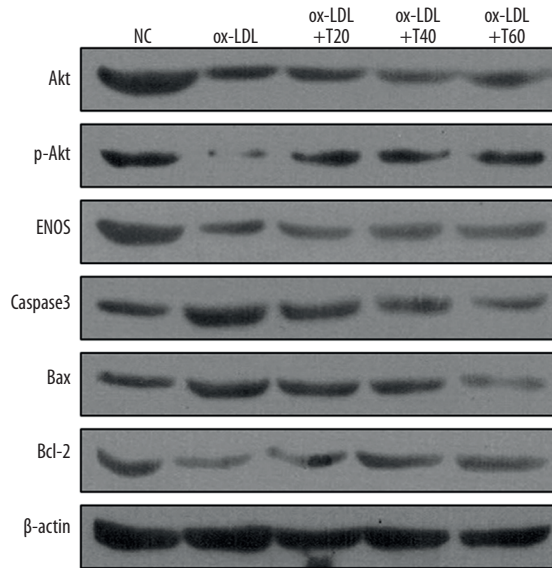
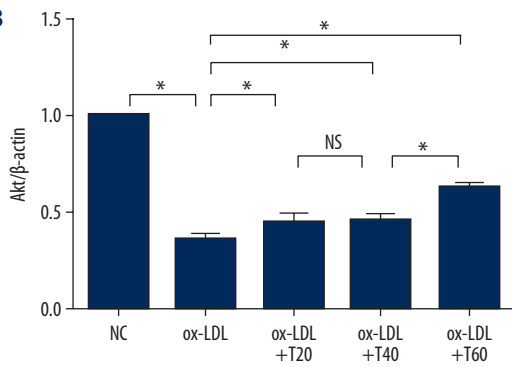


Figure 2. Representative flow cytometric data from HUVECs. The lower left quadrant represents living cells, the lower right quadrant represents early apoptosis cells, the upper left quadrant and the upper right quadrant represent late apoptosis and necrosis cells. (A) NC group; (B) ox-LDL group; (C) ox-LDL+ticagrelor20 group; (D) ox-LDL+ticagrelor40 group; (E) ox-LDL+ticagrelor60 group; (F) Comparison of apoptosis rate in each group. Ticagrelor can dose-dependently reduced ox-LDL induced apoptosis, in the histogram, * Indicates significant difference between the 2 groups at $P < 0.05$.

