Ticagrelor and clopidogrel suppress NF-κB to treat acute coronary syndrome

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Abstract

Background: Inflammatory cytokines are involved in acute coronary syndrome (ACS),and NF-kB is the central regulator of inflammation. Moreover, ticagrelor and clopidogrelcan prevent thrombotic events and improve the care of patients with ACS. Thus, we speculated that ticagrelor and clopidogrel relieve ACS by regulating NF-kB pathway.

Methods: After human umbilical vein endothelial cells (HUVECs) were cultured with ticagrelor or clopidogrel and given lipopolysaccharide (LPS) and CD14, the mRNA levels of related inflammatory factors, the protein level changes of molecules in the NF-kB pathway, and the changes in cell viability, apoptosis and the cell cycle, cell migration, vascular formation and other vital activities were detected using quantitative Polymerase chain reaction (qPCR), Western blotting and immunofluorescence assay, CCK8⊠ flow cytometry, transwell assay, matrigel, respectively. All data was expressed as the mean ± S.D. The statistical significance of data was assessedby an unpaired two-tailed t-test.

Results: Ticagrelor and clopidogrel can suppress the NF-kB pathway by inhibiting the phosphorylation and entry into the nucleus of p65, restraining the degradation of IKBa, improving cell viability, restoring the cell cycle, cell migration and angiogenic ability, and inhibiting apoptosis.

Conclusions: Ticagrelor and clopidogrel alleviate cellular dysfunction through suppressing NF-kB signaling to treat acute coronary syndrome.

Background

Acute coronary syndrome (ACS) is a syndrome in which reduced blood flow in the coronary arteries causes dysfunction or death of the cardiomyocytes[1]. The rupture or erosion of coronary atherosclerotic plaques and secondary to complete or incomplete occlusion thrombosis are the pathological basis of ACS[1]. In the clinic, ACS is usually treated by percutaneous coronary intervention (PCI) combined with antiplatelet agents{Steinhubl, 2007 #16;Jin, 2019 #26}. Ticagrelor and clopidogrel, antiplatelet agglutination agents, cure ACS by targeting the platelet P2Y12 adenosine diphosphate (ADP) receptor to inhibit platelet aggregation and reduce thrombosis[2].

Some studies have indicated that inflammatory cytokines are involved in the initiation and progression of atherosclerosis[3]. In addition to triggering thrombus formation at the site of atherosclerotic plaque rupture, platelets also release proinflammatory mediators and interact with other related cells, while antiplatelet therapy can reduce the levels of inflammatory cytokines[1]. Thus, inflammation plays an important role in ACS. Further research showed that NF-B, a central regulator of inflammation, which is involved in various inflammatory diseases, is associated with susceptibility to ACS[1]. Furthermore, long-term administration of clopidogrel after severe coronary artery injury reduces inflammation via inhibition of NF-B and activator protein 1 activation in pigs[2]. There are few reports on the mechanism of antiplatelet agglutination drug-mediated inhibition of inflammation. Therefore, we hypothesized that antiplatelet agglutination drugs, including ticagrelor and clopidogrel, can regulate the NF-B pathway and

reduce inflammation while targeting the platelet P2Y12 ADP-receptor to inhibit platelet aggregation, alleviating ACS.

To confirm our hypothesis, the mRNA levels of related inflammatory factors, the changes of molecules in the NF-B pathway, and the changes in cell viability, apoptosis, the cell cycle, cell migration, vascular formation and other vital activities were detected after human umbilical vein endothelial cells (HUVECs) were stimulated with lipopolysaccharide (LPS) and CD14, and given ticagrelor or clopidogrel. The results showed that ticagrelor and clopidogrel can inhibit the degradation of IKB and phosphorylation of p65, prevent p65 from entering the nucleus, reduce the production of TNF, IL−1, IL−8, IL−6 and IL−2, and reduce the changes in life activities caused by LPS, such as cell viability, apoptosis, cell cycle, cell migration ability, and vascular formation. These results provide a new theoretical basis for ticagrelor and clopidogrel to cure ACS.

Materials And Methods

Preliminary experiment

Cell proliferation assay

HUVECs (FuHeng Cell Center, Shanghai, China, FH0278) were incubated with ticagrelor (0 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM) clopidogrel (0 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM), separately, for 12 h, 24 h or 48 h. Then cell viability was determined by CCK−8 (Biosharp, BS350B).

