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Ticagrelor, a P2Y12 antagonist, attenuates vascular dysfunction and inhibits atherogenesis in apolipoprotein-E-deficient mice

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ABSTRACT

Background and aims: Ticagrelor reduces cardiovascular events in patients with acute coronary syndrome (ACS). Recent studies demonstrated the expression of P2Y12 on vascular cells including endothelial cells, as well as platelets, and suggested its contribution to atherogenesis. We investigated whether ticagrelor attenuates vascular dysfunction and inhibits atherogenesis in apolipoprotein E-deficient (*apoe*$^{-/-}$) mice.

Methods: Eight-week-old male *apoe*$^{-/-}$ mice were fed a western-type diet (WTD) supplemented with 0.1% ticagrelor (approximately 120 mg/kg/day). Non-treated animals on WTD served as control. Atherosclerotic lesions were examined by *en-face* Sudan IV staining, histological analyses, quantitative RT-PCR analysis, and western blotting. Endothelial function was analyzed by acetylcholine-dependent vasodilation using aortic rings. Human umbilical vein endothelial cells (HUVEC) were used for *in vitro* experiments.

Results: Ticagrelor treatment for 20 weeks attenuated atherosclerotic lesion progression in the aortic arch compared with control ($p < 0.05$). Ticagrelor administration for 8 weeks attenuated endothelial dysfunction ($p < 0.01$). Ticagrelor reduced the expression of inflammatory molecules such as vascular cell adhesion molecule-1, macrophage accumulation, and lipid deposition. Ticagrelor decreased the phosphorylation of JNK in the aorta compared with control ($p < 0.05$). Ticagrelor and a JNK inhibitor ameliorated impairment of endothelium-dependent vasodilation by adenosine diphosphate (ADP) in wild-type mouse aortic segments. Furthermore, ticagrelor inhibited the expression of inflammatory molecules which were promoted by ADP in HUVEC ($p < 0.001$). Ticagrelor also inhibited ADP-induced JNK activation in HUVEC ($p < 0.05$).

Conclusions: Ticagrelor attenuated vascular dysfunction and atherogenesis through the inhibition of inflammatory activation of endothelial cells. These effects might be a potential mechanism by which ticagrelor decreases cardiovascular events in patients with ACS.

1. Introduction

P2Y12 antagonists in combination with aspirin are widely used for the treatment of patients with acute coronary syndrome (ACS) and patients undergoing percutaneous coronary intervention [1]. Ticagrelor is the first reversible oral P2Y12 antagonist, which acts directly on P2Y12 without hepatic biotransformation [2]. Clinical studies demonstrated that ticagrelor reduced vascular events in

Abbreviations: Ach, acetylcholine; ACS, acute coronary syndrome; ADP, adenosine diphosphate; *apoE*$^{-/-}$, apolipoprotein E-deficient; Ctrl, control; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; MOMA-2, monocyte/macrophage marker-2; qPCR, quantitative real-time PCR; SNP, sodium nitroprusside; VCAM-1, vascular cell adhesion molecule-1; WT, wild-type; WTD, western-type diet.

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patients with ACS or a history of myocardial infarction [3,4]. P2Y12-mediated platelet activation plays a central role in thrombosis [5,6], whereas several studies have suggested that P2Y12 expression is not restricted to platelets, and that many cell types including endothelial cells [7,8], vascular smooth muscle cells (VSMC) [9–11] and immune cells [12] express it. Furthermore, recent studies indicate that ADP-P2Y12 signaling directly mediates the expression of inflammatory molecules and inflammation in the vessel wall, leading to the development of atherosclerosis independent of platelet activation [13]. In fact, apolipoprotein E-deficient (*apoe*$^{-/-}$) mice which lack P2Y12 develop smaller atherosclerotic lesions compared with P2Y12-expressing mice [14,15]. Previous studies demonstrated the involvement of P2Y12-mediated signaling such as ADP-induced monocyte chemoattractant protein-1 (MCP-1) expression and mitogenesis in VSMC in atherosclerotic processes [11,16]. Several clinical studies suggested that P2Y12 antagonists such as clopidogrel and ticagrelor exert anti-atherosclerotic effects including improvement of endothelial function, besides their anti-thrombotic effect, in patients with coronary artery disease [17–20]. Also, previous studies have shown that these P2Y12 antagonists attenuate atherogenesis in an atherosclerotic mouse model [21,22], although the number of studies that examined the effects of ticagrelor on the endothelium and the underlying mechanisms is limited.

Endothelial dysfunction is an initial step of atherosclerosis. Vascular inflammation caused by lifestyle-related diseases such as dyslipidemia promotes endothelial dysfunction. Accumulating evidence indicates the reversibility of endothelial dysfunction, suggesting it as a potential therapeutic target [23,24]. In this study, we administered ticagrelor to *apoe*$^{-/-}$ mice and investigated the mechanisms by which ticagrelor attenuates endothelial dysfunction and the development of atherosclerosis.

2. Materials and methods

2.1. Animals and drug administration

Apoe$^{-/-}$ mice (C57BL/6 J background), a widely used mouse model of atherosclerosis with severe hypercholesterolemia [25], were originally purchased from The Jackson Laboratory. Ticagrelor was supplied by Astra-Zeneca. From eight weeks of age, male *apoe*$^{-/-}$ mice were fed a western-type diet (WTD) supplemented with 0.1% ticagrelor (approximately 120 mg/kg/day) for 20 weeks to examine its effects on atherogenesis. To investigate the effect of ticagrelor on endothelial function at the early stage of atherosclerosis, the same dose of ticagrelor was administered to 8-week-old male *apoe*$^{-/-}$ mice for 8 weeks. Non-treated animals on WTD served as the control. Mice were maintained under controlled lighting (12 h light/dark) and temperature (24 °C) conditions. All animal experimental procedures conformed to the guidelines for animal experimentation of Tokushima University.

