UNIVERSITAS NAHDLATUL ULAMA SURABAYA
LEMBAGA PENELITIAN DAN PENGABDIAN KEPADA MASYARAKAT
Kampus A Wonokromo : Jl. SMEA No.57 Tlp. 031-8291920, 8284508 Fax. 031-8298582 – Surabaya 60243
Kampus B RSIJemursari : Jl. Jemursari NO.51-57 Tlp. 031-8479070 Fax. 031-8433670 – Surabaya 60237
Website : unusa.ac.id Email: info@unusa.ac.id

SURAT KETERANGAN

Nomor: 2518/UNUSA-LPPM/Adm.I/XI/2022

Lembaga Penelitian dan Pengabdin Kepada Masyarakat (LPPM) Universitas Nahdlatul Ulama Surabaya menerangkan telah selesai melakukan pemeriksaan duplikasi dengan membandingkan artikel-artikel lain menggunakan perangkat lunak **Turnitin** pada tanggal 22 November 2022.

Judul	: Thrombin inhibition by dabigatran attenuates endothelial dysfunction in diabetic mice
Penulis	: Arief Rahadian , Daiju Fukuda , Hotimah Masdan Salim , Shusuke Yagi , Kenya Kusunose , Hirotsugu Yamada , Takeshi Soeki , Michio Shimabukuro , Masataka Sata
No. Pemeriksaan	: 2022.11.28.1044

Dengan Hasil sebagai Berikut:

Tingkat Kesamaan diseluruh artikel (*Similarity Index*) yaitu 13%

Demikian surat keterangan ini dibuat untuk digunakan sebagaimana mestinya.

Surabaya, 28 November 2022

Ketua LPPM

UNUSA LPPM

Achmad Syafiuddin, Ph.D
NPP: 20071300

Thrombin inhibition by dabigatran attenuates endothelial dysfunction in diabetic mice

by HotimahHotimah

Submission date: 22-Nov-2022 11:25AM (UTC+0700)
Submission ID: 1960957714
File name: Art._Thrombin_inhibition.pdf (2.63M)
Word count: 5679
Character count: 33836

Vascular Pharmacology 124 (2020) 106632

Contents lists available at ScienceDirect

Vascular Pharmacology

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

Thrombin inhibition by dabigatran attenuates endothelial dysfunction in diabetic mice

Arief Rahadian[a,1], Daiju Fukuda[b,*,1], Hotimah Masdan Salim[a], Shusuke Yagi[a], Kenya Kusunose[a], Hirotsugu Yamada[c], Takeshi Soeki[a], Michio Shimabukuro[b,d], Masataka Sata[a]

[a] Department of Cardiovascular Medicine, Tokushima University Graduate School of Biomedical Sciences, Tokushima 770-8503, Japan
[b] Department of Cardio-Diabetes Medicine, Tokushima University Graduate School of Biomedical Sciences, Tokushima 770-8503, Japan
[c] Department of Community Medicine for Cardiology, Tokushima University Graduate School of Biomedical Sciences, Tokushima 770-8503, Japan
[d] Department of Diabetes, Endocrinology and Metabolism School of Medicine, Fukushima Medical University, Fukushima 960-1295, Japan

ARTICLE INFO

Keywords:
Thrombin
Endothelial function
Dabigatran
Inflammation
Diabetes

ABSTRACT

Diabetic patients have coagulation abnormalities, in which thrombin plays a key role. Whereas accumulating evidence suggests that it also contributes to the development of vascular dysfunction through the activation of protease-activated receptors (PARs). Here we investigated whether the blockade of thrombin attenuates endothelial dysfunction in diabetic mice. Induction of diabetes by streptozotocin (STZ) increased the expression of PAR1, PAR3, and PAR4 in the aorta. STZ-induced diabetic mice showed impairment of endothelial function, while the administration of dabigatran etexilate, a direct thrombin inhibitor, significantly attenuated endothelial dysfunction in diabetic mice with no alteration of metabolic parameters including blood glucose level. Dabigatran did not affect endothelium-independent vasodilation. Dabigatran decreased the expression of inflammatory molecules (e.g., MCP-1 and ICAM-1) in the aorta of diabetic mice. Thrombin increased the expression of these inflammatory molecules and the phosphorylation of IκBα, and decreased the phosphorylation of $eNOS^{Ser1177}$ in human umbilical endothelial cells (HUVEC). Thrombin significantly impaired the endothelium-dependent vascular response of aortic rings obtained from wild-type mice. Inhibition of NF-κB attenuated thrombin-induced inflammatory molecule expression in HUVEC and ameliorated thrombin-induced endothelial dysfunction in aortic rings. Dabigatran attenuated the development of diabetes-induced endothelial dysfunction. Thrombin signaling may serve as a potential therapeutic target in diabetic condition.

1. Introduction

Atherosclerosis and subsequent cardiovascular disease are critical complications of diabetes mellitus [1]. Multiple pathophysiological conditions related to diabetes cause vascular inflammation [2], leading to the development of atherosclerosis [3]. Vascular inflammation causes endothelial dysfunction, an initiator of atherosclerosis [4]. Endothelial dysfunction induces the expression of adhesion molecules and chemokines and alters vascular responses, which stimulate monocyte-endothelial cell interactions, leading to the development of atherosclerosis [5]. However, the mechanism that causes endothelial dysfunction in diabetic patients is not fully understood.

Previous studies have reported that patients with diabetes mellitus have coagulation abnormalities [6–8]. For example, hyperglycemia in acute coronary syndrome patients with and without a previous history of diabetes is associated with enhanced local thrombin generation [9]. These studies suggested that hyperglycemia promotes thrombin generation, which is associated with cardiovascular complications, in these patients. The vascular endothelium primarily has protective effects against atherogenesis; however, an imbalance of coagulation causes endothelial dysfunction, platelet and monocyte adhesion, and macrophage activation, as well as blood coagulation, all of which are known to promote atherogenesis [10]. In the coagulation cascade, thrombin plays a key role, whereas accumulating evidence suggests its

Abbreviations: Ach, acetylcholine; HUVEC, human umbilical endothelial cell; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; PAR, protease-activated receptor; qPCR, quantitative RT-PCR; SNP, sodium nitroprusside; STZ, streptozotocin; VCAM, vascular cell adhesion molecule

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

https://doi.org/10.1016/j.vph.2019.106632
Received 15 January 2019; Received in revised form 19 September 2019; Accepted 19 November 2019
Available online 20 November 2019
1537-1891/

contribution to vascular inflammation through protease-activated receptor (PAR)1, PAR3, and PAR4, a family of seven transmembrane G-protein-coupled receptors activated by proteolytic cleavage of the amino-terminal extracellular domain [11,12]. Previous studies reported that activation of PARs by thrombin is associated with the pathophysiology of inflammatory diseases including vascular inflammation [13]. However, few studies have examined the role of thrombin in the development of diabetes-related endothelial dysfunction.

