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Full Paper

Thrombin induces a temporal biphasic vascular response through the differential phosphorylation of endothelial nitric oxide synthase via protease-activated receptor-1 and protein kinase C

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) is a critical regulatory enzyme that controls vascular tone via the production of nitric oxide. Although thrombin also modulates vascular tone predominantly via the activation of protease-activated receptors (PARs), the time course and mechanisms involved in how thrombin controls eNOS enzymatic activity are unknown. eNOS enzymatic activity is enhanced by the phosphorylation of eNOS-Ser1177 and reduced by the phosphorylation of eNOS-Thr495. In this study, we hypothesized that thrombin regulates vascular tone through the differential phosphorylation of eNOS. Using rat descending aorta, we show that thrombin modulates vascular tone in an eNOS-dependent manner via activated PAR-1. We also show that thrombin causes a temporal biphasic response. Protein kinase C (PKC) is associated with second phase of thrombin-induced response. Western blot analysis demonstrated thrombin phosphorylated eNOS-Ser1177 and eNOS-Thr495 in human umbilical vein endothelial cells. A PKC inhibitor suppressed the thrombin-induced phosphorylation of eNOS-Thr495, but not that of eNOS-Ser1177. Our results suggest that thrombin induces a temporal biphasic vascular response through the differential phosphorylation of eNOS via activated PAR-1. Thrombin causes transient vasorelaxation by the phosphorylation of eNOS-Ser1177, and subsequent attenuation of vasorelaxation by the phosphorylation of eNOS-Thr495 via PKC, leading to the modulation of vascular tone.

1. Introduction

The endothelium plays a pivotal roles in regulating vascular tone by synthesizing several factors such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF).[1] In particular, endothelium-derived NO is a critical regulator of vascular homeostasis.[2] Endothelial nitric oxide synthase (eNOS) oxidizes L-arginine to yield NO.[3] The enzymatic activity of eNOS is determined by intracellular calcium concentrations as well as post-translational modification including phosphorylation, palmitoylation, S-glutathionylation, or S-nitrosylation.[4,5] Phosphorylation at Ser1177/1179 (human/bovine), a regulatory site, increases eNOS enzymatic activity, whereas phosphorylation at Thr495/497 (human/bovine) decreased eNOS enzymatic activity.[6] However, little is known about how these two posttranslational modification events may work in concert to regulate eNOS activity and the time-dependent physiological vascular response.

Thrombin, an allosteric multifunctional serine protease, is a key enzyme of the blood coagulation cascade and regulates vascular

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tone.[7] An *ex vivo* study using vascular beds showed that thrombin caused vasorelaxation,[8] whereas another study reported it caused vasoconstriction.[9] Thrombin functions as a signaling molecule via the specific cleavage of protease-activated receptors (PARs) in the cell membrane.[10] Although these changes in vascular tone were shown to be mediated via activated PAR-1,[8,9] the mechanisms involved in the different physiological results in these studies remain to be determined. Thrombin-mediated PAR-1 activation leads to increased intracellular calcium (Ca^{2+}) and protein kinase C (PKC) enzymatic activity.[11] Thus, the activation of PAR-1 controls the phosphorylation levels of eNOS proteins.[12] Indeed, it was reported that activation of PAR-1 resulted in the phosphorylation of eNOS at different sites, Ser1177 and Thr495, and that the time-dependent phosphorylation levels of eNOS-Ser1177 and eNOS-Thr495 were different.[13] This suggests that the different phosphorylation levels of eNOS-Ser1177 and Thr495 might explain the different physiological results.

Here, we provide evidence for a temporal relationship between physiological vascular responses and the posttranslational modification of eNOS by thrombin. In this study, we examined time-dependent vascular responses to thrombin in rat descending aorta as a highly NO-dependent ductal vessel and evaluated the relationship between PKC and thrombin. We also examined the posttranslational modification of eNOS using human umbilical vein endothelial cells (HUVECs). We demonstrate that thrombin causes temporal eNOS-dependent transient vasorelaxation and subsequent attenuation of vasorelaxation via activated PAR-1. We suggest that the mechanism of the thrombin-induced biphasic response is associated with the differential phosphorylation of eNOS. These findings characterizing the thrombin-induced physiological vascular response and posttranslational modification of eNOS might provide a greater understanding of the effects of thrombin on endothelial function.