2.2. Blood pressure and laboratory data

Blood pressure was measured by a tail-cuff system as we described previously [26]. At the time of sacrifice, blood was collected from the heart, and plasma was separated and stored at −80 °C until required. Plasma total cholesterol, HDL-cholesterol, and triglyceride levels were measured at LSI Medience Corporation (Japan).

2.3. Quantification of atherosclerotic lesions

The severity of atherosclerotic lesions in the aorta was assessed as we previously described [26]. In brief, mice were sacrificed with an overdose of pentobarbital, and perfused with 0.9% sodium chloride solution at a constant pressure via the left ventricle. Both the heart and whole aorta were immediately removed. The thoracic aorta was excised, opened longitudinally, and fixed with 10% neutral buffered formalin. To quantify atherosclerotic lesions in the aortic arch, en-face Sudan IV staining was performed, and the percentage of Sudan IV-positive area was measured. The abdominal aorta was removed and snap-frozen in liquid nitrogen for gene expression and western blot analysis.

2.4. Histological and immunohistochemical analyses

Histological and immunohistochemical analyses were performed on frozen sections of the aortic root. The sections (at 5-μm intervals) were stained with oil red O to detect lipid deposition. Also, sections were incubated with anti-vascular cell adhesion molecule-1 (VCAM-1) antibody, anti-intercellular adhesion molecule-1 (ICAM-1) antibody (Abcam), or anti-monocyte/macrophage marker (MOMA-2) antibody (BioRad), followed by the alkaline phosphatase-conjugated secondary antibody (VECTOR Laboratories, Inc.), and stained using a VectorRed AP Substrate Kit (VECTOR Laboratories, Inc.). All sections were counterstained with hematoxylin. The ratio of positive area to plaque area was calculated in three valve lesions in the aortic root and used for comparison [26].

2.5. Vascular reactivity assay

Analysis of vascular reactivity was performed as we described previously [27]. In brief, the descending thoracic aorta was cut into 2-mm rings with special care to preserve the endothelium, and mounted in organ baths filled with modified Krebs–Henseleit buffer (KHB; 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose) aerated with 95% O_2 and 5% CO_2 at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were primed with 31.4 mM KCl, and then precontracted with phenylephrine, producing submaximal (60% of maximum) contraction. After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach; 10^{-9} to 10^{-4} M) and sodium nitroprusside (SNP; 10^{-9} to 10^{-4} M) to obtain cumulative concentration–response curves. In ex-vivo experiments, aortic rings isolated from wild-type (WT) mice were incubated with 100 nM ticagrelor or 100 nM JNK inhibitor (SP600125) for 2 h and then stimulated with 100 μM ADP (Sigma, Aldrich) for 16 h, and vascular reactivity was examined.

2.6. Cell culture experiments

Human umbilical vein endothelial cells (HUVEC) were purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVEC (passages 4–6) were incubated with 0–100 nM ticagrelor or 100 nM JNK inhibitor for 2 h, and then stimulated with 100 μM ADP in EBM-2 (Lonza) containing 2% FBS.

2.7. Quantitative RT-PCR

Total RNA was extracted from the aorta and HUVEC using an illustra RNAspin RNA Isolation Kit (GE Healthcare). cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR (qPCR) was performed on an Mx3000 P (Agilent Technologies) using Power SYBR Green PCR Master Mix (Applied Biosystems). Data are expressed in arbitrary units normalized by *β-actin* or *GAPDH*. The sequences of primers are listed in Supplementary Table 1.