Dabigatran is an oral anticoagulant that directly inhibits thrombin and is prescribed for the prevention of thrombotic complications in patients with atrial fibrillation [14–16]. In addition, recent studies have reported that dabigatran prevented the development of atherosclerosis in a hypercholesterolemic mouse model [17–19]. The results of these studies suggested that the inhibition of thrombin by dabigatran is associated with vascular protection. Therefore, in this study, to address the hypothesis that inhibition of thrombin signaling by dabigatran attenuates endothelial dysfunction in diabetic mice, we administered dabigatran to streptozotocin (STZ)-induced diabetic mice and examined vascular responses. We also performed in vitro studies using endothelial cells and ex vivo experiments using aortic rings to investigate the underlying mechanisms. The results of our study suggest that dabigatran attenuates vascular inflammation and endothelial dysfunction in diabetic mice, and provides a potential therapeutic target for diabetes-related endothelial dysfunction.

2. Methods

2.1. Animal experiments

Wild-type (C57BL/6 J background) mice were purchased from Japan SLC, Inc. STZ (150 mg/kg) or vehicle (citrate buffer) was injected intraperitoneally into 8-week-old male wild-type mice. From 3 days after injection, mice were fed normal chow supplemented with 10 mg/g dabigatran etexilate (approximately 1800 mg/kg/day), a direct thrombin inhibitor, for 3 weeks. The control group received non-supplemented chow. STZ was purchased from Sigma-Aldrich. Dabigatran was provided by Boehringer Ingelheim. Mice were maintained under a 12-h light/dark cycle with free access to chow and water. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by our institutional ethics committee.

2.2. Metabolic parameter analyses

At the time of sacrifice, blood was collected from the heart without fasting into EDTA-containing tubes, and plasma was stored at −80 °C until required. Plasma total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were measured at LSI Medience Corporation (Japan).

2.3. Vascular reactivity assay

Analysis of vascular reactivity was performed as we described previously [20]. In brief, the descending thoracic aortas obtained from each group of mice were cut into 2-mm rings and mounted in organ baths filled with modified Krebs–Henseleit buffer 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. The viability of aortic segments was tested with 31.4 mM KCl. Blood vessel integrity was assessed in response to phenylephrine to induce vasoconstriction followed by vasorelaxation produced by acetylcholine. Vessel rings pre-contracted 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 segments prepared from wild-type mice were incubated with 10 nM thrombin (Sigma-Aldrich) in DMEM containing 2% FBS in the presence or absence of a NF-κB inhibitor, BAY 11–7082 (Sigma-Aldrich), for 4 h before analyses of vascular reactivity.

2.4. Flow cytometry analysis

To investigate effects of dabigatran on endothelial cells, we performed flow cytometry analysis using the aorta. The aorta was fractionated as described previously [21]. Fractionated cells were stained with anti-CD31-Alexa488, anti-ICAM-1-PE/Cy7, and anti-VCAM-1-APC antibodies (Biolegend). Data were acquired on FACSVerse (BD Biosciences) and the percentage of ICAM-1 or VCAM-1 positive endothelial cells were analyzed.

2.5. Cell culture experiment

Human umbilical vein endothelial cells (HUVEC) were purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVEC (passage 4–6) were treated with 1–100 nM thrombin in EBM-2 (Lonza) containing 2% FBS for 4 h in the presence or absence of BAY 11–7082. To investigate the effect of high glucose condition on thrombin-induced endothelial activation, HUVEC which were cultured in EBM-2 or in glucose (50 mM)-supplemented EBM-2, both of which containing 2% FBS, were treated with 10 nM thrombin for 2 h.

2.6. Quantitative RT-PCR

Total RNA was extracted from aortic tissue or 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 using Power SYBR Green PCR Master Mix (Applied Biosystems) on an Mx3000P (Agilent

Table 1
List of PCR primers.

	Sense	Antisense
Mouse		
ICAM-1	5′- TTCACACTGAATGCCAGCTC -3′	5′- GTCTGCTGAGACCCCTCTTG −3′
MCP-1	5′- CCACTCACCTGCTGCTACTCAT −3′	5′- TGGTGATCCTCTTGTAGCTCTCC -3′
PAR-1	5′- AGAGTCGCTTCCACGAAAGTCCTA −3′	5′- GGTCACAAGCGCGGTGATAA −3′
PAR-3	5′- TTCTGCCAGTCACTGTTTGC -3′	5′- AGGTTGGCTTTGCTGAGTTG −3′
PAR-4	5′- GATCCAGCCCTAGACACCCTGA −3′	5′- TGTACCCGCAGGCACATACAA −3′
VCAM-1	5′- CCCGTCATTGAGGATATTGG −3′	5′- GGTCATTGTCACAGCACCAC -3′
β-actin	5′- CCTGAGCGCAAGTACTCTGTGT −3′	5′- GCTGATCCACATCTGCTGGAA −3′
Human		
MCP-1	5′- CCCCAGTCACCTGCTGTTAT −3′	5′- AGATCTCCTTGGCCACAATG −3′
ICAM-1	5′- TGATGGGCAGTCAACAGCTA- 3′	5′- GGGTAAGGTTCTTGCCCACT −3′
VCAM-1	5′- GCTGCTCAGATTGGAGACTCA −3′	5′- CGCTCAGAGGGCTGTCTATC -3′
GAPDH	5′- TGGGTGTGAACCATGAGAAG −3′	5′- GCTAAGCAGTTGGTGGTGC -3′

Technologies). Data are expressed in arbitrary units normalized by β-actin or GAPDH. The sequences of primers are listed in Table 1.