2. Materials and methods

2.1. Animals

The present study was approved by the Ethics Committee for experimental animals at the Faculty of Medicine, Tottori University (the approval numbers: 18-Y-20) and conducted in compliance with ethical principles of the Declaration of Helsinki. Sprague Dawley male rats (bodyweight 350–410 g; age 12–16 weeks; Japan SLC, Shizuoka, Japan) were housed in a temperature-controlled, 12:12-h light–dark cycle room and had unrestricted access to standard chow (CE-2 purchased from CLEA Japan, Inc., Tokyo, Japan) and water. The environmental conditions were maintained at a specific pathogen-free grade. The total number of animals used in this study was 26.

2.2. Reagents

Thrombin (from human plasma) was purchased from Sigma–Aldrich (St Louis, MO). Phenylephrine (PE) was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Gö6983, vorapaxar, and ML-354 were purchased from Cayman Chemical (Ann Arbor, MI). Acetylcholine (ACh) and L-NG-Nitroarginine methyl ester (L-NAME) were purchased from Nacalai Tesque (Kyoto, Japan). Antibodies against eNOS-Thr495 (cat. no. 9574) and eNOS-Ser1177 (cat. no. 9571) were purchased from Cell Signaling Technology Danvers, MA. Antibodies against total eNOS (cat. no. sc-376,751 HRP) and β-actin (cat. no. sc-8432 HRP) were purchased from Santa Cruz Biotechnology (Dallas, TX).

2.3. Vascular experiments

Animals were anesthetized with isoflurane (2.5%), and heparin (500 U/kg) was administered via jugular vein to prevent clotting. The thoracic descending aorta, a highly NO-dependent conduit vessel,[14] was quickly excised. Isolated thoracic aortas were cleaned of connective tissue with special care to avoid endothelium damage and were cut into rings (2–3 mm in length). The aortic rings were mounted isometrically in a tissue bath between a stationary stainless-steel hook and an isometric transducer (Easy Magnus System UC-5A, Iwashiya Kishimoto Medical Instruments, Kyoto, Japan) coupled to an AD converter Lab-Chart (ADInstruments, Sydney, Australia). Aortic rings were equilibrated for at least 60 min at a stable resting tension of 1 g in an organ bath at 37 °C by constant bubbling with a gas mixture of 95% O_2 and 5% CO_2 and containing 5 mL of modified Krebs Henseleit buffer solution consisting of (in mM): 118 NaCl, 25 $NaHCO_3$, 3.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 10 D-glucose (pH 7.4). Vascular reactivity was examined by recording isometric tension. After the maximal and submaximal vasoconstrictive doses of PE were determined and the intact vessels with functional endothelium were defined as the vessels with the maximal relaxation response to ACh more than 85% against the pre-constriction with the maximal constriction dose of PE (1 μM), the subsequent experiments to test the vasorelaxant effects of thrombin were performed against the pre-constriction with the submaximal vasoconstrictive dose of PE (0.3 μM). Then, the relaxation effects were expressed as percentages against the pre-constriction with the submaximal vasoconstrictive dose of PE. We constructed time-response and concentration–response curves for thrombin. To determine the mechanism of thrombin-induced vascular responses, rings were exposed to specific inhibitors or antagonists for 15 min before the addition of PE (0.3 μM). The concentration of specific inhibitors or antagonists used in this study were determined based on their 50% inhibitory concentration.[15–17] To evaluate the endothelium-dependent effects of thrombin on vascular responses, denuded vessels, in which the endothelial layer was removed by gently rubbing the luminal surface with cotton swabs, were used.

2.4. Cell culture

HUVECs were obtained from PromoCell GmbH (Heidelberg, Germany) and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Thermo Fisher Scientific, Waltham, MA)/10% fetal bovine serum (FBS, Gibco BRL) at 37 °C in a 5% CO_2 incubator. Cells from passage 4–6 were grown to approximately 90% confluency and were used for all experiments. Experiments were performed in 6-well plates. The cells were starved by serum deprivation for 2 h in DMEM. After starvation, cells were stimulated with thrombin (1000 mU/ml). The cells were exposed to specific inhibitors for 15 min before the application of thrombin or were untreated.

