

学位論文

Thrombin-Induced Responses Via Protease-Activated Receptor 1 Blocked by  
the Endothelium on Isolated Porcine Retinal Arterioles

(豚摘出網膜動脈におけるプロテイナーゼ活性型受容体1を介したトロンビン起  
因性の反応は血管内皮によりブロックされる)

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**Thrombin-Induced Responses Via Protease-Activated Receptor 1 Blocked  
by the Endothelium on Isolated Porcine Retinal Arterioles**

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## ABSTRACT

*Purpose:* Thrombin, a serine protease, causes organ-specific responses to vessels. However, the mechanism by which thrombin affects the retinal microcirculation remains unclear. We examined the effects of thrombin on the retinal microvasculature and signaling mechanisms.

*Methods:* Porcine retinal arterioles were isolated, cannulated, and pressurized (55 cmH<sub>2</sub>O) without flow in this in vitro study. Videomicroscopy techniques recorded changes in diameter in the retinal arterioles in response to thrombin at concentrations ranging from 0.001 to 20 mU/ml.

*Results:* Extraluminal administration of thrombin induced concentration-dependent vascular responses, i.e., vasoconstriction at low concentrations less than 5 mU/ml and vasorelaxation with high concentrations greater than 5 mU/ml. However, intraluminal administration of thrombin (5 mU/ml) did not constrict the retinal arterioles; in denuded vessels, intraluminal administration constricted the retinal arterioles. Thrombin-induced vasoconstriction was significantly ( $p < 0.01$ ) suppressed by pretreatment with a protein kinase C (PKC) inhibitor and a protease-activated receptor (PAR)-1 inhibitor but not by PAR-2 and PAR-4 inhibitors or denudation. A rho kinase (ROCK) inhibitor also

suppressed thrombin-induced vasoconstriction (5 mU/ml) compared with sodium nitroprusside. Endothelial denudation and pretreatment with an endothelial nitric oxide (NO) synthase inhibitor suppressed vasorelaxation caused by a high concentration of thrombin.

*Conclusions:* A low concentration of thrombin causes vasoconstriction of smooth muscles via PAR-1, PKC, and ROCK, and a high concentration of thrombin possibly causes vasorelaxation of the retinal arterioles via nitric oxide synthase activation in the endothelium. The vascular endothelium might block signaling of thrombin-induced vasoconstriction in the retinal arterioles when administered intraluminally.

**KEYWORDS**

thrombin, protease-activated receptor 1, PAR-1, vasoconstriction, vasorelaxation, endothelium blood retinal barrier

## Introduction

Thrombin, a serine protease, plays an important role in hemostasis by converting fibrinogen into fibrin. Many studies have reported the responses of the vascular system to thrombin. Thrombin causes vasodilation of the coronary (pig, dog, and human)<sup>1-4</sup> and pulmonary (newborn pig) arteries<sup>5</sup> and vasoconstriction of the pulmonary (pig)<sup>6</sup> and cerebral (dog) arteries.<sup>7,8</sup> In the porcine renal interlobar artery, thrombin also causes vasorelaxation at low concentrations and vasoconstriction at high concentrations.<sup>9</sup> Taken together, thrombin causes various responses depending on the vessels in the various organs. Moreover, thrombin mediates various vascular effects via protease-activated receptor (PAR).<sup>10</sup> Since PAR is expressed in the vascular endothelium and smooth muscle,<sup>11-15</sup> the vascular reactivity of thrombin might be induced via PAR on the vessel wall.

Thrombin causes vasoconstriction of bovine retinal arterioles.<sup>16</sup> In addition, thrombin catalyzes the generation of a variety of cytokines, chemokines, and growth factors as a result of stimulating PAR-1 in human retinal pigment epithelial cells,<sup>17,18</sup> and intravitreal thrombin activity is elevated in ocular disorders.<sup>19-22</sup> Based on these factors, elevated serum and intravitreal thrombin concentrations might contribute to the pathophysiology of ocular

disorders via activation of PAR. However, the role of PAR in thrombin-induced vasomotion of the retinal arterioles remains unclear.