2.7. Western blotting

Protein lysates were isolated from aortic tissue or HUVEC 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 membranes were blocked in 5% bovine serum albumin for 1 h at room temperature, followed by incubation with primary antibody against either phosphorylated eNOS^{Ser1177}, eNOS (BD Biosciences), phosphorylated IκBα, IκBα (Cell Signaling Technology), ICAM-1, VCAM-1 (abcam), 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).

2.8. Statistical analysis

All data are expressed as mean ± SEM. Comparison of parameters between two groups was performed with unpaired Student's *t*-test. Differences between multiple groups were analyzed by ANOVA followed by Tukey's post hoc analysis. 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 $P < .05$ was considered significant.

3. Results

3.1. Induction of diabetes promoted expression of PARs in aorta

To investigate the role of thrombin signaling in the development of endothelial dysfunction, we examined the expression of thrombin receptors in the aorta. Induction of diabetes significantly promoted thrombin receptor expression (e.g., PAR1, 3, and 4), as shown in Fig. 1.

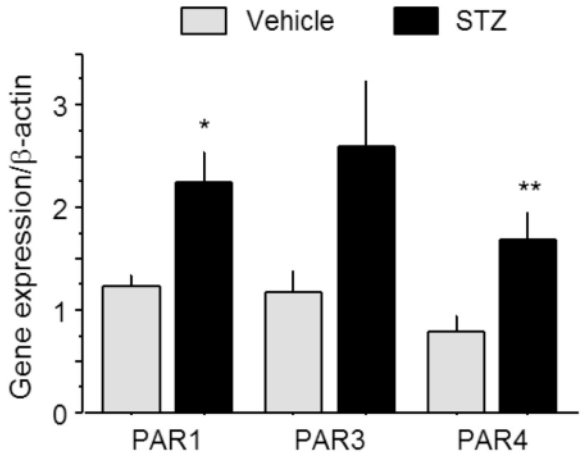

Fig. 1. Induction of diabetes promoted expression of PARs in aorta.
Induction of diabetes increased the expression of PAR1, PAR3, and PAR4, receptors for thrombin, in the aorta. (n = 6–7, per group). STZ; streptozotocin. *; $P < .05$ and **; $P < .01$. All values are mean ± SEM.

3.2. Dabigatran ameliorated endothelial dysfunction in diabetic mice

Endothelial dysfunction is an initial step in atherosclerosis. Therefore, to investigate the effect of dabigatran on endothelial function, we administered dabigatran to STZ-induced diabetic mice. Endothelium-dependent vasodilation in response to Ach was impaired in STZ-induced diabetic mice compared with that in the normoglycemic control group ($P < .001$); however, dabigatran administration significantly ameliorated the impairment of endothelium-dependent vasodilation compared with the non-treated group ($P < .001$) (Fig. 2A). On the other hand, endothelium-independent relaxation in response to SNP did not differ between the dabigatran-treated group and non-treated group (Fig. 2B). In addition, induction of diabetes promoted the expression of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 in the aorta, while dabigatran administration reduced their expression (Fig. 2C-E). Also, the results of flow cytometry analysis demonstrated that dabigatran significantly reduced ICAM-1-positive endothelial cells and tended to reduce VCAM-1-positive endothelial cells in the aorta of diabetic mice (Fig. 2F and G). Administration of dabigatran did not alter plasma glucose and plasma lipid levels in diabetic mice (Table 2).

3.3. Thrombin stimulated pro-inflammatory activation of endothelial cells

Dabigatran is a specific inhibitor of thrombin. Therefore, we performed in vitro experiments using HUVEC to examine the effect of thrombin on endothelial cells. Treatment with thrombin dose-dependently increased the expression of inflammatory molecules such as MCP-1, ICAM-1, and VCAM-1 in HUVEC (Fig. 3A). Increase in VCAM-1 and VCAM-1 expression in HUVEC was also confirmed in the protein level (Fig. 3B). Thrombin significantly attenuated the phosphorylation of eNOS at Ser1177 in HUVEC ($P < .05$). On the other hand, thrombin increased the phosphorylation of IκBα ($P < .01$), suggesting activation of the NF-κB pathway in this cell type (Fig. 3C). We further examined the effect of thrombin under high glucose condition. High glucose condition ptomoted the expression of ICAM-1 and MCP-1 in thrombin-treated HUVEC, suggesting that high glucose condition enhances thrombin-induced inflammatory activation of endothelial cells (Fig. 3D).

To investigate the involvement of NF-κB signaling in thrombin-induced pro-inflammatory activation of endothelial cells, we treated HUVEC with thrombin in the presence of a NF-κB inhibitor, BAY11–7082. BAY11–7082 ameliorated thrombin-induced expression of inflammatory molecules in this cell type (Fig. 4A-C). To confirm the effect of thrombin on endothelial function, we incubated aortic segments obtained from wild-type mice with thrombin, and examined the vascular response. Thrombin markedly reduced endothelium-dependent vascular relaxation, which was blocked by a NF-κB inhibitor, BAY11–7082 (Fig. 4D). However, thrombin did not alter endothelium-independent vascular relaxation (Fig. 4E).

4. Discussion

Diabetes causes endothelial dysfunction which is an initial step of atherosclerosis [2]. Previous studies suggested that endothelial dysfunction could be a potential therapeutic target for the prevention of vascular disease in these patients [22], although effective prevention is not established. In this study, we examined whether dabigatran, a direct thrombin inhibitor, attenuates endothelial dysfunction, using a diabetic mouse model. We found that dabigatran ameliorated the development of endothelial dysfunction and vascular inflammation in STZ-induced diabetic mice. In vitro experiments using HUVEC demonstrated that thrombin promotes the expression of inflammatory molecules at least partially via the NF-κB pathway. Furthermore, incubation with thrombin impaired the vascular response to Ach in mouse aortic rings,