Formal experiment

Cell culture and treatment

HUVECs were cultured in F12K supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin Solution at 37°C with 5% CO_2.

HUVECs were incubated with DMSO (as control), ticagrelor, clopidogrel, DMSO plus LPS and CD14, ticagrelor plus LPS and CD14, and clopidogrel plus LPS and CD14, separately, for 16 h. The concentrations of these compounds are shown in the table 1.

Cell proliferation assay

Cells were seeded in 96 well culture plates (2000 cells/well). After the cells were incubated with the indicated compounds for 16 h. Finally, cell viability was tested with CCK8 reagent. We evaluated cell viability by measured the absorbance at 450 nm.

Western blot assay

Whole cell extracts were lysed in RIPA Lysis buffer (Beyotime, P0013B) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Then protein concentration of lysates was determined by BCA protein concentration determination kit (Beyotime, P0010). Cell lysates containing equal amount protein were resolved on a 10%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Millipore, IPVH00010). After separate incubation with rabbit anti-p65 (CST, #8242), rabbit anti-p-p65 (CST, #3033), rabbit anti-MMP2 (proteintech, 10373–2-AP), rabbit anti-MMP9 (proteintech, 10375–2-AP), rabbit anti-E-cadherin (proteintech, 20874–1-AP), rabbit anti-IKB (abcam, Ab32518), rabbit anti-ICAM−1 (proteintech, 10831–1-AP), rabbit anti-VCAM−1 (Affinity, DF6082), rabbit anti-E-selectin (proteintech, 20894–1-AP), rabbit anti-GAPDH, mouse anti-P-selectin (proteintech, 60322–1-Ig), mouse anti-MCP−1 (Affinity, BF0678), followed by horseradish peroxidase-conjugated secondary antibody, the membranes were visualized by ECL chemiluminescence.

RNA Extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Ambion, 15596–026), and reverse transcription was accomplished with HiScript Reverse Transcriptase (VAZYME, R101–01/02). The reverse transcription products were amplified with SYBR Green Master Mix (VAZYME, Q111–02) according to the manufacturer's instructions. The data were normalized according to the level of GAPDH expression in each individual sample. The qPCR primers are listed in Table 2.

Immunofluorescence assay

The cells were incubated with the indicated compounds and then fixed for 15 min in 4% paraformaldehyde in 1×phosphate-buffered saline (PBS) pH 7.4. The fixed cells were permeabilized for 20 min with 0.5% Triton X−100 in 1×PBS and then blocked in 1× PBS with 1% bovine serum albumin for 30 min. The cells were incubated with the appropriate primary rabbit anti-p65 (CST, 8242S) and then stained with Alexa Fluor Cy3-labeled goat anti-rabbit immunoglobulin G (BOSTER, BA1032) and DAPI (Beyotime, C1002), separately. The subcellular localization of p65 was visualized using inverted fluorescence microscope (magnification, ×400).

Apoptosis assay

After incubated with the indicated compounds for 16 h, the cells were harvested and stained with APC/7-AAD apoptosis kit (SUNGENE BIOTECH, AO2001−11A-H), and then were analyzed by flow cytometry.

Cell cycle assay

After incubated with the indicated compounds for 16 h, the cells were harvested and stained with cell cycle kit (KeyGEN BioTECH, KGA512), and then were analyzed by flow cytometry.

Cell migration assay

The cells were incubated with the indicated compounds for 16 h, and then cell migration was assessed using transwell assay (magnification, ×200). Cells are stained using the 0.5% hexamethylpararosaniline solution.

Matrigel assay

The cells were incubated with the indicated compounds for 16 h, and then cultured for 6 h in 24-well plates coated with matrigel. The cells were imaged under an inverted microscope (Nikon, ECLIPSE Ts2) at 100 magnification and the network length and width was quantified.

Statistical analyses

All data were expressed as the mean ± S. D. The statistical significance of data was assessed by an unpaired two-tailed t-test. A value of $p < 0.05$ was used as the standard for statistical significance.

