Atherosclerosis 275 (2018) 124–132

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

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

Byambasuren Ganbaatar [a], Daiju Fukuda [b, *], Hotimah Masdan Salim [a], Sachiko Nishimoto [a], Kimie Tanaka [c], Yasutomi Higashikuni [d], Yoichiro Hirata [e], Shusuke Yagi [a], Takeshi Soeki [a], Masataka Sata [a]

[a] Department of Cardiovascular Medicine, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, 770-8503, Japan
[b] Department of Cardio-Diabetes Medicine, Institute of Biomedical Science, Tokushima University Graduate School, Tokushima, 770-8503, Japan
[c] Division for Health Service Promotion, The University of Tokyo, Tokyo, 113-0033, Japan
[d] Department of Cardiovascular Medicine, The University of Tokyo, Tokyo, 113-8655, Japan
[e] Department of Pediatrics, The University of Tokyo Hospital, Tokyo, 113-8655, Japan

ARTICLE INFO

Article history:
Received 17 October 2017
Received in revised form
28 May 2018
Accepted 30 May 2018
Available online 31 May 2018

Keywords:
Atherosclerosis
Inflammation
Endothelial function
P2Y12
Ticagrelor

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.

* Corresponding author. Department of Cardio-Diabetes Medicine, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima, 770-8503, Japan.
E-mail address: daiju.fukuda@tokushima-u.ac.jp (D. Fukuda).

https://doi.org/10.1016/j.atherosclerosis.2018.05.053
0021-9150/

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.