Because blood only flows in the vascular lumen under normal physiologic conditions, thrombin can cause a direct response on the endothelium. In most studies, the effects of thrombin on the endothelium have been evaluated in cultured endothelial cells, i.e., thrombin increases production of nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>), potent vasodilators of retinal arterioles, in cultured endothelial cells.<sup>3,23</sup> However, extraluminal administration of thrombin also causes vasoconstriction of the retinal arterioles.<sup>16</sup> Thus, the intraluminal effects of thrombin might differ from the extraluminal effects of thrombin on the endothelium of the retinal arterioles. The current study examined the intraluminal and extraluminal effects of thrombin and investigated the signaling mechanisms via PAR involved in the vasomotor activity of the retinal microvasculature. We hypothesized that only extraluminal administration of thrombin can cause the response and not intraluminal administration, because the permeability of the inner blood-retinal barrier (BRB) to molecules is below 1 kDa<sup>24</sup> and the molecular weight (MW) of thrombin is 33.6 kDa.

## **Material and methods**

### *Animal preparation*

The Animal Care Committee of Asahikawa Medical University approved all animal protocols, which were conducted according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Eyeballs from white pigs of either sex (age, 22-24 weeks; weight, 100-150 kg) were obtained from local slaughterhouses and transported to the laboratory immediately in a humid chamber on ice.

### *Isolation and cannulation of microvessels*

The procedures used to identify, isolate, cannulate, pressurize, and visualize the retinal vasculature have been described previously.<sup>25-28</sup>

One retinal arteriole was isolated in each porcine eye and cannulated, and the pressure in the lumen was increased to 55 cmH<sub>2</sub>O without flow.<sup>29</sup> After stable basal tone developed without any precontractors, the changes in the vascular diameter in response to pharmacologic agents were recorded using videomicroscopy techniques.<sup>25</sup>

***Experimental protocol***

Porcine retinal arterioles were bathed in physiologic saline solution with albumin (PSS-albumin, 0.1%) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl<sub>2</sub>, 1.17 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM ethylenediaminetetraacetic acid (EDTA), and 3.0 mM 3-(N-morpholino) propanesulfonic acid maintained at a 36°C to 37°C to allow development of basal tone for 30 to 40 minutes.

In previous studies using our methods regarding the intravitreal concentration of thrombin in ocular disorders,<sup>19-22</sup> we assessed the response of the retinal arterioles to extraluminally administered thrombin at concentrations ranging from 0.001 to 20 mU/ml within 15 minutes. In the first series of studies, we compared the thrombin-induced vasomotor activity between intraluminal and extraluminal administration. We performed intraluminal administration as described previously.<sup>28</sup> In the second series of studies, to determine the participation of thrombin receptor subtypes, the vessels were pretreated with PAR-1, -2, and -4, and the specific thrombin antagonists RWJ 56110 (10 μM PAR-1),<sup>30</sup> FSLLRY-NH<sub>2</sub> (10 μM PAR-2),<sup>31</sup> tcY-NH<sub>2</sub> (1 μM PAR-4),<sup>32</sup> and hirudin (50 mATU/ml), the specific



thrombin antagonist (twice the thrombin concentration).<sup>9</sup> The vessels were incubated with respective blockers for 30 minutes, and to determine the response of the retinal arterioles to extraluminally administered thrombin at concentrations ranging from 0.001 to 20 mU/ml.