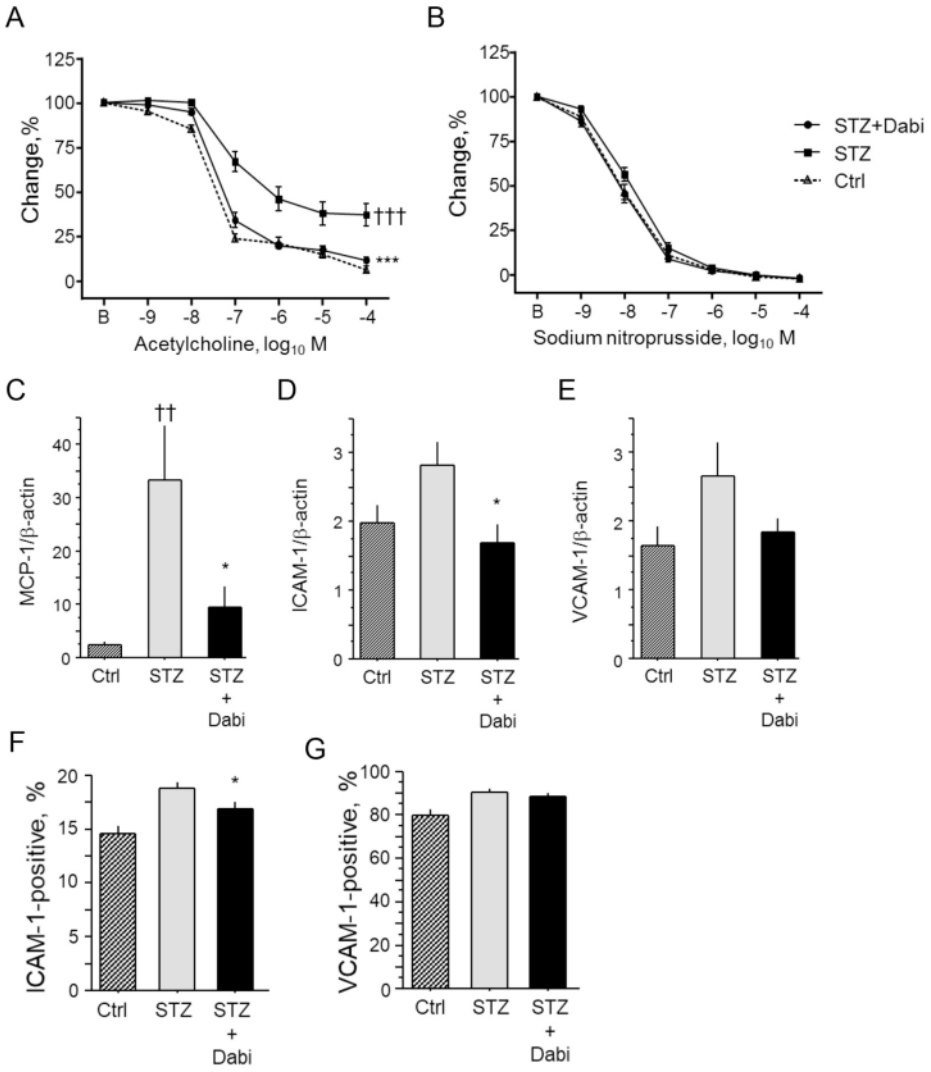

Fig. 2. Dabigatran ameliorated endothelial dysfunction in diabetic mice.
(A and B) Vascular reactivity to Ach (A) or SNP (B) was determined using aortic segments isolated from dabigatran- or non-treated diabetic mice and non-diabetic control mice. Induction of diabetes by STZ injection impaired the endothelium-dependent vascular response, while dabigatran administration to diabetic mice for 3 weeks ameliorated this response ($P < .001$). Vasorelaxation in response to SNP did not differ among the three groups. ($n = 9–14$, per group). (C-E) The expression of inflammatory molecules was examined by qPCR using abdominal aorta. Induction of diabetes by STZ injection increased the expression of MCP-1 (C), ICAM-1 (D), and VCAM-1 (E). Administration of dabigatran attenuated their expression ($n = 8–9$, per group). (F and G) Flow cytometry analysis demonstrated that dabigatran decreased ICAM-1 or VCAM-1-positive endothelial cells (n = 6–7, per group). ††; $P < .01$ and †††; $P < .001$ vs. non-diabetic control group, and *; $P < .05$ and ***; $P < .001$ vs. STZ group. Ctrl; non-diabetic control and Dabi; dabigatran. All values are mean ± SEM.

Table 2
Effects of dabigatran on metabolic parameters.

	Vehicle ($n = 14$)	STZ ($n = 12$)	STZ + Dabi ($n = 9$)	P-value
Body weight, g	21.6 ± 0.6	16.0 ± 0.6***	16.8 ± 0.3***	$P < .001$
Blood glucose, mg/dl	117.6 ± 3.0	528.0 ± 34.9***	533.1 ± 33.0***	$P < .001$
Triglyceride, mg/dl	26.8 ± 3.0	65.2 ± 11.0**	49.8 ± 9.5	$P < .01$
Total cholesterol, mg/dl	62.4 ± 4.3	70.8 ± 9.3	72.4 ± 8.2	*NS*
HDL cholesterol, mg/dl	64.4 ± 7.6	82.6 ± 8.3	79.3 ± 10.8	*NS*

All values are mean ± SEM. Dabi, dabigatran; HDL, high density lipoprotein.
; $P < .01$ and *; $P < .001$ vs. vehicle.