Results

Ticagrelor and clopidogrel inhibit the expression of inflammatory cytokines induced by LPS

Inflammation plays an important role in the initiation and progression of atherosclerosis, indicating the involvement of inflammatory cytokines in ACS[4]. To test whether ticagrelor and clopidogrel affect the production of inflammatory cytokines, HUVECs were incubated with ticagrelor and clopidogrel at different concentrations (0 μM, 5 μM, 10 μM, 20 μM, 50 M, 100 M) for 12, 24 and 48 h separately, without affecting cell viability (Figure 1A). Then, HUVECs were cultured with different combinations of compounds, DMSO, ticagrelor, clopidogrel, DMSO plus LPS and CD14, ticagrelor plus LPS and CD14, and clopidogrel plus LPS and CD14, separately, for 16 h. The results showed that ticagrelor and clopidogrel inhibited the expression of TNF, IL−1, IL−6, IL−8, and IL−2 induced by LPS (Figure 1B−1F). These data suggested that ticagrelor and clopidogrel inhibited the production of inflammatory cytokines.

Ticagrelor and clopidogrel suppress NF-B signaling

Studies have shown that NF-B is closely related to ACS, and NF-B plays a key role in inflammation[1], [5]. We wondered if ticagrelor and clopidogrel acted on NF-B. The variations of molecules in the NF-B pathway were tested after HUVECs were stimulated with LPS and given ticagrelor or clopidogrel. As a consequence, ticagrelor and clopidogrel inhibited p65 phosphorylation (Figure 2A−2B) and IKB degradation (Figure 2C−2D). After HUVECs were treated with ticagrelor and clopidogrel, the amount of nuclear-translocated p65 was significantly reduced (Figure 2E). These findings demonstrated that ticagrelor and clopidogrel inhibited the production of inflammatory factors by suppressing the NF-B pathway.

Ticagrelor and clopidogrel alleviate cellular dysfunction through suppressing NF-B signaling

NF-B was shown to be involved in various biological processes, such as cell proliferation, the cell cycle, apoptosis and cell migration, by regulating the expression of various genes[6], [7], [8], [3]. Thus, we tested the cell proliferation, cell cycle, apoptosis and cell migration of HUVECs after culture with the indicated compounds (Figure 3A−3G). As shown in Figure 3A−3G, ticagrelor and clopidogrel inhibited apoptosis and the decrease in cell viability caused by LPS (Figure 3A−3C) and restored the cell migration (Figure 3D−3E) and the cell cycle disrupted by LPS (Figure 3F−3G). These data implied that ticagrelor and clopidogrel prevented LPS from damaging cells.

Activated NF-B regulates the expression of a range of genes, including intercellular adhesion molecule−1 (ICAM−1), vascular cell adhesion molecule (VCAM−1), E-selectin, P-selectin, and monocyte chemoattractant protein−1 (MCP−1), which can act directly or indirectly on microvascular endothelial cells or blood cells or mediate their interactions, leading to microcirculation disorders[9], [4]. Therefore, we detected their expression level and the angiogenic capacity of HUVECs treated with DMSO, ticagrelor, clopidogrel, DMSO plus LPS and CD14, ticagrelor plus LPS and CD14, and clopidogrel plus LPS and CD14. We found that ticagrelor and clopidogrel reduced the expression of ICAM−1, E-selectin, P-selectin, VCAM−1 and MCP−1 induced by LPS. (Figure 4A−4D). Furthermore, HUVECs incubated with DMSO, ticagrelor, clopidogrel, ticagrelor plus LPS and CD14, and clopidogrel plus LPS and CD14 formed complex tubular structures, whereas the total width of the tubes was significantly increased in the cells compared with those incubated with DMSO plus LPS and CD14 (Figure 4G). Our data indicated that ticagrelor and clopidogrel restored the ability of HUVECs to generate blood vessels.

In addition, E-cadherin plays a crucial role in mediating cell adhesion and maintaining the integrity of the vascular endothelial layer. In contrast, MMP2 and MMP9 can degrade the extracellular matrix and basal membrane, damaging the vascular endothelial layer[10], [11], [8]. Hence, we also tested the expression of E-cadherin, MMP2 and MMP9. As shown in Figure 2A−2D, ticagrelor and clopidogrel increased the expression of E-cadherin, which was reduced by LPS and restrained the expression of MMP2 and MMP9 induced by LPS. Ticagrelor and clopidogrel protected the endothelial layer from damage by NF-B.

Overall, our findings reveal that ticagrelor and clopidogrel alleviate cellular dysfunction by suppressing NF-B signaling.

Discussion

ACS is a type of acute coronary heart disease. Mismanagement of this disease will cause high morbidity and mortality. The symptoms of the disease are paroxysmal chest pain, chest tightness, arrhythmia, heart failure and even sudden death, seriously reducing the longevity and quality of life of patients[12]. Therefore, research on the treatment of this disease will have clinical and social significance.

