Thrombin inhibition by dabigatran attenuates endothelial dysfunction in diabetic mice

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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.

Key words; thrombin, endothelial function, dabigatran, inflammation, diabetes

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

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 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/6J 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 hours 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 hours 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 hours.

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 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 hour 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 hour. 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 $P < 0.05$ 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 Figure 1.

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 ($P < 0.001$); however, dabigatran administration significantly ameliorated the impairment of endothelium-dependent vasodilation compared with the non-treated group ($P < 0.001$) (Figure 2A). On the other hand, endothelium-independent relaxation in response to SNP

did not differ between the dabigatran-treated group and non-treated group (Figure 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 (Figure 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 (Figure 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 (Figure 3A). Increase in VCAM-1 and VCAM-1 expression in HUVEC was also confirmed in the protein level (Figure 3B). Thrombin significantly attenuated the phosphorylation of eNOS at Ser1177 in HUVEC ($P < 0.05$). On the other hand, thrombin increased the phosphorylation of IκBα ($P < 0.01$), suggesting activation of the NF-κB pathway in this cell type (Figure 3C). We further examined the effect of thrombin under high glucose condition. High glucose condition promotes 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 (Figure 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 (Figure 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 (Figure 4D). However, thrombin did not alter endothelium-independent vascular relaxation (Figure 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, 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 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 anti-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.

Competing interests

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Figure legends

Figure 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. *; $P < 0.05$ and **; $P < 0.01$. All values are mean ± SEM.

Figure 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 ($P < 0.001$). 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). ††; $P < 0.01$ and †††; $P < 0.001$ vs. non-diabetic control group, and *; $P < 0.05$ and ***; $P < 0.001$ vs. STZ group. Ctrl; non-diabetic control and Dabi; dabigatran. All values are mean ± SEM.

Figure 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 hours 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 = 8). **(C)** The effect of thrombin on the phosphorylation of eNOS and IκBα was examined by western blotting. Thrombin treatment for 60 minutes 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). *; $P < 0.05$ and ***; $P < 0.001$ vs. non-treatment. ††; $P < 0.01$ and †††; $P < 0.001$ vs. thrombin. All values are mean ± SEM.

Figure 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. †††; $P < 0.001$ vs. non-treatment, and *; $P < 0.05$, **; $P < 0.01$ and ***; $P < 0.001$ vs. thrombin-treatment. NT; non-treatment. All values are mean ± SEM.

Table 1. List of PCR primers

	Sense	Antisense
Mouse		
F4/80	5’- TGCATCTAGCAATGGACAGC -3’	5’- GCCTTCTGGATCCATTTGAA -3’
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’

1

Table2. 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<0.001*
Blood glucose, mg/dl	117.6±3.0	528.0±34.9***	533.1±33.0***	*P<0.001*
Triglyceride, mg/dl	26.8±3.0	65.2±11.0**	49.8±9.5	*P<0.01*
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.