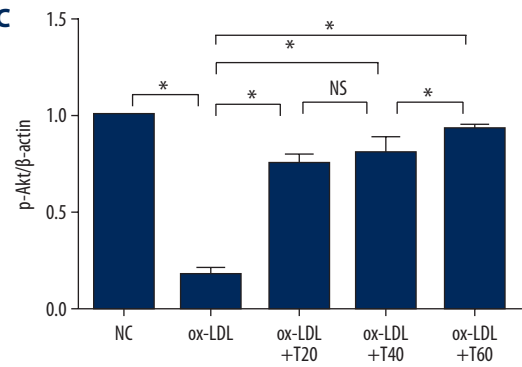
A



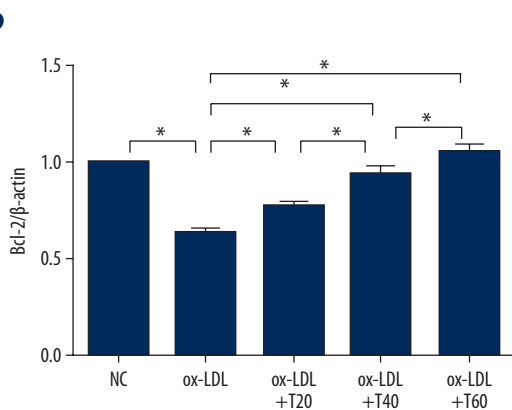
B



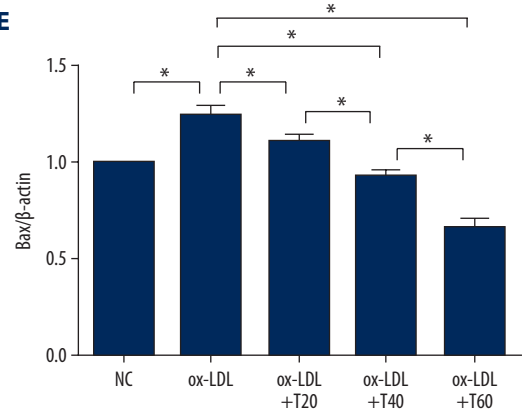
C



D



E



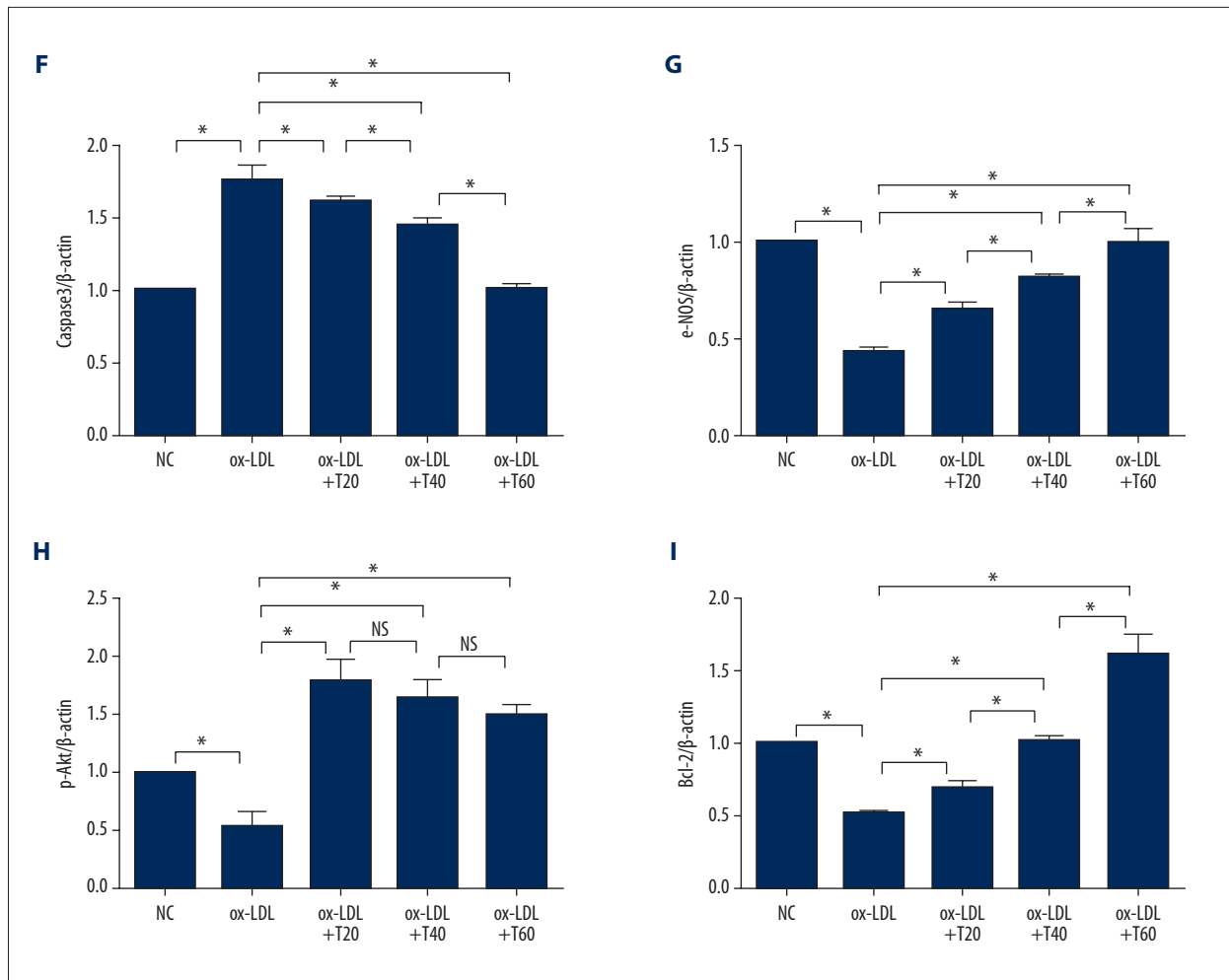


Figure 3. (A) Representative photographs of Western blot. Comparison of protein expression levels of Akt (B), p-Akt (C), Bcl-2 (D), Bax (E), caspase-3 (F), and eNOS (G) determined by Western blot. (H) p-Akt/Akt; (I) Bcl-2/Bax.