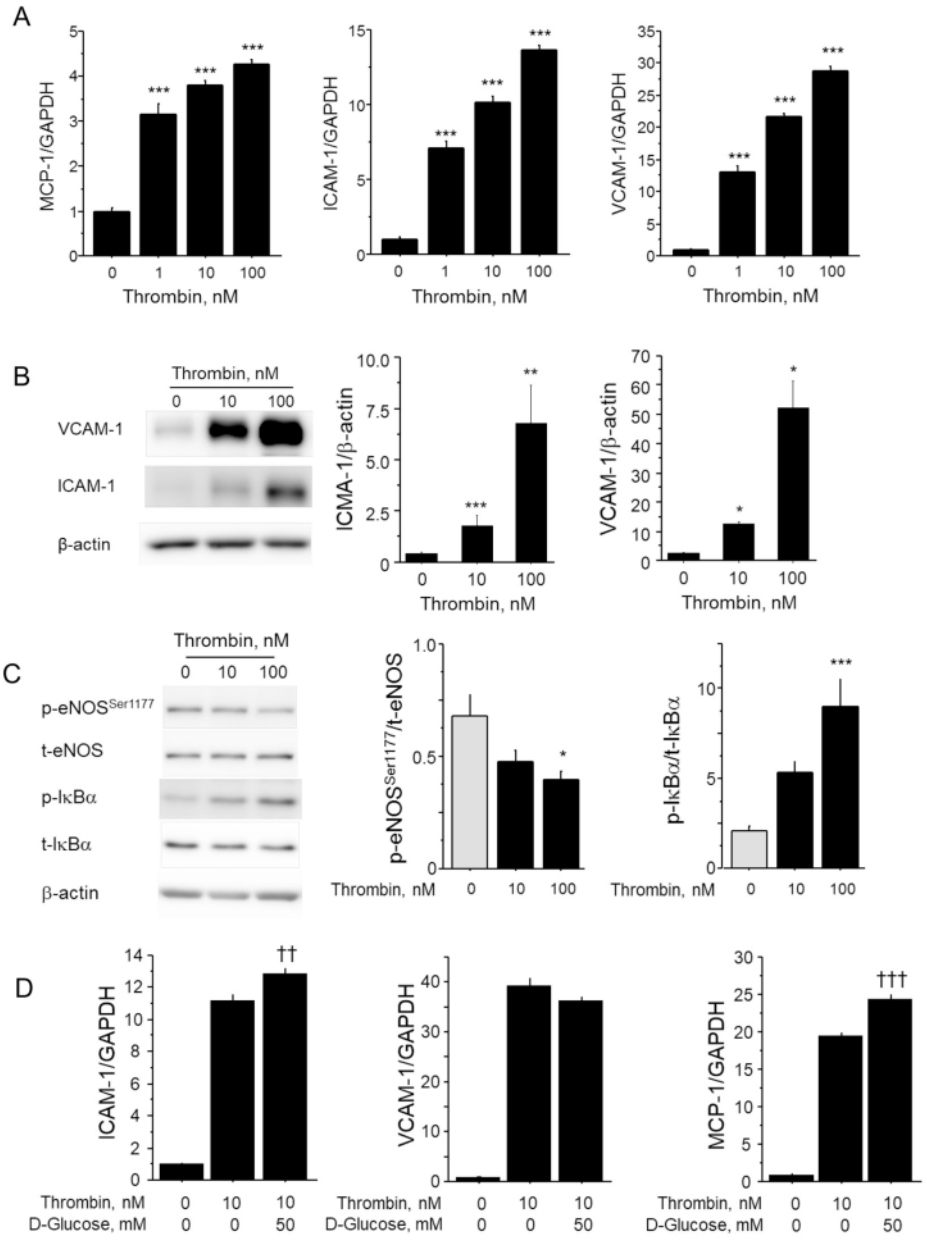

Fig. 3. Thrombin promoted pro-inflammatory activation of endothelial cells.
(A) The effect of thrombin on inflammatory molecule expression in HUVEC was examined by qPCR. Thrombin treatment for 4 h increased the expression of MCP-1, ICAM-1, and VCAM-1 in HUVEC ($n = 4$). (B) The results of western blotting also demonstrated the increase in ICAM-1 and VCAM-1 expression in thrombin-treated HUVECs in the protein level (n = 4). (C) The effect of thrombin on the phosphorylation of eNOS and IκBα was examined by western blotting. Thrombin treatment for 60 min decreased eNOS phosphorylation and increased IκBα phosphorylation (n = 6). (D) Incubation of HUVEC with thrombin in high glucose condition promotes thrombin-induced expression of ICAM-1 and MCP-1, suggesting that high glucose condition enhances proinflammatory property of thrombin (n = 6). *; $P < .05$ and ***; $P < .001$ vs. non-treatment. ††; $P \leq .01$ and †††; $P \leq .001$ vs. thrombin. All values are mean ± SEM.

although a NF-κB inhibitor attenuated this response. These results suggest that dabigatran attenuates endothelial dysfunction in diabetic mice by inhibiting vascular inflammation, and that thrombin serves as a potential therapeutic target for diabetes-related endothelial dysfunction.

Diabetic patients have increased risk of vascular complications. The vascular complications associated with atherosclerosis are the most serious manifestations in patients with diabetes. Although multifactorial in etiology [23], recent studies demonstrated that a hypercoagulable state in diabetic patients, which results from enhanced thrombin generation, for example, is associated with atherosclerotic complications in these patients [6–10]. Thrombin plays a key role in the