; $P < 0.01$ and *; $P < 0.001$ vs. vehicle

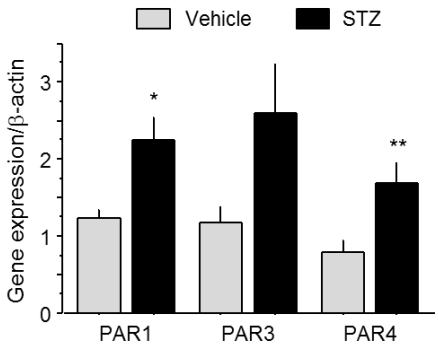

Figure 1

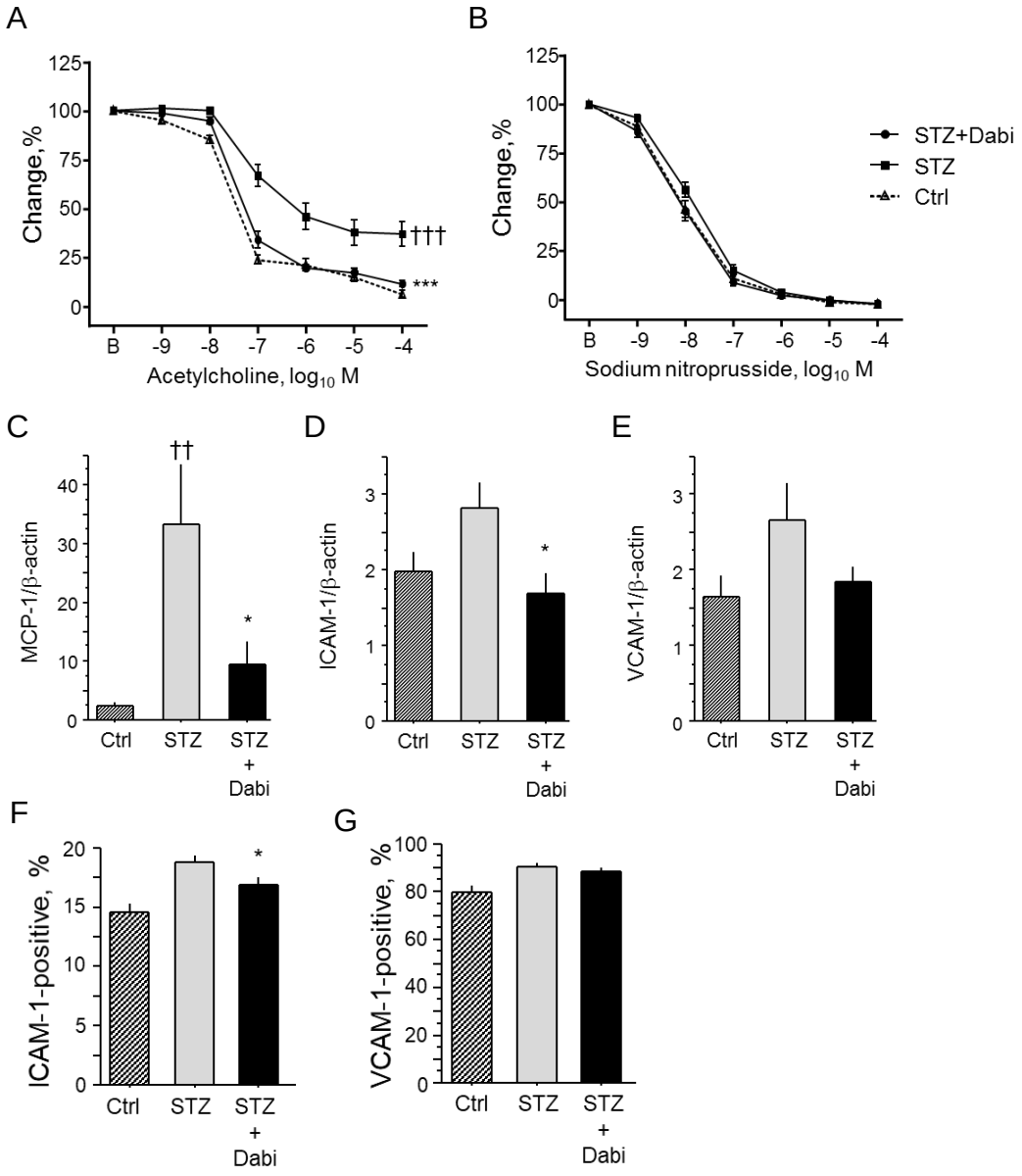
A
B
Change, %
Acetylcholine, log10 M
Sodium nitroprusside, log10 M
STZ+Dabi
STZ
Ctrl
†††
***
C
D
E
MCP-1/β-actin
ICAM-1/β-actin
VCAM-1/β-actin
††
*
F
G
ICAM-1-positive, %
VCAM-1-positive, %
Ctrl
STZ
STZ + Dabi