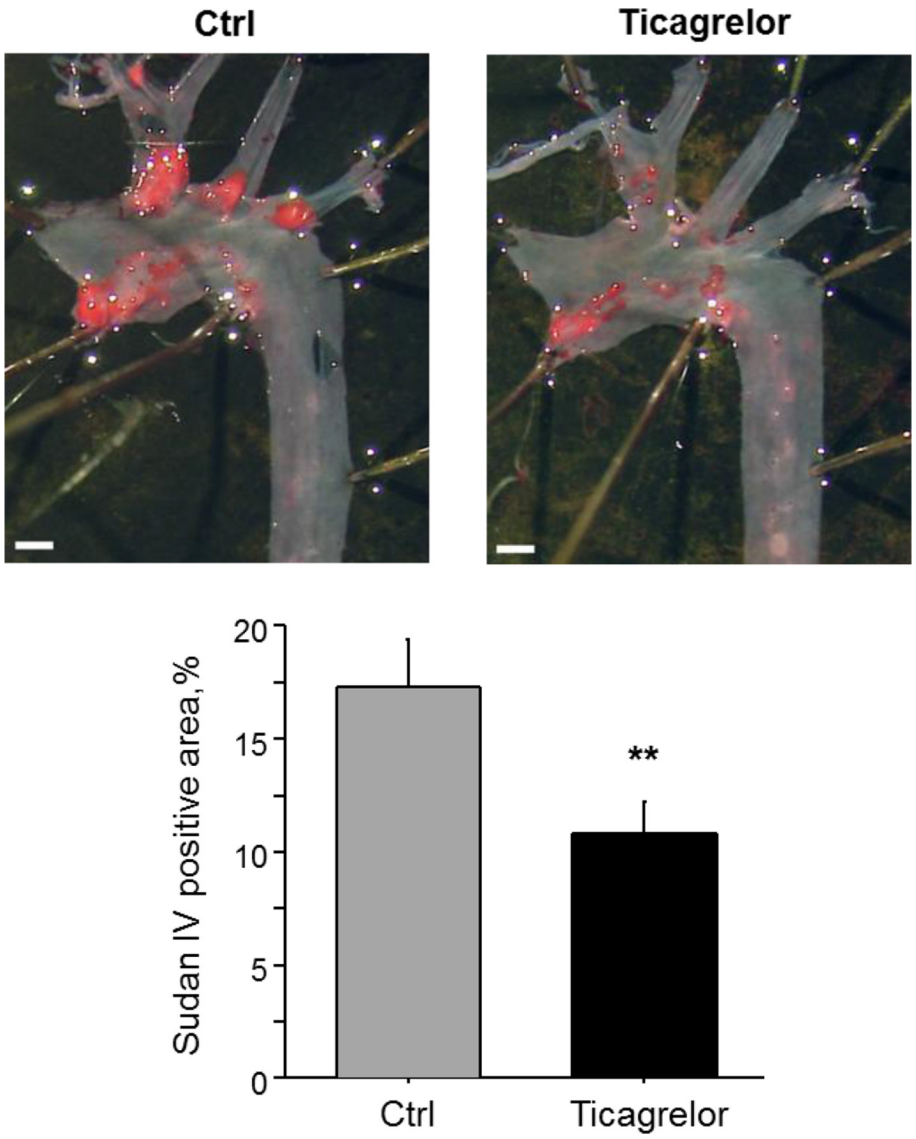


Fig. 1. Ticagrelor attenuated atherogenesis in *apoe*$^{-/-}$ mice.
The results of *en face* Sudan IV staining demonstrated that ticagrelor administration to *apoe*$^{-/-}$ mice for 20 weeks significantly reduced the development of atherosclerosis in the aortic arch (n = 11–14, per group). Bar: 1 mm **; $p < 0.01$ *vs.* Ctrl group. Ctrl; control. All values are mean ± SEM.

2.8. Western blot analysis

Protein lysates were isolated from HUVEC or aortic tissue using RIPA buffer (Wako Pure Chemical Industries, Ltd.) containing a protease inhibitor cocktail (Takara Bio Inc.) and phosphatase inhibitors (Roche LifeScience). Proteins were separated by SDS-PAGE and transferred to polyvinilidine difluoride membranes (Hybond-P; GE Healthcare). The membrane was blocked in 5% bovine serum albumin for 1 h at room temperature, followed by incubation with primary antibody against either phospho-SAPK/JNK, SAPK/JNK (Cell Signaling Technology), or β-actin (Sigma) at 4 °C overnight. After blots were washed in TBS containing 1% Tween-20, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Chemicon) for 1 h. Expression of β-actin was used as an internal control to confirm equivalent total protein loading. Antibody distribution was visualized with ECL-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-1000, Fuji Film).