In the third series of studies, to determine the role of the endothelium in the

thrombin-induced responses of the retinal vessels, we removed the endothelium using previously reported techniques.<sup>33</sup> Briefly, the vessels were filled with nonionic detergent,

CHAPS (0.4%) intraluminally, for 1 to 2 minutes to destroy the endothelial cells. We then assessed the response of the retinal arterioles to thrombin at concentrations ranging from

0.001 to 20 mU/ml. The specific inhibitors of NO synthase and hydrogen peroxide (10  $\mu$ M NG-nitro-L-arginine methyl ester [L-NAME]),<sup>25,26,34</sup> cyclooxygenase and hydrogen

peroxide (10  $\mu$ M indomethacin),<sup>26,35,36</sup> and Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels and

endothelium-derived hyperpolarizing factor (EDHF) (combination of 0.1  $\mu$ M apamin and

0.1  $\mu$ M charybdotoxin [ChTX] 0.1  $\mu$ M)<sup>37-40</sup> were used to assess the contributions of NO,

PGI<sub>2</sub>, and K<sub>Ca</sub>. In the final series of studies, to assess the contributions of extracellular Ca<sup>2+</sup>,

protein kinase C (PKC), and Rho-associated protein kinase (ROCK), factors important for

vascular smooth muscle contractions, to thrombin-induced vasoactivity, Ca<sup>2+</sup>-free PSS, the

broad-spectrum PKC inhibitor Gö-6983 (3  $\mu\text{M}$ ),<sup>41</sup> or the non-selective ROCK inhibitor H1152 (10  $\mu\text{M}$ )<sup>41</sup> was administered. Gö-6983 was administered using the same methods as in the second study. In the study of extracellular  $\text{Ca}^{2+}$  and ROCK, after  $\text{Ca}^{2+}$ -free PSS or H1152 was administered, the resting diameter changes over 20 minutes were recorded. After establishing a new stable tone with  $\text{Ca}^{2+}$ -free PSS or H1152, these vessels were exposed to thrombin (5 mU/ml) and the changes in the vascular diameter were monitored for another 15 minutes. Because  $\text{Ca}^{2+}$ -free PSS and H1152 reduce vascular tone, in another set of experiments, for comparison, the vessels were incubated with the endothelium-independent vasodilator sodium nitroprusside (SNP) (30  $\mu\text{M}$ ) for 20 minutes before administration of thrombin. Arterioles with tone were exposed first to thrombin (5 mU/ml) for 15 minutes to establish stable vasoconstriction and then incubated with  $\text{Ca}^{2+}$ -free PSS or H1152 (10  $\mu\text{M}$ ) for another 20 minutes.

### ***Chemicals***

Thrombin was obtained from Roche (Mannheim, Germany). RWJ 56110, FSLLRY-NH<sub>2</sub>, tcY-NH<sub>2</sub>, and H1152 were obtained from Tocris Bioscience (Bristol, UK). Other drugs

were obtained from Sigma-Aldrich (St. Louis, MO). Gö-6983 was in dimethylsulfoxide (DMSO). Other drugs were dissolved in PSS. The final concentrations of DMSO were less than 0.1% in the vessel bath.<sup>26</sup> As a pilot study, vehicle control did not affect the diameter of the retinal arterioles.

### ***Analysis***

At the end of the functional experiment, the vessel was relaxed with EDTA (1 mM)  $\text{Ca}^{2+}$ -free PSS to obtain the maximal diameter at 55 cmH<sub>2</sub>O.<sup>25</sup> The diametric changes observed during responses to thrombin were normalized to the resting vessel diameter after development of basal tone and are expressed as the percentage changes in diameter. The data are expressed as the mean  $\pm$  standard error of the mean;  $n$  represents the number of vessels studied. Distribution was analyzed by the Kolmogorov-Smirnov test. The Student's  $t$ -test was used for the statistical comparisons of changes in the resting tone caused by the inhibitors. One-way analysis of variance followed by Turkey's test was applied to determine the significant differences in the controls ( $p < 0.05$ ).

## Results

### *Responses of retinal arterioles to thrombin*

All vessels ( $n = 138$ ) had similar levels of basal tone, resting diameters, and maximal diameters, i.e., constricted to  $61.4 \pm 5.9\%$  of the maximal diameter,  $59.0 \pm 8.1$  micrometers, and  $96.2 \pm 10.3$  micrometers, respectively, under the same conditions. Thrombin induced the maximal responses of the retinal arterioles within 2 to 4 minutes and a concentration-dependent vascular response, i.e., initial vasoconstriction at a low concentration ( $\leq 5$  mU/ml) and then vasorelaxation at a high concentration ( $> 5$  mU/ml) (Figure 1). The thrombin-induced response was elicited only once on one vessel, and a higher concentration ( $> 30$  mU/ml) of thrombin did not result in any responses on retinal arterioles (data not shown).