Figure 2

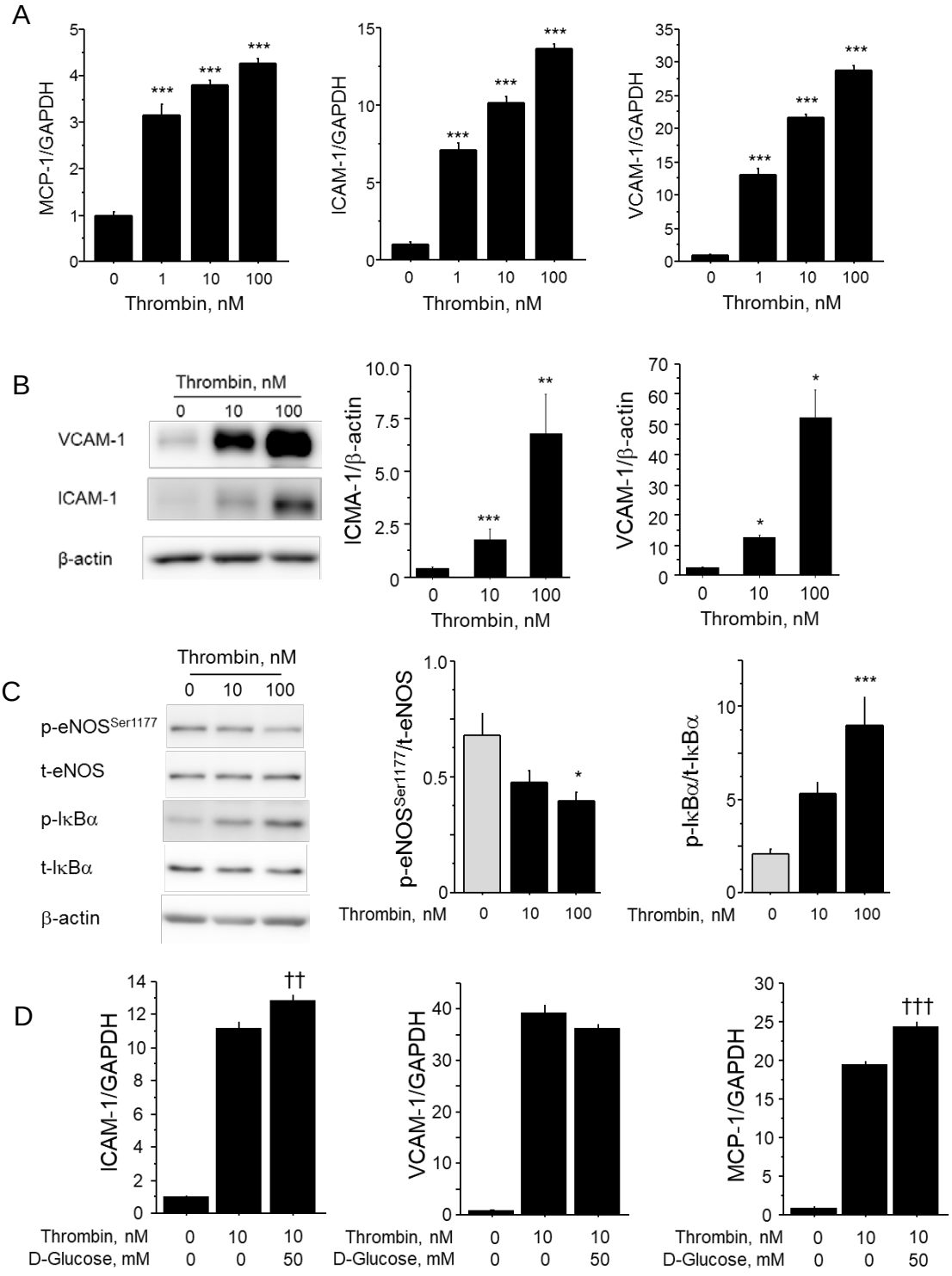
A
MCP-1/GAPDH
ICAM-1/GAPDH
VCAM-1/GAPDH
Thrombin, nM
0
1
10
100
***
B
Thrombin, nM
0
10
100
VCAM-1
ICAM-1
β-actin
ICMA-1/β-actin
VCAM-1/β-actin
**
*
C
p-eNOSSer1177
t-eNOS
p-IκBα
t-IκBα
β-actin
p-eNOSSer1177/t-eNOS
p-IκBα/t-IκBα
D
ICAM-1/GAPDH
VCAM-1/GAPDH
MCP-1/GAPDH
††
†††
Thrombin, nM
D-Glucose, mM