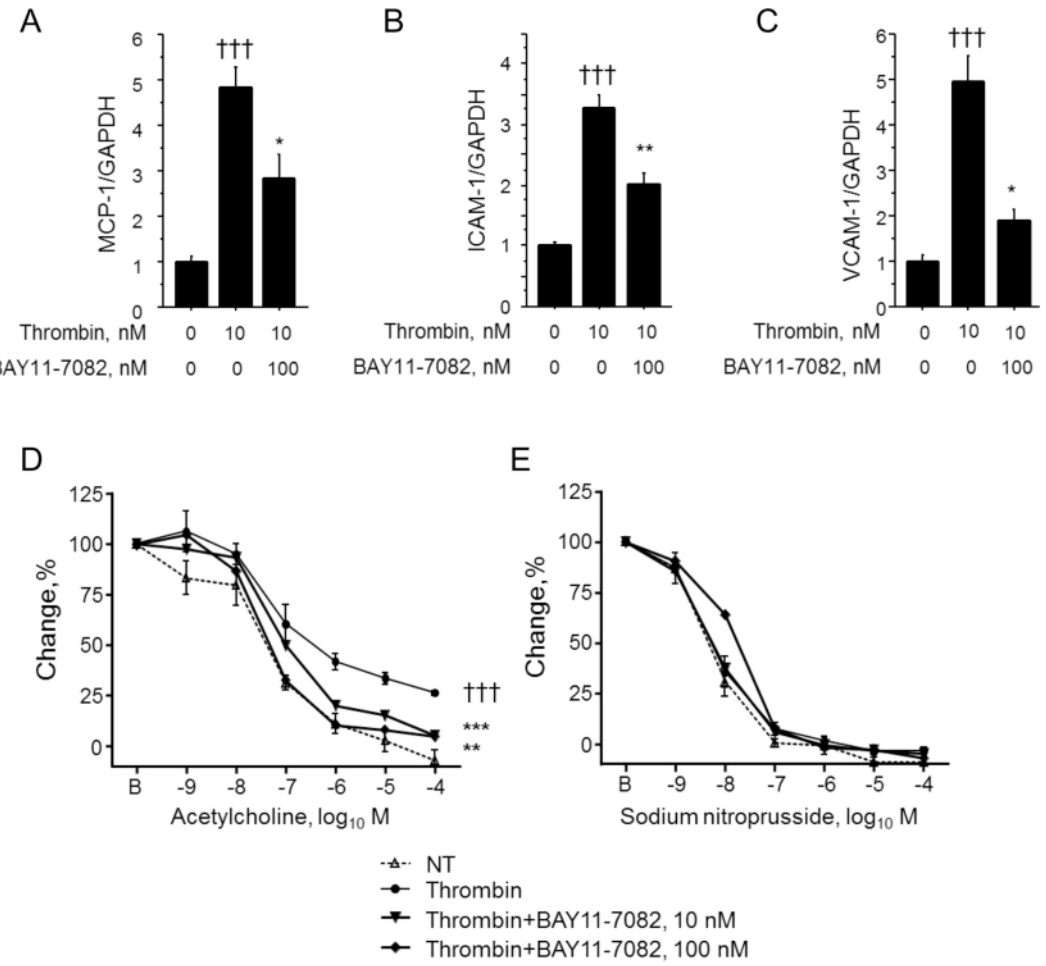

Fig. 4. NF-κB inhibitor attenuated effects of thrombin on endothelial cells.
(A-C) The effect of a NF-κB inhibitor, BAY11–7082, on thrombin-induced endothelial cell activation was examined by qPCR. BAY11–7082 inhibited the expression of MCP-1 (A), ICAM-1 (B), and VCAM-1 (C) which were promoted by thrombin in HUVEC. (D and E) Aortic segments obtained from wild-type mice were incubated with thrombin in the presence or absence of a NF-κB inhibitor, BAY11–7082, and then, vascular reactivity to Ach (D) or SNP (E) was examined. Thrombin significantly inhibited endothelium-dependent vascular relaxation, while BAY11–7082 attenuated thrombin-induced endothelial dysfunction. Neither thrombin nor BAY11–7082 affected endothelium-independent vascular relaxation. †††; $P < .001$ vs. non-treatment, and *; $P < .05$, **; $P < .01$ and ***; $P < .001$ vs. thrombin-treatment. NT; non-treatment. All values are mean ± SEM.

coagulation cascade by cleaving fibrinogen to fibrin, while accumulating evidence indicates that thrombin has direct effects on the endothelium, independent of blood coagulation. Thrombin increases inflammatory molecule expression, recruitment of inflammatory cells [24], generation of reactive oxygen species [25], and vascular tone [26] in endothelial cells, all of which disturb homeostasis of the vasculature, causing vascular inflammation and deterioration of endothelial cell function. Prolonged incubation with thrombin has also been reported to inhibit NO synthesis, which has a critical impact on endothelial function [27]. Considering these pro-inflammatory roles of thrombin in vascular biology, targeting thrombin signaling may offer a potential therapeutic target.

Dabigatran is the first oral anticoagulant that directly inhibits thrombin. Dabigatran prevents stroke and systemic thromboembolic events in patients with atrial fibrillation [14–16]. In addition to these anti-thrombotic effects, together with the increasing evidence of pro-inflammatory properties of thrombin, the effect of dabigatran on atherogenesis has attracted much attention. Several studies have demonstrated that dabigatran prevents the development and destabilization of atherosclerotic plaques in apolipoprotein *E*-deficient mice [17–19]. Furthermore, a previous study demonstrated that dabigatran attenuated endothelial dysfunction in a hyperlipidemic mouse model [17]. However, few studies have investigated the effect of dabigatran on endothelial function in a diabetic condition.

In our present study, induction of diabetes impaired the endothelium-dependent vascular response and increased the expression of inflammatory molecules (e.g., MCP-1 and ICAM-1), all of which were ameliorated by the administration of dabigatran, without an alteration of blood glucose level. These results suggest that dabigatran attenuates vascular inflammation and preserves endothelial function. Although the mouse model was different, our results are in line with previous studies demonstrating anti-inflammatory and vasoprotective properties of dabigatran. We also found that induction of diabetes increased the expression of PARs in the aorta. Previous studies have demonstrated that PAR1, 3, and 4 mediate non-thrombotic effects of thrombin such as vascular regulation [12,28]. Especially, a recent study showed that

PAR4 plays a pivotal role in vasculopathy in a diabetic condition [29]. Therefore, promotion of PAR expression might also play roles in the development of thrombin-induced endothelial dysfunction in diabetic mice. In our in vitro experiments, thrombin markedly promoted the expression of inflammatory molecules and reduced the phosphorylation of eNOS. These findings are consistent with previous studies [27]. In this study, we further found that high glucose condition enhances thrombin-induced inflammatory activation of endothelial cells in in vitro experiments. Also, thrombin activates the NF-κB pathway, and an inhibitor of NF-κB suppressed pro-inflammatory effects of thrombin in endothelial cells. These results suggest the involvement of NF-κB signaling in the pro-inflammatory properties of thrombin in endothelial cells. Previous studies demonstrated that thrombin-induced PAR activation promotes NF-κB signaling [30,31]. NF-κB signaling promotes the expression of inflammatory molecules and oxidative stress, leading to the deterioration of eNOS function [32,33]. Thus, our study suggests that inhibition of thrombin-PAR signaling by dabigatran may provide a therapeutic option for diabetes-induced endothelial dysfunction. On the other hand, several previous studies reported vasodilation effect of thrombin [34,35]. Marked differences in species, vascular beds, vascular viability, incubation time and dose might explain this discrepant results [26,34]. In addition, several signaling pathways are suggested for thrombin, however, it is not fully understood [36,37]. Therefore, further studies are needed to elucidate the effect of thrombin on vascular tone. In this study we focused on inflammatory effects of thrombin, whereas a recent study demonstrated that thrombin inhibition with dabigatran preserves endothelial barrier integrity, resulting in atheroprotection [38]. In contrast, one recent study reported that long-term inhibition of thrombin by dabigatran may increase atherosclerotic and atherothrombotic risk [39]. Further studies are required to reveal the effect and underlying mechanisms of dabigatran on vascular function.

Finally, in this study, we used STZ-induced diabetic mice. This is a widely used mouse model for diabetes, however this model is more representative for type 1 diabetes. In clinical studies, the effect of coagulation system on vascular complication have been mainly investigated in type 2 diabetic patients. Therefore, this is one of the important limitations for our study.