2.5. Western blotting

Protein lysates were isolated from HUVECs using RIPA buffer (Nacalai Tesque) containing a protease inhibitor cocktail (cOmplete™, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma–Aldrich). Protein quantification was determined using the bicinchoninic acid method. Proteins were separated by SDS–PAGE and electro-transferred to PVDF membranes, which were removed and blocked with PVDF Blocking reagent for Can Get Signal® (purchased from TOYOBO, Osaka, Japan). The expressions of total and phospho-proteins were

measured in HUVECs. Antibodies against eNOS-pSer1177/Thr495 (1:1000 dilution), eNOS (1:4000 dilution), and β-actin (1:2000 dilution) diluted with Can Get Signal®. The membrane was incubated with primary antibodies at 4 °C overnight. The membrane was incubated with secondary antibodies (Cell Signaling Technology) and visualized with enhanced chemiluminescence (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific). The exposure images were captured and analyzed by the ChemiDoc MP imaging system (Bio-Rad, Tokyo, Japan). Intensities of bands were quantified using NIH ImageJ Software.

2.6. Statistical analysis

All data are presented as the mean ± SEM. Statistical analyses were performed using R software (version 3.4.0; The R foundation for Statistical Computing, Vienna, Austria). Statistical significance between two groups was assessed by the Student's *t*-test ($\alpha = 0.05$). Statistical significance among multiple groups was assessed by one-way analysis of variance and Bonferroni test at a significance level of 5%. To analyze the effect of inhibitors or antagonists on the time response curve of thrombin, two-way repeated measures ANOVA was performed, and a P-value < 0.05 was considered statistically significant. To analyze the trend for the phosphorylation levels of eNOS depending on time, Jonckheere-Terpstra trend test was performed, and a P-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of thrombin on isolated aortic rings

We confirmed that thrombin caused no vasoconstriction in both intact and endothelium-denuded aortic rings. Therefore, to determine the effect of thrombin on vascular tone, the aortic rings were exposed to different concentrations of thrombin (10–3000 mU/mL) after submaximal vasoconstriction by PE (0.3 μM). Thrombin (1000 mU/mL) after submaximal vasoconstriction by phenylephrine (0.3 μM) caused transient vasorelaxation, followed by a gradual return of vascular tone to control levels (time-response curve shown in Fig. 1A and concentration–response curve in Fig. 1B). These thrombin-induced vascular responses were eliminated by endothelium denudation or pre-treatment with L-NAME (100 μM), a NOS inhibitor (Fig. 2). Thrombin-induced vascular responses were significantly reduced by vorapaxar (1 μM),[15] a PAR-1 inhibitor, but not ML-354 (300 nM),[16] a PAR-4 inhibitor, compared with controls (P < 0.001) (Fig. 2). When analyzed at each time point, compared with controls, thrombin-induced vascular responses were significantly reduced by vorapaxar at the time points of 40–180 s (P < 0.001) and by ML-354 at those of 40–60 s (P < 0.01). Pre-treatment with Gö6983 (1 μM),[17] a PKC inhibitor, significantly increased vasorelaxation and decreased attenuation of vasorelaxation in thrombin-induced vascular responses compared with controls (time response curve shown in Fig. 3A; P < 0.001). In the presence of Gö6983 (1 μM), thrombin-induced maximal vasorelaxation was significantly greater than that in controls (Fig. 3B). Moreover, subsequent thrombin-induced attenuation of vasorelaxation was significantly slowed as compared with controls, whereas the transient vasorelaxation time was not significantly changed (Fig. 3C).

3.2. Thrombin induces the phosphorylation of eNOS in HUVECs

We performed *in vitro* experiments using HUVECs to examine the effects of thrombin on the phosphorylation of eNOS. The