At present, the number of patients with ACS continues to grow, and antiplatelet therapy is still one of the main treatments of ACS[13], [14]. Ticagrelor and clopidogrel are platelet aggregation inhibitors that are antiplatelet agents. Ticagrelor reversibly interacts with the P2Y12 ADP receptor, which inhibits ADP-induced platelet aggregation by blocking P2Y12 receptors and does not need to be metabolically activated in the liver[15], [16]. Clopidogrel is a prodrug. The metabolite 2-epoxy-clopidogrel is formed by the oxidation of clopidogrel and then forms active metabolites (mercaptan derivatives) through hydrolysis. Clopidogrel selectively inhibits the binding of ADP to its platelet receptor and the subsequent activation of ADP-mediated glycoprotein GPlllb/llla complex, thus inhibiting platelet aggregation[1]. Clopidogrel must undergo biological transformation to inhibit platelet aggregation, but relevant active metabolites have not been isolated. We discovered that ticagrelor and clopidogrel protect the endothelial layer by increasing the expression of E-cadherin decreased by LPS and restraining the expression of MMP2 and MMP9 induced by LPS. Thus, ticagrelor and clopidogrel not only inhibit platelet aggregation but also protect endothelial cells. This finding is consistent with the fact that in clinical practice, ticagrelor and clopidogrel are used to prevent thrombosis and improve the nursing care of patients with acute coronary syndrome after percutaneous coronary intervention.

In recent years, studies have shown that NF-B and VCAM−1 are closely related to the formation of atherosclerosis, causing deviant cell proliferation and inflammatory response, thereby affecting the stability of plaques. Moreover, NF-B was found to be increased in ACS patients. NF-B can transcriptionally regulate key proinflammatory cytokines, chemokines, adhesion molecules, immune recognition receptors and some enzymes in the inflammatory response and directly participate in inflammatory processes such as inflammatory cell infusion and aggregation[4], injuring vascular endothelial cells[10], [11], [8]. Our study demonstrated that ticagrelor and clopidogrel negatively regulate the NF-B pathway via inhibiting IKB degradation and p65 phosphorylation, markedly reducing the amount of nuclear-translocated p65, thereby inhibiting cell apoptosis and restoring the cell cycle, cell proliferation, cell migration and formation of blood vessels, protecting cells from the damage by NF-B. Therefore, we speculated that, in addition to inhibiting platelet aggregation, ticagrelor and clopidogrel can also treat ACS by reducing the functional impairment of vascular endothelial cells through suppressing NF-B.

Conclusions

Ticagrelor and clopidogrel negatively regulate the NF-B pathway via inhibiting the degradation of IKB and the phosphorylation and entry into the nucleus of p65 to alleviate cellular dysfunction and ACS, which provides a new theoretical basis for ticagrelor and clopidogrel to cure ACS.

Abbreviations

ACS: acute coronary syndrome; HUVECs: human umbilical vein endothelial cells; qPCR: quantitative polymerase chain reaction; LPS: lipopolysaccharide; PCI: percutaneous coronary intervention; ADP: adenosine diphosphate.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

Not applicable.

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Authors' contributions

Zhuyin Jia and Guosheng Fu conceived the study and designed the experiments, Zhuyin Jia performed the cell proliferation assay, western blot, qRT-PCR, apoptosis, and cell cycle assay with assistance from

Yiwei Huang, and Xiaojun Ji. Yiwei Huang performed the immunofluorescence assay, Jiaju Sun performed the migration and invasion assay. Zhuyin Jia and Guosheng Fu wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript.

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Not applicable.

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Tables

Table 1. The concentrations of these compounds

Compounds	Concentrations
Ticagrelor	20 µM
Clopidogrel	20 µM
LPS	10 ng/mL
CD14	1 µg/mL