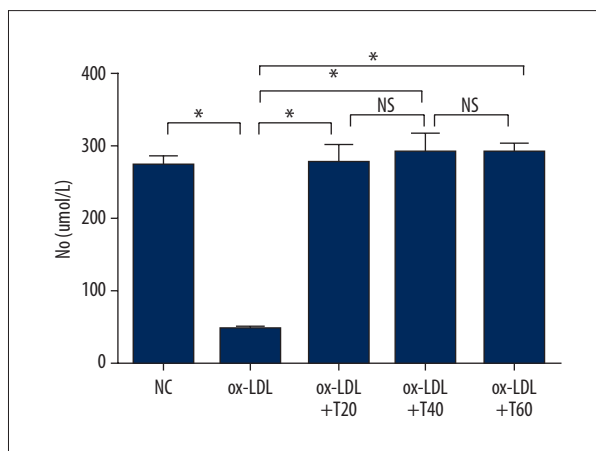


Figure 4. NO concentration determined by enzyme-linked immunosorbent assay (ELISA) in each group. * Indicates significant difference between 2 groups at $P < 0.05$.

might be 50 $\mu\text{g}/\text{mL}$ and 24 hours. We also found that ticagrelor can dose-dependently attenuate ox-LDL-induced apoptosis in HUVECs. In addition, these changes might be explained by alterations in apoptosis and antioxidant pathways.

Ticagrelor is an antagonist for G protein coupled receptor P2Y₁₂, which is activated by adenosine diphosphate. It not only inhibits platelet activation and aggregation by antagonizing P2Y₁₂ receptor, but also has several anti-inflammatory effects. Li et al. [9] showed that the atherosclerotic lesion in apoE^{-/-}/P2Y₁₂^{-/-} double knockout mice was significantly reduced compared to control mice. A previous study on ticagrelor also found that ticagrelor has a protective effect in endothelial function through decreasing circulating epidermal growth factor, which leads to the activation of eNOS in the vascular endothelium [10]. Ticagrelor can inhibit the activity of equilibrative nucleoside transporter 1 (ENT1), which inhibits cellular adenosine uptake and elevated adenosine content in the blood [11]. In addition, many *in vitro* studies have also found

that ticagrelor can induce adenosine triphosphate release from human red blood cells [12]. A recently animal experiment found that ticagrelor promoted atherosclerotic plaque stability in a mouse model of advanced atherosclerosis, which was induced by a reduction of ox-LDL uptake in RAW 264.7 macrophages [13]. However, the anti-atherosclerosis mechanisms of this compound remained largely unclear.

Apoptosis in endothelial cell has been implicated in the development of early period of atherosclerosis. Previous studies have showed that the increased apoptosis rate of endothelial cells was observed in atheromatous plaques, leading to monocyte adhesion and the formation of foam cell, which may play an important role in the maintenance and development of atherosclerosis [14]. Thus, it is urgent to identify the effective protection against endothelial cell apoptosis. In our study, we found that ticagrelor dose-dependently reversed ox-LDL-induced apoptosis rate in our model. To further clarify the potential mechanisms, Akt and its associated apoptosis pathways were detected.

Abnormal expression levels of apoptotic factors were generally associated with the process of apoptosis, these factors consisted of anti-apoptotic Bcl-2, pro-apoptotic Bax, and so on. One of the most classical apoptosis pathways is that Bax forms oligomers, which transfer from cytoplasm to mitochondrial membrane, leading to mitochondrial membrane depolarization [15]. Subsequently, cytochrome C is released from mitochondria into the cytoplasm and triggers the activation of caspase pathway, thus promoting cell apoptosis [16]. Upon apoptotic stimulation, the expression level of Bax is usually increased, which will lead to a decreased expression level of Bcl-2 [17].

In our study, our results demonstrated that ox-LDL led to increased expression levels of Bax and caspase-3, and decreased the expression level of Bcl-2 in our model, and the Bcl-2/Bax ratio was significantly decreased, and these changes were improved following exposure to ticagrelor. Furthermore, ticagrelor attenuated the ox-LDL-induced apoptosis in HUVECs. Consequently, we found that compared with the NC groups, the expression levels of Akt and p-Akt were significantly decreased in ox-LDL groups. However, the expression levels of Akt and p-Akt were significantly elevated in ticagrelor group, which suggested that ticagrelor improved ox-LDL-induced apoptosis

in our model mainly through the Akt signaling pathway. We speculated that ticagrelor can activate the Akt signaling pathway, which plays an important role of anti-apoptotic effects.

Nanhwan et al. [18] found that, unlike clopidogrel, ticagrelor can dose-dependently reduce the area of myocardial infarction, and dependent on adenosine-receptor activation with downstream upregulation of endothelial nitric oxide synthase and COX2 activity. However, clopidogrel didn't have similar effects of ticagrelor. Gündüz et al. [7] also found that ticagrelor sensitizes endothelial cell barrier function by inducing Ca²⁺ influx and activating downstream endothelial cell contractile machinery. We also found that ticagrelor has antioxidant properties. As compared to the ox-LDL group, eNOS and NO were significantly increased in the drug intervention group. The result might be correlated with the Akt signaling pathway, due to p-Akt can stimulate eNOS activation and NO increase [19]. From this result, ticagrelor can reduce the ox-LDL-induced damage to HUVECs oxidative stress and protect the function of endothelial cells through Akt/eNOS signaling pathway.