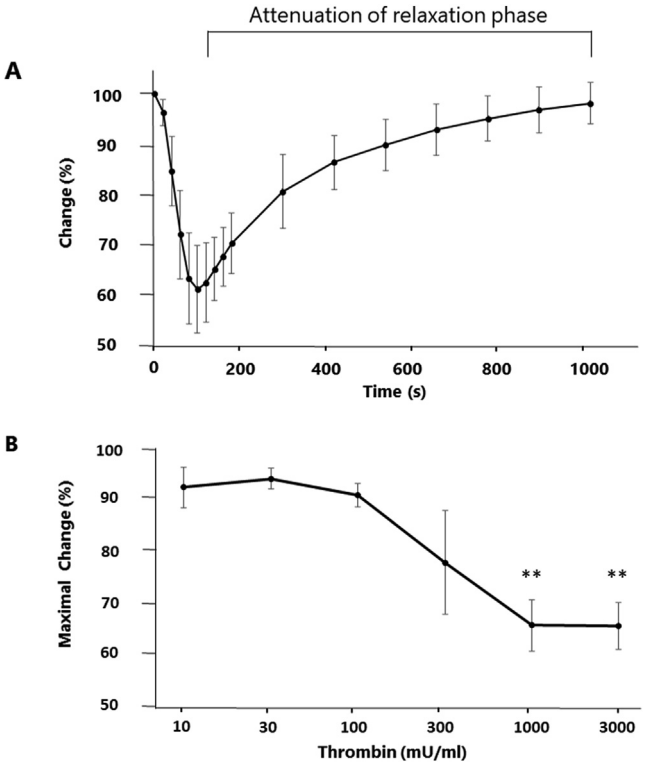


Fig. 1. The vascular response to thrombin after submaximal vasoconstriction induced by phenylephrine (PE). A: Time response curve of thrombin (1000 mU/ml) after submaximal vasoconstriction by PE (0.3 μM) (n = 10). Thrombin reproducibly caused transient vasorelaxation, followed by a gradual return of vascular tone to control levels (subsequent attenuation of vasorelaxation). B: Concentration response curve of thrombin (10–3000 mU/mL) after submaximal vasoconstriction by PE (n = 4). Thrombin-induced maximal vasorelaxation was saturated at a concentration of 1000 mU/ml. The sampling intervals were every 20 or 120 s **P < 0.001 versus a concentration of 10 mU/mL.

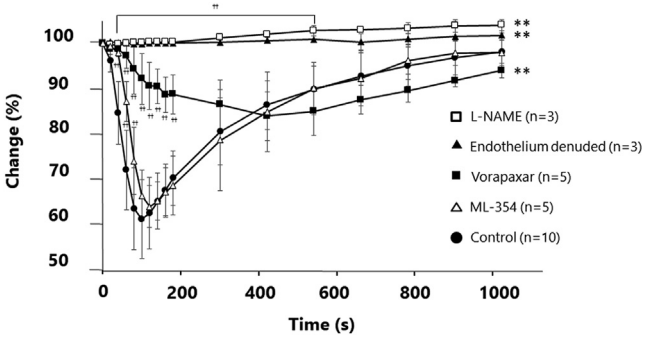
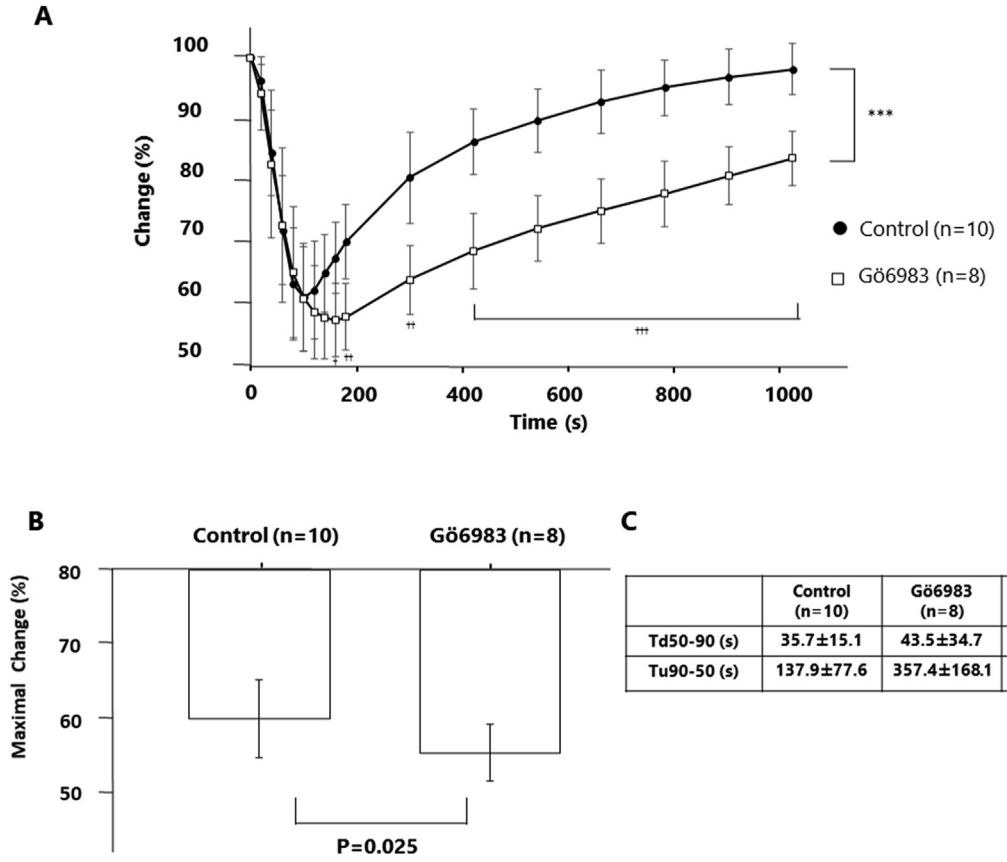