Table 2. The qPCR primers

Name	Primer	Sequence
homo GAPDH	Forward	5'- TCAAGAAGGTGGTGAAGCAGG -3'
	Reverse	5'- TCAAAGGTGGAGGAGTGGGT -3'
Homo TNFa	Forward	5'-CCCATGTTGTAGCAAACCCTC -3'
	Reverse	5'-AGAGGACCTGGGAGTAGATGA -3'
	Reverse	5'-GTGCCTCTTTGCTGCTTTC -3'
Homo IL-1	Forward	5'-CGAATCTCCGACCACCACTA -3'
	Reverse	5'-AGCCTCGTTATCCCATGTGT -3'
Homo IL-6	Forward	5'-GGTCCAGTTGCCTTCTCCC -3'
	Reverse	5'-GTGCCTCTTTGCTGCTTTC -3'
Homo IL-2	Forward	5'-CAACTCCTGTCTTGCATTGC-3'
	Reverse	5'-TGTGAGCATCCTGGTGAGTT -3'
Homo IL-8	Forward	5'-GACATACTCCAAACCTTTCCACCCC-3'
	Reverse	5'-CAAAAACTTCTCCACAACCCTCTGC -3'

Figures
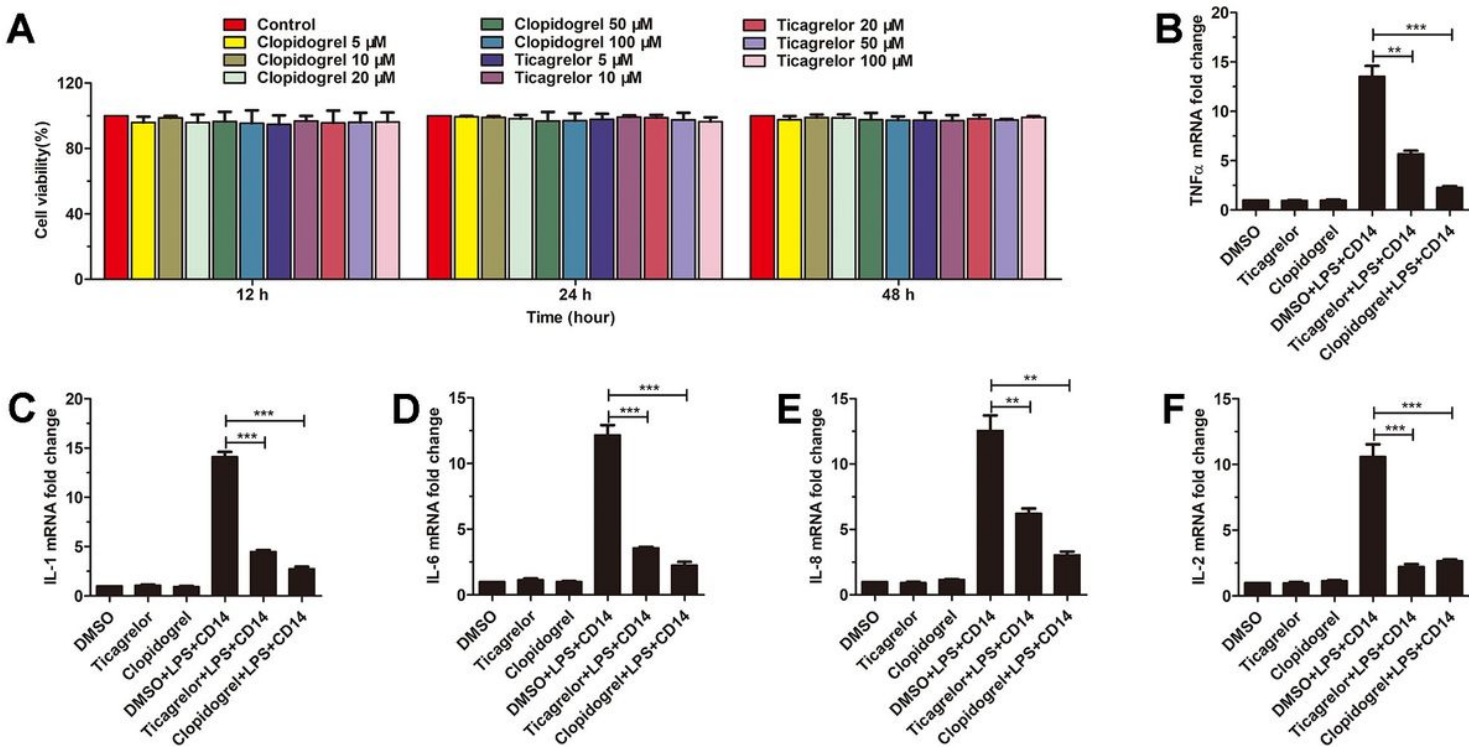
Figure 1