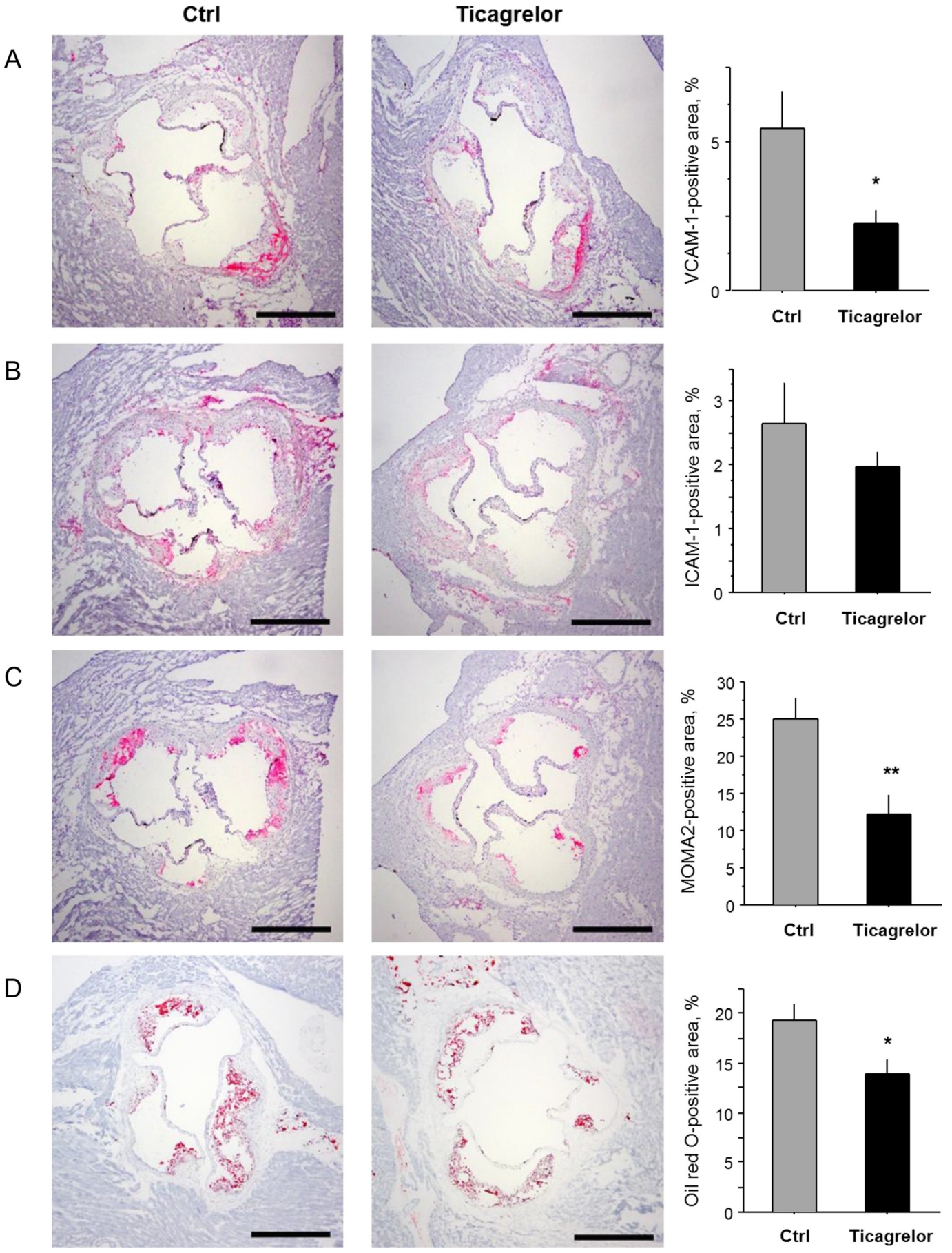
2.9. Statistical analysis

All results are expressed as mean ± SEM. Comparison of parameters between two groups was performed with unpaired Student's t-test. Comparisons of dose–response curves were made by two-factor repeated-measures ANOVA, followed by Tukey's *post hoc* test for comparison between groups. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Ticagrelor attenuated atherosclerosis in apoe$^{-/-}$ mice

To investigate the effect of ticagrelor on atherogenesis, *apoe*$^{-/-}$ mice were fed WTD supplemented with 0.1% ticagrelor for 20 weeks. Ticagrelor did not alter metabolic parameters in *apoe*$^{-/-}$ mice, as shown in Supplementary Table 2. Sudan IV staining showed that ticagrelor treatment decreased the development of


Ctrl
Ticagrelor
A
B
C
D
VCAM-1-positive area, %
ICAM-1-positive area, %
MOMA2-positive area, %
Oil red O-positive area, %
Ctrl
Ticagrelor
*
**

atherosclerotic lesions in the aortic arch compared with the control (17.2 ± 2.1 vs 10.8 ± 1.3%, $p < 0.01$) (Fig. 1).

3.2. Ticagrelor attenuated endothelial dysfunction in *apoe*$^{-/-}$ mice

To investigate the mechanism by which ticagrelor attenuates atherogenesis, we examined the effects of ticagrelor on endothelial dysfunction, an initial step in vascular inflammation and atherogenesis. The results of immunostaining demonstrated that administration of ticagrelor for 8 weeks reduced the expression of adhesion molecules such as VCAM-1 and ICAM-1 in atherosclerotic plaques. Associated with the reduction of these inflammatory molecules, ticagrelor attenuated the accumulation of macrophages as determined by the expression of MOMA-2 and lipid deposition in the lesions (Fig. 2). Furthermore, ticagrelor decreased mRNA expression of *Vcam1* and *Icam1* ($p < 0.05$, respectively), and tended to decrease the expression of *Mcp1* ($p = 0.07$) in the abdominal aorta compared with the control (Fig. 3A).

Endothelium-dependent vasodilation in response to Ach was significantly impaired in *apoe*$^{-/-}$ mice after 8-week WTD feeding compared with that in age- and sex-matched WT mice fed normal chow. However, ticagrelor administration for 8 weeks ameliorated the impairment of endothelium-dependent vasodilation (Fig. 3B). On the other hand, endothelium-independent relaxation in response to SNP did not differ between the ticagrelor-treated group and control group (Fig. 3C). Ticagrelor administration inhibited phosphorylation of JNK in the atherosclerotic aorta, suggesting that ticagrelor inhibits JNK activation (Fig. 3D). Metabolic parameters did not differ between the ticagrelor-treated group and control group (Supplementary Table 3).

3.3. Ticagrelor inhibited ADP-induced expression of inflammatory molecules in HUVEC

The effects of ADP on the expression of inflammatory molecules in HUVEC were examined by qPCR. ADP promoted the expression of inflammatory molecules such as *Vcam1*, *Icam1*, and *Mcp1* in HUVEC, while pre-treatment with ticagrelor inhibited these responses (Fig. 4A–C). The results of western blotting indicated that ADP stimulated the phosphorylation of JNK, suggesting that ADP promotes JNK activation in endothelial cells. Ticagrelor inhibited JNK activation induced by ADP (Fig. 4D).

3.4. Inhibition of JNK ameliorated endothelial dysfunction

To confirm the contribution of JNK signaling to ADP-induced endothelial dysfunction, HUVEC were treated with ADP in the presence or absence of a JNK inhibitor (SP600125). ADP promoted the expression of *Vcam1* and *Mcp1*, while SP600125 significantly attenuated ADP-induced inflammatory molecule expression in HUVEC (Fig. 5A). Furthermore, endothelium-dependent vascular reactivity was impaired in aortic rings treated with ADP. However, pre-treatment with ticagrelor or SP600125 ameliorated this impairment (Fig. 5B). The vascular response to SNP was unaffected by ADP even in the presence/absence of ticagrelor or SP600125 (Fig. 5C).