Conclusions

In conclusion, our data demonstrate that ticagrelor can dose-dependently reduce endothelial apoptosis via a pleiotropic effect on the Akt signaling pathway, which affects the apoptosis pathway.

Limitations

Despite our novel findings, the study had some limitations. Firstly, we did not detect the expression of cleaved-caspase-3. Secondly, we did not detect the apoptosis rate and necrosis rate in HUVECs with a less dose of ox-LDL. Thirdly, we did not elucidate the effects of ticagrelor on the activation of the Akt pathway by using Akt inhibitors, future studies focusing on the application of Akt inhibitors are warranted. Fourthly, detection of antioxidant pathway related proteins was insufficient in our experiments, future studies focusing on the detection of antioxidant pathways are warranted.

Conflicts of interest

None.

References:

- Schiro A, Wilkinson FL, Weston R et al: Endothelial microparticles as conveyors of information in atherosclerotic disease. *Atherosclerosis*, 2014; 234: 295–302
- Wang M, Wang Y, Weil B et al: Estrogen receptor beta mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia. *Am J Physiol Regul Integr Comp Physiol*, 2009; 296: 972–78
- Burgering BM, Medema RH: Decisions on life and death: FOXO forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol*, 2003; 73: 689–701
- Teng R: Ticagrelor: Pharmacokinetic, pharmacodynamic and pharmacogenetic profile: An update. *Clin Pharmacokinet*, 2015; 54: 1125–38

5. Motovska Z, Hlinomaz O, Kala P et al: 1-year outcomes of patients undergoing primary angioplasty for myocardial infarction treated with prasugrel versus ticagrelor. *J Am Coll Cardiol*, 2018; 71: 371–81
6. Kim HK, Jeong MH, Lim KS et al: Effects of Ticagrelor on neointimal hyperplasia and endothelial function, compared with clopidogrel and prasugrel, in a porcine coronary stent restenosis model. *Int J Cardiol*, 2017; 240: 326–31
7. Gündüz D, Tanislav C, Schlüter KD et al: Effect of ticagrelor on endothelial calcium signalling and barrier function. *Thromb Haemost*, 2017; 117: 371–81
8. Ren H, Li M, Feng L et al: Effects of clopidogrel on vascular proliferation and apoptosis in an atherosclerotic rabbit model. *J Cardiovasc Pharmacol*, 2010; 55: 617–24
9. Li D, Wang Y, Zhang L et al: Roles of purinergic receptor P2Y₂, G protein-coupled 12 in the development of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*, 2012; 32: 81–89
10. Vieceli Dalla Sega F, Fortini F, Aquila G et al: Ticagrelor improves endothelial function by decreasing circulating epidermal growth factor (EGF). *Front Physiol*, 2018; 9: 337
11. Armstrong D, Summers C, Ewart L et al: Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. *Cardiovasc Pharmacol Ther*, 2014; 19: 209–19
12. Ohman J, Kudira R, Albinsson S et al: Ticagrelor induces adenosine triphosphate release from human red blood cells. *Biochem Biophys Res Commun*, 2012; 418: 754–58
13. Preusch MR, Rusnak J, Staudacher K et al: Ticagrelor promotes atherosclerotic plaque stability in a mouse model of advanced atherosclerosis. *Drug Des Devel Ther*, 2016; 10: 2691–99
14. Li H, Horke S, Förstermann U: Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis*, 2014; 237: 208–19
15. Boatright KM, Salvesen GS: Mechanisms of caspase activation. *Curr Opin Cell Biol*, 2003; 15: 725–31
16. Tait SW, Green DR: Mitochondria and cell death: Outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol*, 2010; 11: 621–32
17. Besbes S, Mirshahi M, Pocard M et al: New dimension in therapeutic targeting of BCL-2 family proteins. *Oncotarget*, 2015; 6: 12862–71
18. Nanhwan MK, Ling S, Kodakandla M et al: Chronic treatment with ticagrelor limits myocardial infarct size: An adenosine and cyclooxygenase-2-dependent effect. *Arterioscler Thromb Vasc Biol*, 2014; 34: 2078–85
19. Chung BH, Kim JD, Kim CK et al: Icaritin stimulates angiogenesis by activating the MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways in human endothelial cells. *Biochem Biophys Res Commun*, 2008; 376: 404–8