Ticagrelor and clopidogrel inhibit the LPS-induced expression of TNFα, IL-1, IL-6, IL-8, and IL-2. A: HUVECs were treated with different concentrations of ticagrelor and clopidogrel, separately, for 12 h, 24 h or 48 h. Cell viability was detected by CCK-8 assays at the indicated time points. B-C: HUVECs were treated with DMSO, ticagrelor, clopidogrel, DMSO plus LPS and CD14, ticagrelor plus LPS and CD14, and clopidogrel plus LPS and CD14, separately, for 16 h. The mRNA levels of TNFα (B), IL-1 (C), IL-6 (D), IL-8 (E), and IL-2 (F) were detected by qPCR (n=3; *, p<0.05; **, p<0.01; and ***, p<0.001).
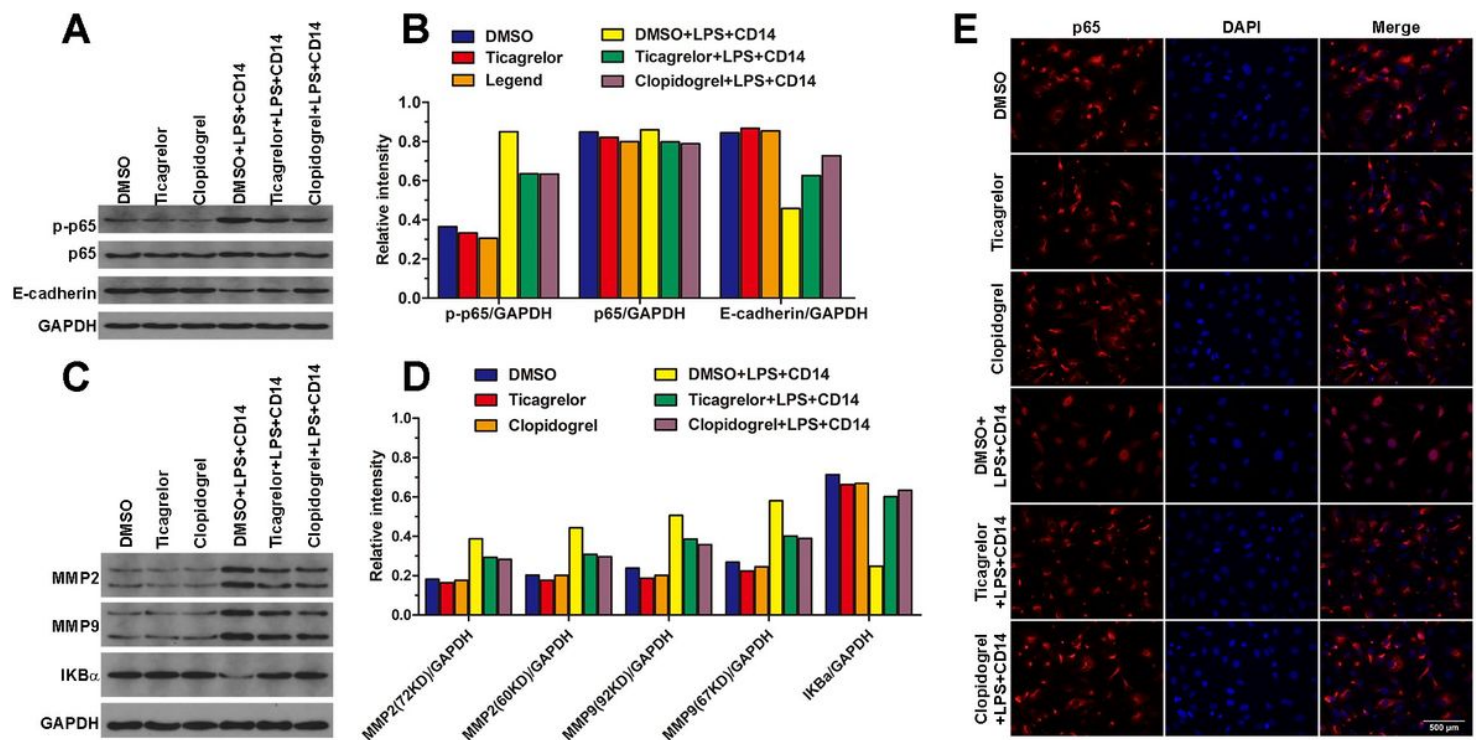
Figure 2