4. Discussion

The present study demonstrated that treatment with ticagrelor, a P2Y12 antagonist, reduced the development of atherosclerotic lesions in *apoe*$^{-/-}$ mice. Ticagrelor decreased the expression of inflammatory molecules in the aorta and ameliorated the vascular response to Ach, suggesting that ticagrelor has protective effects on endothelial function in *apoe*$^{-/-}$ mice. Atherosclerosis is a chronic inflammatory disease, which involves various cellular and molecular processes [28–30]. It is widely accepted that endothelial damage interrupts homeostasis of the vasculature, and initiates atherosclerotic processes including endothelial permeability, platelet aggregation, leukocyte adhesion, and cytokine production [23]. Therefore, impairment of endothelial function is an early marker of atherosclerosis and a potential therapeutic target for the prevention of atherosclerotic diseases [24]. The results of recent clinical trials demonstrated that ticagrelor reduced vascular events in patients with ACS or a history of myocardial infarction [3,4]. The results of our study may explain the mechanism of the beneficial outcome of ticagrelor, at least partially.

P2Y12 was originally found in platelets and plays a key role in platelet activation, which results in platelet aggregation and coagulation [6]. However, several studies have demonstrated that vascular cells such as endothelial cells express P2Y12[7, 8]. Platelet-independent roles of P2Y12 have therefore attracted much attention. A recent study reported that vessel wall P2Y12 deficiency but not platelet P2Y12 deficiency attenuated atherosclerotic lesions in the aortic sinus and brachiocephalic artery in *apoe*$^{-/-}$ mice [15]. Previous clinical studies demonstrated that treatment with clopidogrel, another widely used P2Y12 antagonist, improves endothelium-dependent vascular reactivity and decreases pro-inflammatory molecules in humans [19,20,31]. These studies suggested beneficial effects of P2Y12 inhibition on endothelial function. Since its clinical approval, a substantial number of studies have reported the superiority of ticagrelor compared with clopidogrel in reducing cardiovascular events [17,18]. Several studies demonstrated that ticagrelor has protective effects on the endothelium, and that the effects were more robust compared with those of clopidogrel [17,18]. However, the mechanism by which P2Y12 inhibition by these drugs ameliorates endothelial dysfunction is not fully understood.

In this study, ticagrelor decreased the expression of inflammatory molecules in the aorta and attenuated endothelial dysfunction in *apoe*$^{-/-}$ mice. We found that ticagrelor decreased the phosphorylation of JNK, which is an important regulator of vascular inflammation and endothelial function, in treated animals [32,33]. In *in vitro* experiments, ADP stimulated the expression of inflammatory molecules in HUVEC, and a JNK inhibitor, SP600125, abolished these responses. Ticagrelor also inhibited ADP-induced JNK phosphorylation in HUVEC. Furthermore, our *ex vivo* experiment using aortic rings isolated from WT mice confirmed that ticagrelor or SP600125 attenuated impairment of endothelium-dependent vasodilation. These results partially explain that inhibition of ADP-mediated P2Y12 signaling by ticagrelor ameliorates endothelial dysfunction. Several studies have suggested that other P2Y receptors also regulate endothelial function. For example, ADP-mediated P2Y1 signaling promotes human endothelial cell migration, suggesting that it may stimulate re-endothelialization after vascular injury [34]. The ADP-P2Y receptors signaling pathways in the endothelium remain incompletely characterized. Further studies are needed to elucidate the role of these signaling pathways in endothelial cells, which could provide a new concept for endothelial protection.

Fig. 2. Ticagrelor attenuated expression of inflammatory molecules in atherosclerotic plaques in *apoe*$^{-/-}$ mice.
Representative images of immunostaining against VCAM-1 (A), ICAM-1 (B) and MOMA-2 (C), and oil red O staining (D) of atherosclerotic lesions in the aortic root in *apoe*$^{-/-}$ mice treated with ticagrelor for 8 weeks. Ticagrelor decreased the expression of VCAM-1 and ICAM-1. Ticagrelor also reduced macrophage accumulation and lipid deposition in plaques. (n = 7, per group). Bar: 500 μm $^{*}p < 0.05$, $^{**}p < 0.01$. Ctrl; control. All values are mean ± SEM.