Fig. 2. The effects of removing endothelium or that of pretreatment with L-NAME, vorapaxar, or ML-354 on vascular responses to thrombin dependent on time. L-NAME, vorapaxar, or ML-354 were applied 15 min before the application of PE (0.3 μM), and then the application of thrombin (1000 mU/ml) after submaximal vasoconstriction by PE. Thrombin-induced biphasic responses were eliminated by endothelium denudation or pre-treatment with L-NAME (100 μM), a NOS inhibitor compared with control (P < 0.001, repeated measures of ANOVA). Both endothelium denudation and pre-treatment with L-NAME were significantly different compared with controls, from 40 to 540 s (P < 0.001, one-way ANOVA). Thrombin-induced vascular responses were significantly reduced in the presence of vorapaxar (1 μM), a PAR-1 inhibitor, but not ML-354 (300 nM), a PAR-4 inhibitor, compared with controls (P < 0.001, repeated measures of ANOVA). Vorapaxar was significantly different compared with controls from 40 to 180 s (P < 0.001, one-way ANOVA). ML-354 was significantly different compared with controls at 40 and 60 s (P < 0.01, one-way ANOVA). **P < 0.001 versus controls. ††P < 0.001 versus controls at each time point.



	Control (n=10)	Gö6983 (n=8)	P value
Td50-90 (s)	35.7±15.1	43.5±34.7	0.53
Tu90-50 (s)	137.9±77.6	357.4±168.1	0.002

Fig. 3. The effects of pretreatment with Gö6983, a PKC inhibitor, on vascular responses to thrombin. Gö6983 (1 μM) were applied 15 min before the application of PE (0.3 μM), and then the application of thrombin (1000 mU/ml) after submaximal vasoconstriction by PE. A: Time response curve in the presence of Gö6983. Gö6983 significantly increased vasorelaxation and decreased attenuation of vasorelaxation in thrombin-induced vascular responses compared with controls ($P < 0.0001$, repeated measures of ANOVA). Moreover, Gö6983 was significantly different compared with controls, from 160 to 1020 s ($P < 0.01$ at 160 s, $P < 0.001$ at 180 and 300 s, $P < 0.0001$ from 420 to 1020 s). B: Maximal vasorelaxation change by pretreatment with Gö6983. Thrombin-induced maximal vasorelaxation was significantly greater ($P = 0.025$) than that in controls. C: Time analysis of thrombin-induced vasorelaxation and subsequent attenuation of vasorelaxation by pretreatment with Gö6983. We further evaluated the time-dependent, biphasic relaxation response to thrombin. In detail, we analyzed the initial downslope phase of vasorelaxation responses to thrombin by measuring the time intervals (Td50-90) between the half-maximal and the 90% maximal relaxation points in the downslope response. Likewise, as for the subsequent upslope phase of attenuated vasorelaxation responses to thrombin, those (Tu90-50) between the 90% maximal relaxation and the half-maximal points in the upslope response were evaluated. In the vasorelaxation phase, the duration of Td50-90 was not significantly different, whereas in the attenuation of vasorelaxation phase, the duration of Tu90-50 was significantly longer than that of controls ($P = 0.002$). *$P < 0.01$ versus controls. **$P < 0.001$ versus controls. ***$P < 0.0001$ versus controls. †$P < 0.01$ versus controls at each time point. ††$P < 0.001$ versus controls at each time point. †††$P < 0.0001$ versus controls at each time point.