Figure 3

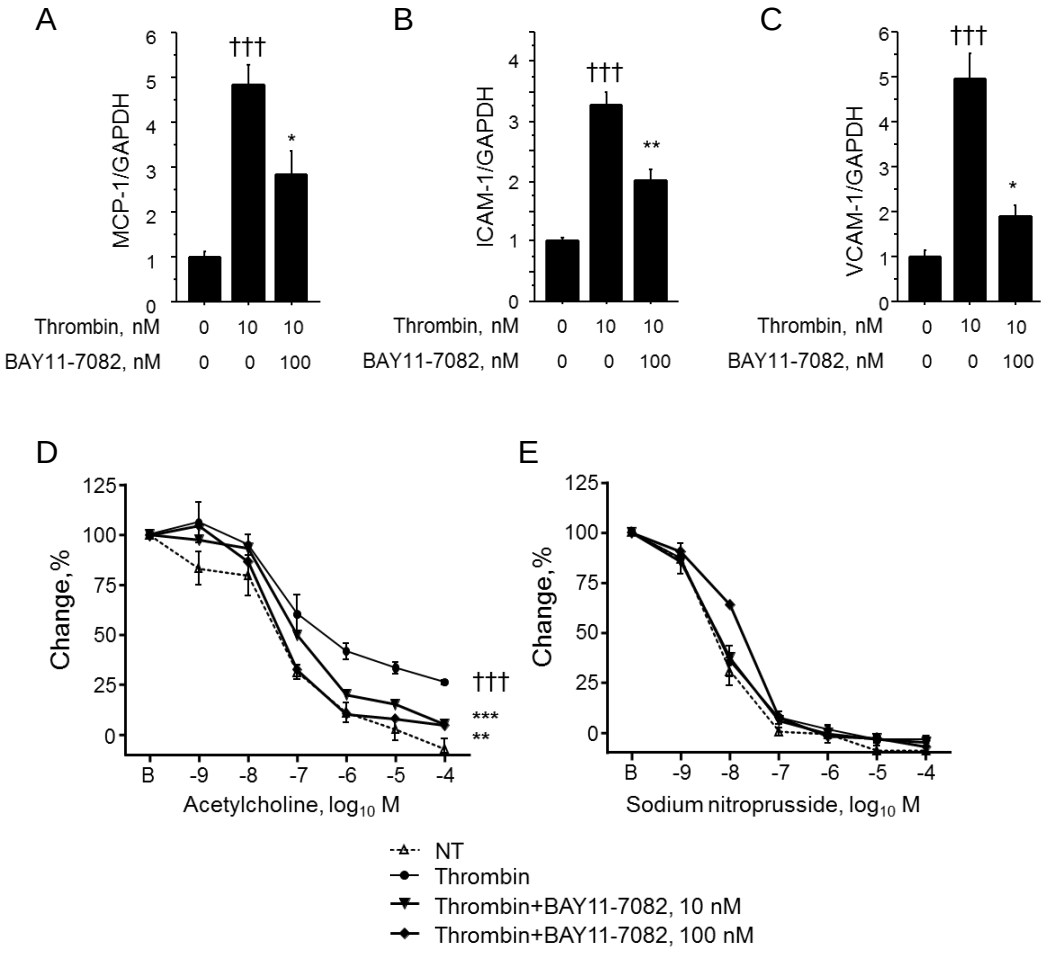
A
MCP-1/GAPDH
†††
*
Thrombin, nM 0 10 10
BAY11-7082, nM 0 0 100
B
ICAM-1/GAPDH
†††
**
Thrombin, nM 0 10 10
BAY11-7082, nM 0 0 100
C
VCAM-1/GAPDH
†††
*
Thrombin, nM 0 10 10
BAY11-7082, nM 0 0 100
D
Change, %
†††
***
**
B -9 -8 -7 -6 -5 -4
Acetylcholine, log10 M
E
Change, %
B -9 -8 -7 -6 -5 -4
Sodium nitroprusside, log10 M
NT
Thrombin
Thrombin+BAY11-7082, 10 nM
Thrombin+BAY11-7082, 100 nM

Figure 4