Ticagrelor and clopidogrel inhibit p65 phosphorylation. A-D: The cells were incubated with the indicated compounds for 16 h, and the cell lysates were analyzed by western blotting. E: The cells were incubated with the indicated compounds for 16 h. The localization of p65 in the nuclear and cytoplasmic compartments was detected by fluorescence microscopy.
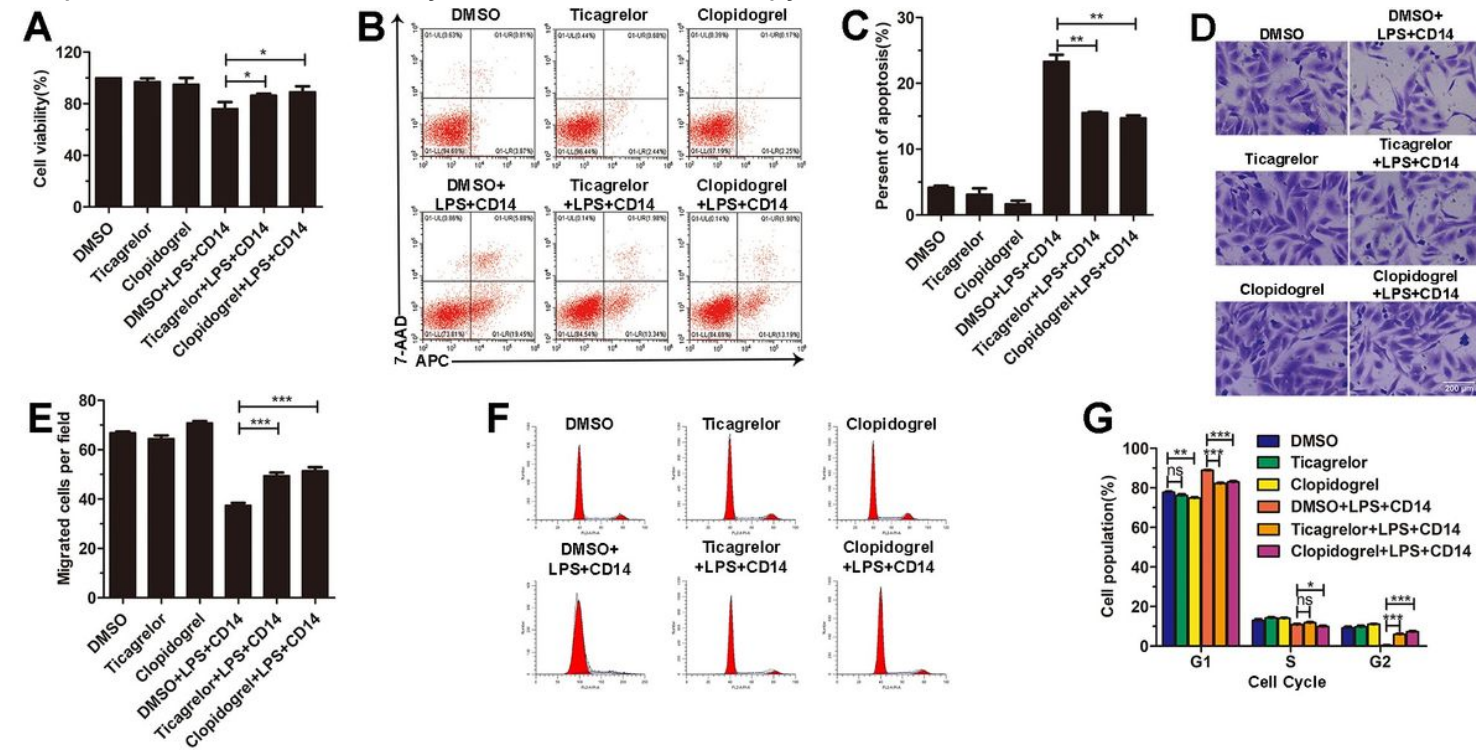
Figure 3