phosphorylation of eNOS at both regulatory sites (Thr495 and Ser1177) was measured by western blotting. To confirm the effect of PKC activation on the phosphorylation of eNOS by thrombin, we pretreated HUVECs with a selective PKC inhibitor, Gö6983 (1 μM) for 15 min prior to stimulation with thrombin (1000 mU/ml). The phosphorylation levels of eNOS-Thr495 showed a significant increasing trend by thrombin (1000 mU) ($P < 0.05$, Jonckheere-Terpstra trend test) and those at the time point of 1 min was significantly increased compared with those at the time point of 0 min (Fig. 4A and C). On the other hand, the increasing trend of phosphorylation levels of eNOS-Thr495 by thrombin was abolished by the pre-treatment of Gö6983 (1 μM) and, at the time point of 1 min, there was a significant reduction of the phosphorylation levels of eNOS-Thr495 in the Gö6983 group compared with the control group (Fig. 4C). Contrary to the trend observed at eNOS-Thr495 in the control, there was no significant trend of the phosphorylation levels of eNOS-Ser1177 by thrombin while a significant increase of those was observed at the time point of 1 min compared with 0 min (Fig. 4B and D). There were no significant differences in the phosphorylation levels of eNOS-Ser1177 between the control and Gö6983 groups (Fig. 4D).

4. Discussion

The main objective of our study was to determine the temporal physiological vascular response induced by thrombin. We found that: (1) thrombin caused a temporal biphasic vascular response (transient vasorelaxation and subsequent attenuation of vasorelaxation); (2) these temporal biphasic responses were dependent on NOS and activated PAR-1; (3) PKC was associated with subsequent thrombin-induced attenuation of vasorelaxation; and (4) the mechanism of temporal biphasic responses to thrombin was the differential phosphorylation of eNOS-Ser1177 and eNOS-Thr495. These results suggest that thrombin induces a temporal biphasic vascular response through the differential phosphorylation of eNOS dependent on time. Subsequent thrombin-induced attenuation of vasorelaxation was caused by the phosphorylation of eNOS-Thr495 via PKC.

eNOS is a critical regulatory enzyme that controls vascular tone via the production of NO.[18] Thrombin also modulates vascular tone, predominantly through the activation of PARs.[19] Therefore, this suggests thrombin may affect the function of eNOS via PARs. According to some reports, PAR-1 and PAR-4 are directly activated by