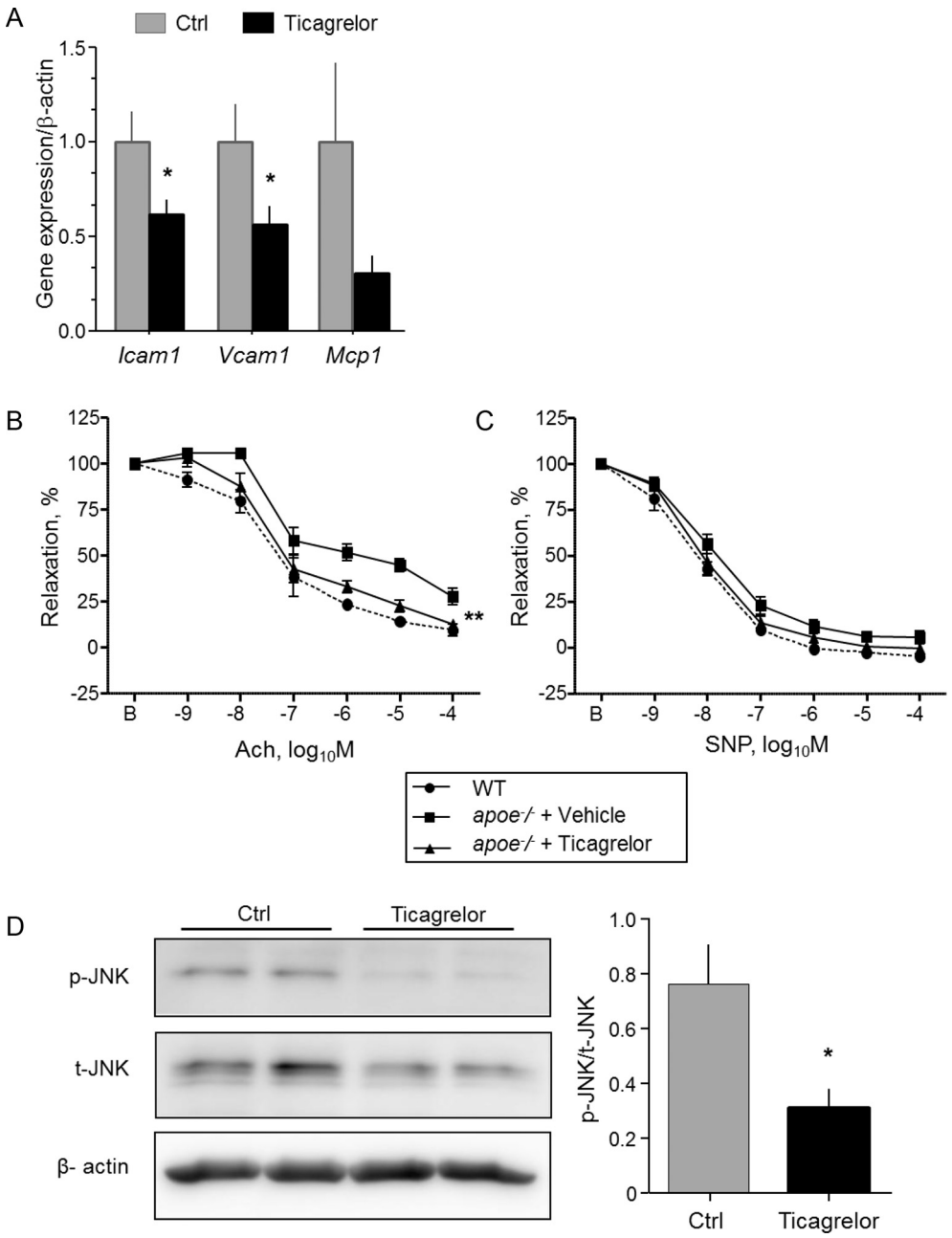


Fig. 3. Ticagrelor attenuated endothelial dysfunction in *apoe*$^{-/-}$ mice.
(A) Results of qPCR demonstrated that administration of ticagrelor for 8 weeks decreased the expression of inflammatory molecules in the atherosclerotic abdominal aorta compared with control (n = 8, per group). (B and C) Vascular reactivity to Ach or SNP was determined using aortic rings obtained from ticagrelor-treated or control *apoe*$^{-/-}$ mice at the early stage of atherosclerosis. Control *apoe*$^{-/-}$ mice showed an impaired endothelial response compared with age- and sex-matched WT mice fed normal chow. Ticagrelor administration for 8 weeks ameliorated endothelium-dependent vasodilation compared with that in control *apoe*$^{-/-}$ mice (B). Vasorelaxation in response to SNP did not differ among the three groups (C). (n = 8, per group). (D) Western blot analysis showed that ticagrelor suppressed JNK activation in the aorta of ticagrelor-treated *apoe*$^{-/-}$ mice compared with the control group (n = 7, per group). $^{*}p < 0.05$, $^{**}p < 0.01$. Ctrl; control. All values are mean ± SEM.

Ticagrelor has vascular protective effects which are beyond the ADP-P2Y12 pathway. Not only platelets but also damaged or stressed tissues including endothelial cells release ADP [35], which is converted to adenosine by CD39/CD73 on endothelial cells [36,37]. Adenosine has protective effects on the endothelium; however, the local adenosine level is immediately reduced by its internalization into cells via equilibrative nucleoside transporter 1 (ENT1) [5]. Recent studies showed that ticagrelor inhibits ENT1, leading to an increase in the local concentration of adenosine [38–40]. Therefore, P2Y12-independent effects of ticagrelor also contribute to the vasodilation and are associated with its superiority.

A previous study showed that ticagrelor stabilized advanced atherosclerotic plaques in *apoe*$^{-/-}$ mice as determined by necrotic core size, fibrous cap thickness, and macrophage accumulation in plaques [22]. In that study, ticagrelor did not reduce already existing atherosclerotic lesions, whereas ticagrelor inhibited the activation of macrophages, leading to the suppression of vascular inflammation. Another study showed that ticagrelor reduced plasma CRP level and the expression of inflammatory cytokines