Ticagrelor and clopidogrel inhibit the LPS-induced changes in cell apoptosis, cycle and migration ability. A: HUVECs were incubated with the indicated compounds for 16 h, and then, cell viability was determined by CCK-8 assays. B-C: Flow cytometric analysis of APC/7-AAD staining was used to analyze apoptosis in HUVECs following incubation with the indicated compounds for 16 h. D-E: After HUVECs were incubated with the indicated compounds for 16 h, cell migration was assessed using a transwell assay, and cells were stained using hexamethylpararosaniline solution. F-G: HUVECs were incubated with the indicated compounds for 16 h and stained with propidium iodide. DNA content was analyzed by flow cytometry (n=3; ns, not significant, $p > 0.05$; *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$)
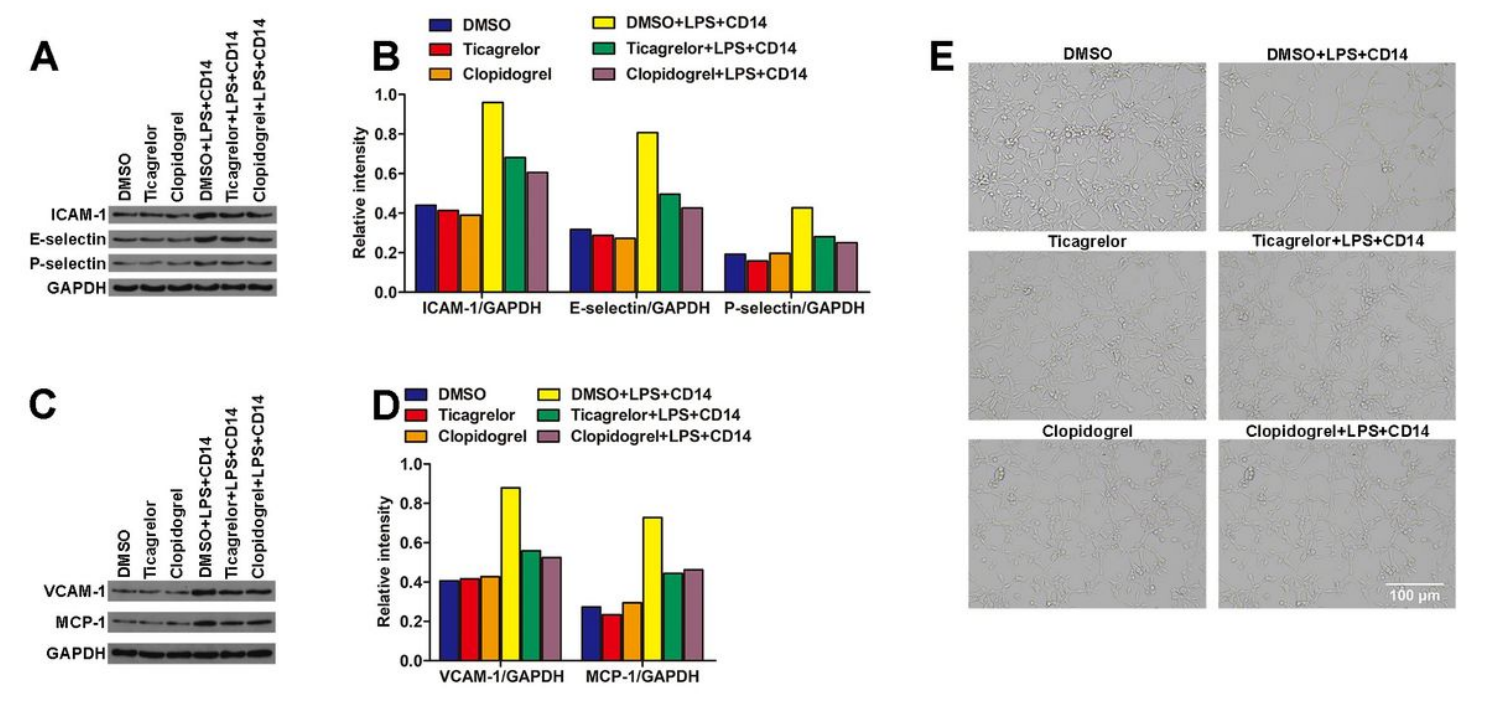
Figure 4

Ticagrelor and clopidogrel restored the LPS-caused reduced ability of HUVECs to form tubular networks. HUVECs were incubated with DMSO (as a control), ticagrelor (20 µM), clopidogrel (20 µM), DMSO plus LPS (10 ng/mL) and CD14 (1 µg/mL), ticagrelor (20 µM) plus LPS (10 ng/mL) and CD14 (1 µg/mL), and clopidogrel (20 µM) plus LPS (10 ng/mL) and CD14 (1 µg/mL), separately, for 16 h. Then, the cell lysates were immunoblotted with the indicated antibodies (A-D), and tube formation assays were performed using Matrigel as described in the Materials and Methods (E).