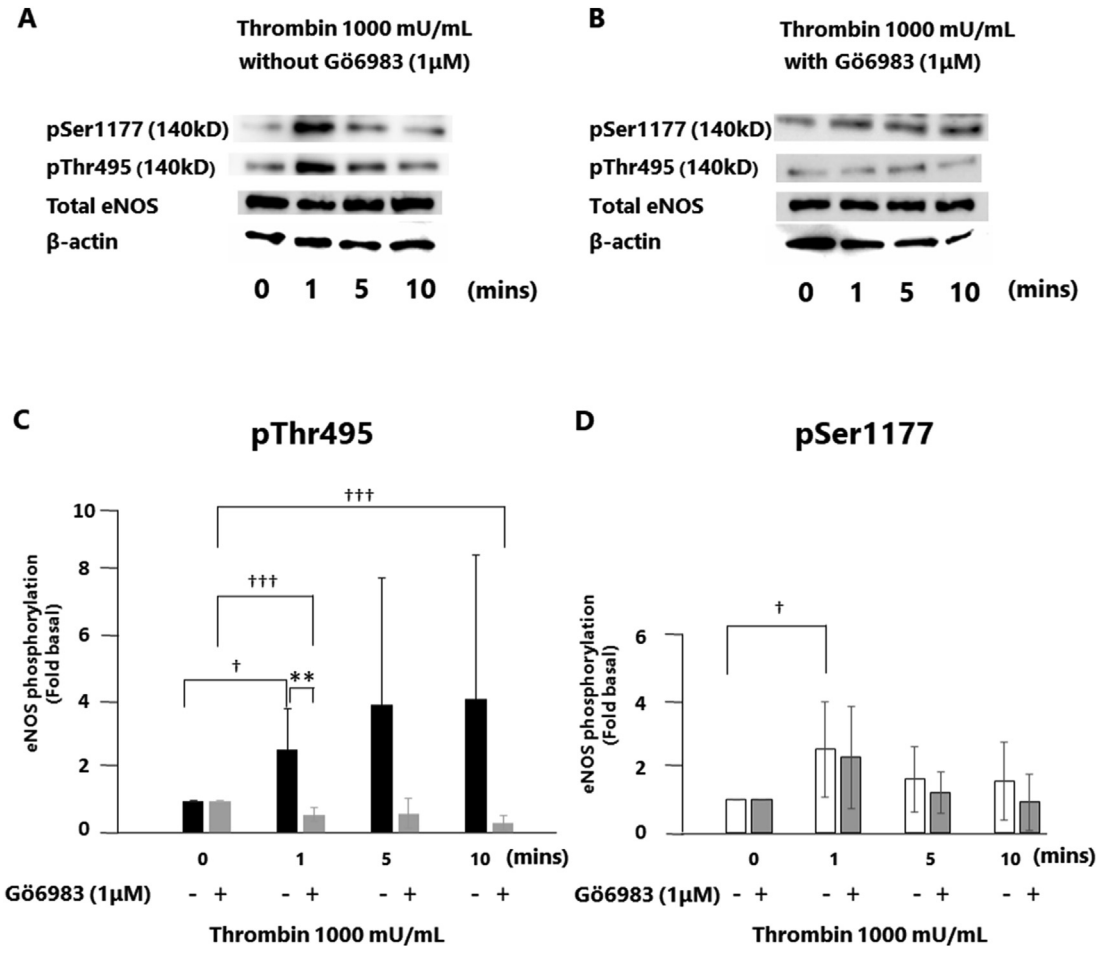


Fig. 4. The effects of thrombin on the phosphorylation of eNOS in HUVECs. The cells were exposed to Gö6983 (1 μM), a specific PKC inhibitor, for 15 min before the application of thrombin or were untreated. A, B: Representative images of experiments using Gö6983 or not. Thrombin (1000 mU/ml) stimulated phosphorylation at eNOS-Thr495 and eNOS-Ser1177 (A). Gö6983 inhibited the phosphorylation of eNOS-Thr495 (B). C, D: Thrombin (1000 mU/ml) significantly phosphorylated eNOS-Thr495 and eNOS-Ser1177 at the 1-min time points compared with 0-min (n = 6). Gö6983 significantly inhibited the phosphorylation levels of eNOS-Thr495 at 1-min time points by stimulation with thrombin compared with controls (n = 6) (C). Moreover, in thrombin-treated cells, Gö6983 significantly reduced the phosphorylation levels of eNOS-Thr495 at 1-min and 10-min time points compared with those at 0 min (C). On the other hand, in Gö6983-pretreated cells, there was no significant change in the phosphorylation levels of eNOS-Ser1177 at each timepoint compared with controls (n = 6) (D). **P < 0.001 between Gö6983-treated and -untreated groups. †P < 0.05 versus at 0 min in Gö6983-untreated groups. †††P < 0.0001 versus at 0 min in Gö6983-treated groups.

thrombin.[10] On the other hand, PAR-2 can respond to thrombin only through transactivation by PAR-1.[20] Similarly, PAR-3 functions as a cofactor for PAR4 activation.[21] In this study, to investigate the direct function of thrombin on vascular response, we examined roles of PAR-1 and PAR-4, but not PAR-2 or PAR-3. Indeed, our study showed that thrombin modulated vascular tone in an eNOS-dependent manner via activation of mainly PAR-1. We also showed that thrombin caused a temporal biphasic response (transient vasorelaxation and subsequent attenuation of vasorelaxation). This biphasic response was observed similarly in another study using renal interlobar arteries.[22] Although their report demonstrated a concentration-dependency,[22] we showed a concentration-independent mechanism. The concentration-dependency of vascular responses to thrombin may differ depending upon the type of vessel. Vascular relaxation is regulated by eNOS-derived NO and eNOS-independent EDHF.[23] Endothelium-dependent relaxation is predominantly regulated by NO in large conduit arteries and by EDHF in small resistance vessels.[14] Indeed, there are inconsistencies in the vascular response to thrombin in arteries other than large conduit arteries. For example, thrombin caused vasorelaxation in coronary arteries,[8,24,25] and vasoconstriction in pulmonary or cerebral arteries.[9,26] These differences in studies might reflect a relatively stronger effect of eNOS-independent EDHF than NO. In our study, to minimize the effects of EDHF on vascular relaxation, we used the thoracic descending aorta. We demonstrated a temporary biphasic response to thrombin using a vascular bed that has relatively high NO dependence. However, how thrombin affects EDHF is unknown. Thus, further studies are required to examine the relationship between thrombin and EDHF.