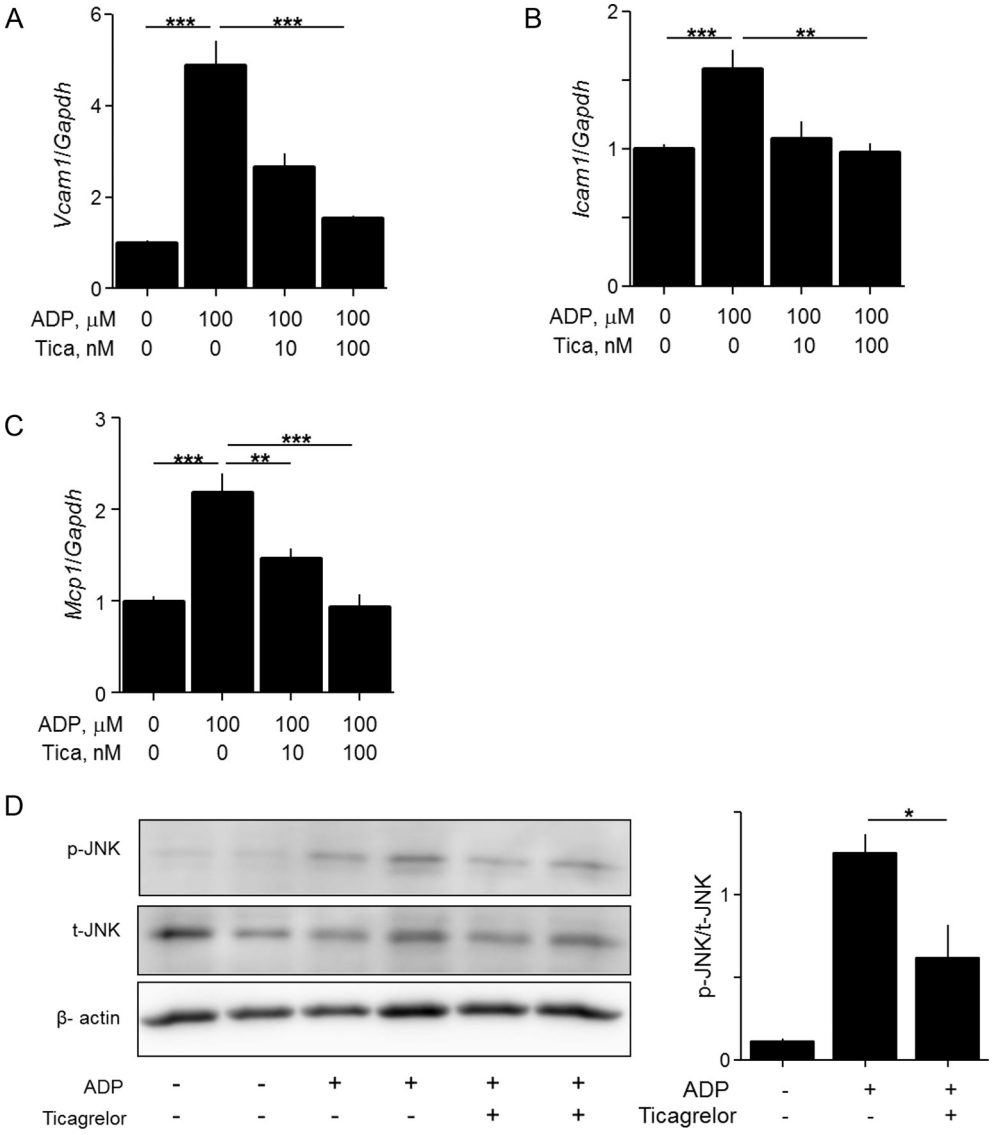


Fig. 4. Ticagrelor inhibited ADP-induced inflammatory molecule expression in endothelial cells.
(A–C) HUVEC pre-incubated with 0–100 nM ticagrelor for 2 h were stimulated with 100 μM ADP for 4 h. The results of qPCR demonstrated that ADP increased the expression *Vcam1* (A), *Icam1* (B), and *Mcp1* (C) in HUVEC. Ticagrelor inhibited the expression of these inflammatory molecules (n = 8, per group). (D) HUVEC pre-incubated with 100 nM ticagrelor for 2 h were stimulated with 100 μM ADP for 15 min. ADP induced JNK activation in HUVEC, which was significantly inhibited by ticagrelor (n = 6, per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Tica; ticagrelor. All values are mean ± SEM.

such as tumor necrosis factor-α and interleukin-6 in the aortic wall in diabetic *apoe*$^{-/-}$ mice [41]. Several clinical studies have also demonstrated anti-inflammatory effects of ticagrelor. Ticagrelor reduced carotid atherosclerotic plaque inflammation as determined by ^{18}F-fluorodeoxyglucose positron emission tomographic imaging [42]. Furthermore, ticagrelor decreased circulating levels of inflammatory molecules in type 2 diabetic patients with non–ST-segment elevation ACS requiring stent implantation [43]. These clinical and animal studies suggest that ticagrelor has anti-inflammatory effects, which may be associated with its vascular protection properties. Thus, ticagrelor seems to have protective effects on the vasculature through a broad range of cellular and molecular mechanisms. Further studies are needed to elucidate the mechanism by which ticagrelor suppresses vascular inflammation and atherogenesis.

There are several limitations of this study. First, we decided our dosage with reference to a previous paper [22]. We did not measure the plasma concentration of ticagrelor directly. Second, the degree of inflammation of atherosclerotic lesions in the ticagrelor-treated group and the control group was equivalent in this study, as determined by the results of Sudan IV staining and immunohistochemical analysis. Therefore, our results could not reveal the causal role of the reduction of inflammation by ticagrelor in the suppression of atherosclerotic lesions. Third, we only examined the P2Y12-dependent effect of ticagrelor, although P2Y12-independent effects of ticagrelor, such as inhibition of ENT1, are also reported. These effects might contribute to the results of this study. Also, our present study was performed using an animal model and cells at limited time points. Therefore, some of the beneficial effects of ticagrelor observed in this study might not necessarily be expected in clinical situations.