5. Conclusions

In conclusion, the results of our study indicated that dabigatran attenuated endothelial dysfunction in diabetic mice. Considering the pro-inflammatory roles of thrombin in vascular biology and enhanced coagulation in diabetic patients, the inhibition of thrombin signaling by dabigatran may offer a promising therapeutic option for treating diabetes-related endothelial dysfunction.

Funding

This work was partially supported by JSPS Kakenhi Grants (Number 19K08584 to D.F., and Number 19H03654 to Ma.S), SENSHIN Medical Research Foundation (D.F.), Takeda Science Foundation (D.F. and Ma.S.), and the Vehicle Racing Commemorative Foundation (Ma.S.). This work was also financially supported by Boehringer Ingelheim, Japan. The funders had no role in the study design, data collection, and analysis, or preparation of the manuscript.

Declaration of Competing Interest

The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School is supported in part by unrestricted research grants from Boehringer Ingelheim, Japan. Other authors declare that they have no conflict of interest.

Acknowledgments

All authors are grateful to E. Uematsu and S. Okamoto (Tokushima Univ.) for their expert technical assistance. Authors also thank M. Kitamura and A. Watanabe (Support Center for Advanced Medical Science, Tokushima University Graduate School of Biomedical Sciences) for their expert skills for operating cell analyzer.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] L. Niskanen, A. Turpeinen, I. Penttila, M.I. Uusitupa, Hyperglycemia and compositional lipoprotein abnormalities as predictors of cardiovascular mortality in type 2 diabetes: a 15-year follow-up from the time of diagnosis, Diabetes Care 21 (11) (1998) 1861–1869.
[2] U. Hink, H. Li, H. Mollnau, M. Oelze, E. Matheis, M. Hartmann, M. Skatchkov, F. Thaiss, R.A. Stahl, A. Warnholtz, et al., Mechanisms underlying endothelial dysfunction in diabetes mellitus, Circ. Res. 88 (2) (2001) E14–E22.
[3] P. Libby, P.M. Ridker, A. Maseri, Inflammation and atherosclerosis, Circulation 105 (9) (2002) 1135–1143.
[4] R. Ross, Atherosclerosis–an inflammatory disease, N. Engl. J. Med. 340 (2) (1999) 115–126.
[5] J. Davignon, P. Ganz, Role of endothelial dysfunction in atherosclerosis, Circulation 109 (23 Suppl 1) (2004) III27–32.
[6] A. Ceriello, R. Giacomello, G. Stel, E. Motz, C. Taboga, L. Tonutti, M. Pirisi, E. Falleti, E. Bartoli, Hyperglycemia-induced thrombin formation in diabetes. The possible role of oxidative stress, Diabetes 44 (8) (1995) 924–928.
[7] P. Ferroni, D. Della-Morte, A. Pileggi, M.G. Valente, F. Martini, F. La Farina, R. Palmirotta, L.F. Meneghini, T. Rundek, C. Ricordi, et al., Impact of statins on the coagulation status of type 2 diabetes patients evaluated by a novel thrombin-generation assay, Cardiovasc. Drugs Ther. 26 (4) (2012) 301–309.
[8] A. Tripodi, A. Branchi, V. Chantarangkul, M. Clerici, G. Merati, A. Artoni, P.M. Mannucci, Hypercoagulability in patients with type 2 diabetes mellitus detected by a thrombin generation assay, J. Thromb. Thrombolysis 31 (2) (2011) 165–172.
[9] A. Undas, I. Wiek, E. Stepien, K. Zmudka, W. Tracz, Hyperglycemia is associated with enhanced thrombin formation, platelet activation, and fibrin clot resistance to lysis in patients with acute coronary syndrome, Diabetes Care 31 (8) (2008) 1590–1595.
[10] K. Croce, P. Libby, Intertwining of thrombosis and inflammation in atherosclerosis, Curr. Opin. Hematol. 14 (1) (2007) 55–61.
[11] S.R. Coughlin, Thrombin signalling and protease-activated receptors, Nature 407 (6801) (2000) 258–264.
[12] K. Hirano, The roles of proteinase-activated receptors in the vascular physiology and pathophysiology, Arterioscler. Thromb. Vasc. Biol. 27 (1) (2007) 27–36.
[13] M.J. Rabiet, J.L. Plantier, E. Dejana, Thrombin-induced endothelial cell dysfunction, Br. Med. Bull. 50 (4) (1994) 936–945.
[14] M. Brambatti, H. Darius, J. Oldgren, A. Clemens, H.H. Noack, M. Brueckmann, S. Yusuf, L. Wallentin, M.D. Ezekowitz, S.J. Connolly, et al., Comparison of dabigatran versus warfarin in diabetic patients with atrial fibrillation: results from the RE-LY trial, Int. J. Cardiol. 196 (2015) 127–131.
[15] S.J. Connolly, M.D. Ezekowitz, S. Yusuf, J. Eikelboom, J. Oldgren, A. Parekh, J. Pogue, P.A. Reilly, E. Themeles, J. Varrone, et al., Dabigatran versus warfarin in patients with atrial fibrillation, N. Engl. J. Med. 361 (12) (2009) 1139–1151.
[16] G.J. Hankey, J.W. Eikelboom, Dabigatran etexilate: a new oral thrombin inhibitor, Circulation 123 (13) (2011) 1436–1450.
[17] I.O. Lee, M.T. Kratz, S.H. Schirmer, M. Baumhakel, M. Bohm, The effects of direct thrombin inhibition with dabigatran on plaque formation and endothelial function in apolipoprotein E-deficient mice, J. Pharmacol. Exp. Ther. 343 (2) (2012) 253–257.
[18] S. Pingel, V. Tiyerili, J. Mueller, N. Werner, G. Nickenig, C. Mueller, Thrombin inhibition by dabigatran attenuates atherosclerosis in ApoE deficient mice, Arch. Med. Sci. 10 (1) (2014) 154–160.
[19] M.R. Preusch, N. Ieronimakis, E.S. Wijelath, S. Cabbage, J. Ricks, F. Bea, M. Reyes, J. van Ryn, M.E. Rosenfeld, Dabigatran etexilate retards the initiation and progression of atherosclerotic lesions and inhibits the expression of oncostatin M in apolipoprotein E-deficient mice, Drug Des. Devel. Ther. 9 (2015) 5203–5211.
[20] S. Matsumoto, M. Shimabukuro, D. Fukuda, T. Soeki, K. Yamakawa, H. Masuzaki, M. Sata, Azilsartan, an angiotensin II type 1 receptor blocker, restores endothelial function by reducing vascular inflammation and by increasing the phosphorylation ratio Ser(1177)/Thr(497) of endothelial nitric oxide synthase in diabetic mice, Cardiovasc. Diabetol. 13 (2014) 30.
[21] F.K. Swirski, M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J.L. Figueiredo, R.H. Kohler, A. Chudnovskiy, P. Waterman, et al., Identification of splenic reservoir monocytes and their deployment to inflammatory sites, Science 325 (5940) (2009) 612–616.