The enzymatic activity of eNOS is determined by the intracellular calcium concentration and phosphorylation of eNOS.[27,28] Elevated intracellular calcium levels activate eNOS activity by binding to calmodulin (CaM).[29] The phosphorylation of eNOS-Ser1177 enhances eNOS enzymatic activity whereas the phosphorylation of eNOS-Thr495 reduces eNOS activity.[6] The temporal biphasic vascular response to thrombin observed in the current study may be explained by the temporal differences in these two phosphorylation events. In our *ex vivo* study, inhibition of PKC slowed thrombin-induced attenuation of vasorelaxation, suggesting PKC contributes to attenuation of vasorelaxation through decreased eNOS enzymatic activity. Indeed, our *in vitro* experiments using HUVECs showed that a PKC inhibitor suppressed the thrombin-induced phosphorylation of

eNOS-Thr495, but not that of eNOS-Ser1177. These results suggest that thrombin induces vasorelaxation by the activation of CaM and the phosphorylation of eNOS-Ser1177, and that the subsequent attenuation of vasorelaxation is promoted by the phosphorylation of eNOS-Thr495 via PKC. In agreement with our results, thrombin was reported to elevate intracellular calcium levels by increasing the influx of extracellular Ca^{2+} and the intracellular release of Ca^{2+} in endothelial cells.[30] Moreover, although the phosphorylation of eNOS-Thr495 was not shown, Motley et al. reported an *in vitro* study where thrombin caused a rapid and transient phosphorylation of eNOS-Ser1177 by a Ca^{2+}-dependent mechanism.[31] Although the PKC-dependent phosphorylation of eNOS-Thr495 has not been reported, the same group showed the *in vitro* phosphorylation of eNOS-Ser1177 via activated PAR1 by PAR1 agonists peaked at 0.5 min, whereas that of eNOS-Thr495 peaked at 2 min, indicating the phosphorylation of eNOS-Ser1177 and eNOS-Thr495 are temporally different.[13] Furthermore, although temporal changes in the phosphorylation of eNOS-Thr495 have not been demonstrated, PKC activated by VEGF was reported to phosphorylate eNOS-Thr495 *in vitro*.[32] Collectively, these suggest that thrombin-induced biphasic responses are caused by the differential phosphorylation of eNOS: i.e. thrombin-induced transient vasorelaxation is associated with an increase in eNOS enzymatic activity mediated by CaM and/or the phosphorylation of eNOS-Ser1177, and the subsequent thrombin-induced attenuation of vasorelaxation is mediated by a decrease in eNOS enzymatic activity by the phosphorylation of eNOS-Thr495 via PKC.

Endothelial dysfunction is a hallmark of cardiovascular diseases (CVD) associated with pathological conditions involving vasoconstriction, thrombosis, and inflammation.[33,34] A hypercoagulable state, such as the overproduction of thrombin, also increases the risk of thrombosis or CVD.[35,36] Although endothelial dysfunction and the overproduction of thrombin lead to CVD, the mechanism of how thrombin reduces endothelial function is poorly understood. It was reported that the phosphorylation of eNOS-Thr495 might trigger eNOS uncoupling,[37,38] which results in endothelial dysfunction. We demonstrated that thrombin caused the PKC-mediated phosphorylation of eNOS-Thr495 via activated PAR1. The phosphorylation of eNOS-Thr495 may be an intrinsic switch mechanism that determines whether eNOS generates NO or reactive oxygen species (ROS).[38] Collectively, a hypercoagulable state with enhanced thrombin may induce eNOS uncoupling or production of ROS through the PKC-mediated phosphorylation of eNOS-Thr495, ultimately leading to CVD. Furthermore, PKC inhibitors may prevent endothelial dysfunction in hypercoagulable states. However, because this was an acute phase study, further studies are required to examine the mechanism of how thrombin induces endothelial dysfunction.

In conclusion, thrombin induces temporal biphasic vascular responses through the differential phosphorylation of eNOS via activated PAR1. Thrombin causes transient vasorelaxation by the phosphorylation of eNOS-Ser1177 and subsequent attenuation of vasorelaxation by eNOS-Thr495 via PKC, leading to the modulation of vascular tone. A hypercoagulable state may promote eNOS uncoupling through the PKC-mediated phosphorylation of eNOS-Thr495.

Declaration of competing interest

The authors indicated no potential conflicts of interest.

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