In conclusion, the results of our study demonstrated that ticagrelor decreased the development of atherosclerotic lesions in *apoe*$^{-/-}$ mice. Suppression of the JNK pathway through ADP-

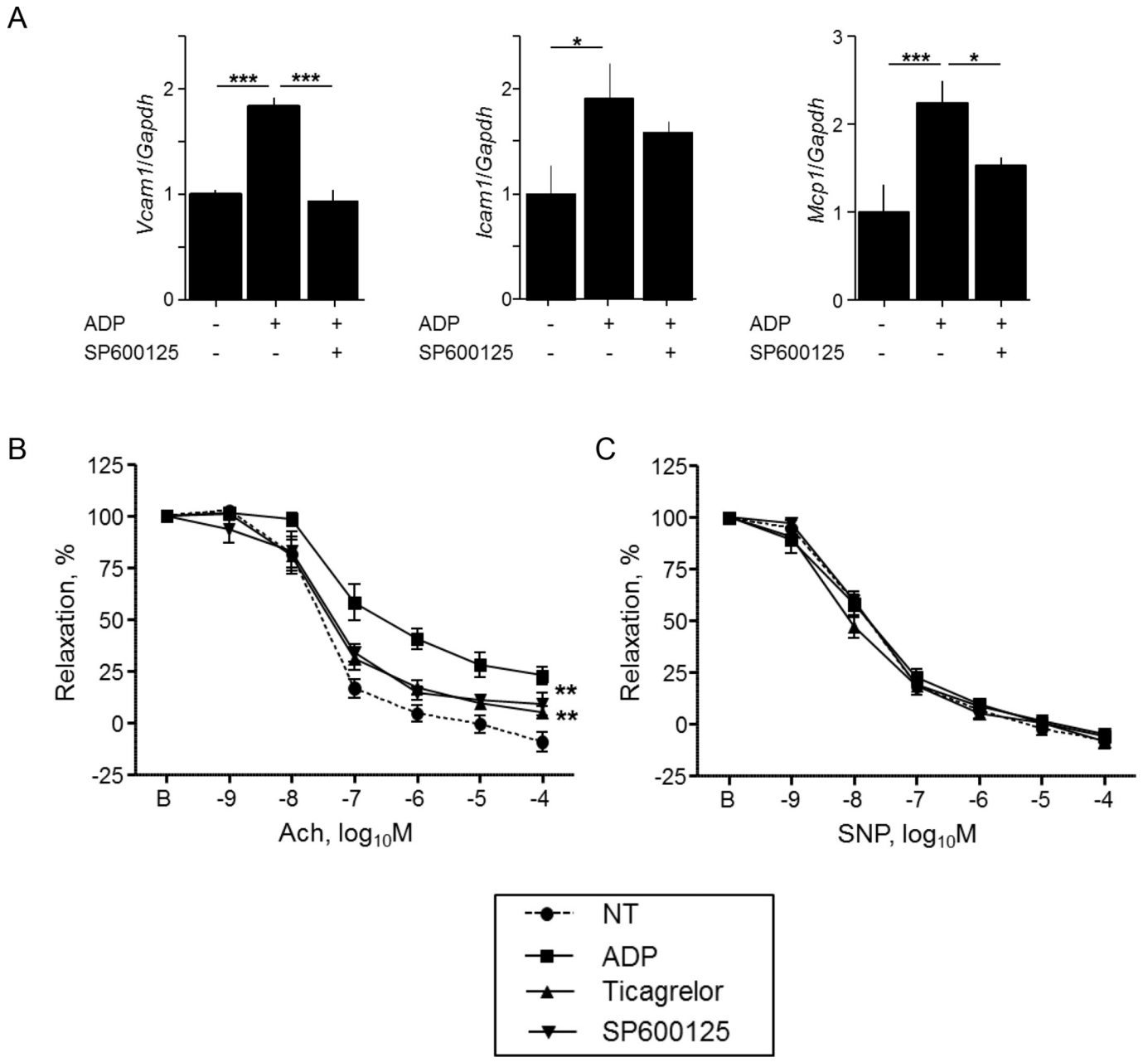


Fig. 5. Inhibition of JNK attenuated ADP-induced endothelial cell activation.
(A) HUVEC were incubated with 100 nM SP600125 for 2 h, and then stimulated with 100 μM ADP for 4 h. The results of qPCR showed that a JNK inhibitor decreased *Mcp1* and *Vcam1* expression in HUVEC (n = 8, per group). $^{*}p < 0.05$, $^{***}p < 0.001$. (B and C) To examine the impact of ticagrelor or an inhibitor of JNK (SP600125) on endothelial function, vascular reactivity to Ach or SNP was measured using aortic rings obtained from WT mice. Aortic rings were pre-incubated with 100 nM ticagrelor or 100 nM SP600125 for 2 h and stimulated with 100 μM ADP for 16 h. ADP impaired endothelium-dependent vascular reactivity, while ticagrelor or SP600125 ameliorated this impairment (B). ADP, ticagrelor, or SP600125 did not affect endothelium-independent vasodilation (C). (n = 8, per group). NT; no treatment. $^{**}p < 0.01$ *vs.* ADP. All values are mean ± SEM.

mediated P2Y12 signaling by ticagrelor partially contributed to endothelial cell protection. These results may partially explain the findings of vascular protective effects of ticagrelor in recent clinical studies. Further studies to investigate the platelet-independent effects of P2Y12 may provide better understanding and a therapeutic strategy for atherosclerosis.

Conflicts of interest

The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School, is supported in part by unrestricted research grants from Boehringer Ingelheim, Tanabe-Mitsubishi, Kowa, and Actelion. The other authors declare no conflict of interest.

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Author contributions

D.F. designed the experiments, interpreted the results, and prepared the manuscript. B.G. performed most of the experiments and prepared the manuscript. H.M.S., S.N., Y. Higashikuni, K.T., and Y. Hirata assisted with *in vivo* experiments. S.Y. and T.S. contributed to critical reading of the manuscript. M.S. interpreted the data and prepared the manuscript. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.05.053.

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