[22] Y. Higashi, K. Noma, M. Yoshizumi, Y. Kihara, Endothelial function and oxidative stress in cardiovascular diseases, Circ. J. 73 (3) (2009) 411–418.
[23] J.A. Kim, M. Montagnani, K.K. Koh, M.J. Quon, Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms, Circulation 113 (15) (2006) 1888–1904.
[24] R. Bizios, L. Lai, J.W. Fenton 2nd, A.B. Malik, Thrombin-induced chemotaxis and aggregation of neutrophils, J. Cell. Physiol. 128 (3) (1986) 485–490.
[25] J.I. Borissoff, J.J. Otten, S. Heeneman, P. Leenders, R. van Oerle, O. Soehnlein, S.T. Loubele, K. Hamulyak, T.M. Hackeng, M.J. Daemen, et al., Genetic and pharmacological modifications of thrombin formation in apolipoprotein e-deficient mice determine atherosclerosis severity and atherothrombosis onset in a neutrophil-dependent manner, PLoS One 8 (2) (2013) e55784.
[26] D.N. Derkach, E. Ihara, K. Hirano, J. Nishimura, S. Takahashi, H. Kanaide, Thrombin causes endothelium-dependent biphasic regulation of vascular tone in the porcine renal interlobar artery, Br. J. Pharmacol. 131 (8) (2000) 1635–1642.
[27] X.F. Ming, C. Barandier, H. Viswambharan, B.R. Kwak, F. Mach, L. Mazzolai, D. Hayoz, J. Ruffieux, S. Rusconi, J.P. Montani, et al., Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction, Circulation 110 (24) (2004) 3708–3714.
[28] S.R. Coughlin, Protease-activated receptors in hemostasis, thrombosis and vascular biology, J. Thromb. Haemost. 3 (8) (2005) 1800–1814.
[29] G. Pavic, M. Grandoch, S. Dangwal, K. Jobi, B.H. Rauch, A. Doller, A. Oberhuber, P. Akhyari, K. Schror, J.W. Fischer, et al., Thrombin receptor protease-activated receptor 4 is a key regulator of exaggerated intimal thickening in diabetes mellitus, Circulation 130 (19) (2014) 1700–1711.
[30] P.C. Delekta, I.J. Apel, S. Gu, K. Siu, Y. Hattori, L.M. McAllister-Lucas, P.C. Lucas, Thrombin-dependent NF-kB activation and monocyte/endothelial adhesion are mediated by the CARMA3.Bcl10.MALT1 signalosome, J. Biol. Chem. 285 (53) (2010) 41432–41442.
[31] A. Rahman, K.N. Anwar, A.L. True, A.B. Malik, Thrombin-induced p65 homodimer binding to downstream NF-kappa B site of the promoter mediates endothelial ICAM-1 expression and neutrophil adhesion, J. Immunol. 162 (9) (1999) 5466–5476.
[32] P. Cirillo, V. Angri, S. De Rosa, G. Cali, G. Petrillo, F. Maresca, G.L. D'Ascoli, P. Maietta, L. Brevetti, M. Chiariello, Pro-atherothrombotic effects of leptin in human coronary endothelial cells, Thromb. Haemost. 103 (5) (2010) 1065–1075.
[33] A.J. Donato, G.L. Pierce, L.A. Lesniewski, D.R. Seals, Role of NFkappaB in age-related vascular endothelial dysfunction in humans, Aging (Albany NY) 1 (8) (2009) 678–680.
[34] J.J. Bosnjak, K. Terata, H. Miura, A. Sato, A.C. Nicolosi, M. McDonald, S.A. Manthei, T. Saito, O.A. Hatoum, D.D. Gutterman, Mechanism of thrombin-induced vasodilation in human coronary arterioles, Am. J. Physiol. Heart Circ. Physiol. 284 (4) (2003) H1080–H1086.
[35] D.D. Ku, Coronary vascular reactivity after acute myocardial ischemia, Science 218 (4572) (1982) 576–578.
[36] H. Kataoka, J.R. Hamilton, D.D. McKemy, E. Camerer, Y.W. Zheng, A. Cheng, C. Griffin, S.R. Coughlin, Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells, Blood 102 (9) (2003) 3224–3231.
[37] E.D. Motley, K. Eguchi, M.M. Patterson, P.D. Palmer, H. Suzuki, S. Eguchi, Mechanism of endothelial nitric oxide synthase phosphorylation and activation by thrombin, Hypertension 49 (3) (2007) 577–583.
[38] H.J. Choi, N.E. Kim, J. Kim, S. An, S.H. Yang, J. Ha, S. Cho, I. Kwon, Y.D. Kim, H.S. Nam, et al., Dabigatran reduces endothelial permeability through inhibition of thrombin-induced cytoskeleton reorganization, Thromb. Res. 167 (2018) 165–171.
[39] A. Scridon, A. Marginean, A. Hutanu, L. Chinezu, D. Gheban, M. Perian, A. Vantu, D. Ghertescu, P.C. Fisca, R.C. Serban, et al., Vascular protease-activated receptor 4 upregulation, increased platelet aggregation, and coronary lipid deposits induced by long-term dabigatran administration - results from a diabetes animal model, J. Thromb. Haemost. 17 (3) (2019) 538–550.

Thrombin inhibition by dabigatran attenuates endothelial dysfunction in diabetic mice

ORIGINALITY REPORT

13%	13%	10%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

2%

★ Yutaka Kawabata, Tetsuzo Wakatsuki, Koji Yamaguchi, Daiju Fukuda et al. "Association of Microluminal Structures Assessed by Optical Coherence Tomography With Local Inflammation in Adjacent Epicardial Adipose Tissue and Coronary Plaque Characteristics in Fresh Cadavers", Circulation Journal, 2022

Publication

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		