### *Comparison of responses between intraluminal and extraluminal administration of thrombin*

Extraluminal administration of thrombin (5 mU/ml) caused vasoconstriction, but intraluminal administration of thrombin (5 mU/ml) did not cause any changes in the vessel

diameter. After removing the endothelium, intraluminal administration of thrombin (5 mU/ml) resulted in similar vasoconstriction with extraluminal administration of thrombin (5 mU/ml) (Figure 2).

### ***Contribution of PAR subtype to thrombin-induced vasoconstriction***

Pretreatment with the PAR-1 inhibitor RWJ56110 and the specific thrombin antagonist hirudin significantly ( $p < 0.01$ ) suppressed the constriction resulting from thrombin administration; the highest rate of vasoconstriction caused by thrombin (5 mU/ml) remained at 30% (Figure 3). However, the PAR-2 inhibitor FSLLRY-NH<sub>2</sub> and the PAR-4 inhibitor tcY-NH<sub>2</sub> did not affect the response to thrombin.

### ***Role of the endothelium***

Endothelial denudation and pretreatment with L-NAME significantly ( $p < 0.05$ ) suppressed vasorelaxation caused by a high concentration of thrombin, but pretreatment with indomethacin and a combination of apamin and ChTX did not suppress the vasorelaxation (Figure 4A, B). To evaluate the thrombin-induced vasorelaxation, the diameter at the concentration of 20 mU/ml was normalized to the diameter at the concentration at 5 mU/ml

with basal tone and maximal vasorelaxation and expressed as the percentage of the maximal relaxation (Figure 4B). No procedures suppressed the thrombin-induced vasoconstriction (Figure 4A).

### ***Role of PKC***

Pretreatment with the PKC inhibitor Gö-6983 significantly ( $p < 0.05$ ) suppressed the constriction in response to thrombin (5 mU/ml) (Figure 5).

### ***Role of Rho kinase***

Thrombin-induced vasoconstriction after H1152 and  $\text{Ca}^{2+}$ -free PSS were administered was suppressed significantly ( $p < 0.01$ ) compared with thrombin-induced vasoconstriction after SNP was administered (Figure 6A). Thrombin (5 mU/ml) caused similar vasoconstriction in all groups of vessels (Figure 6B). The retinal arterioles constricted by thrombin (5 mU/ml) relaxed with 15-minute exposure to  $\text{Ca}^{2+}$ -free PSS and ROCK inhibitor H1152.

## **Discussion**

Many studies of the effect of thrombin on vascular reactivity have reported that thrombin induced vasodilation in the coronary<sup>1-4</sup> and pulmonary arteries.<sup>5</sup> In contrast, other studies have reported that thrombin induced vasoconstriction in the pulmonary<sup>6</sup> and cerebral arteries<sup>7,8</sup> or caused a biphasic response, i.e., both vasorelaxation at a low concentration and vasoconstriction at a high concentration in the renal interlobar artery.<sup>9</sup> These studies have indicated that thrombin can cause both vasoconstriction and vasorelaxation depending on the vascular bed. In bovine retinal arterioles (~200  $\mu\text{m}$ ) after precontraction with prostaglandin  $\text{F}_{2\alpha}$ , thrombin (40 mU/ml) caused vasoconstriction.<sup>16</sup> The current study showed that thrombin caused a dose-dependent biphasic response, i.e., after vasoconstriction of the retinal arterioles at a low concentration ( $\leq 5$  mU/ml) and vasorelaxation at a high concentration ( $> 5$  mU/ml) (Figure 1). The current study also showed that intraluminal administration of thrombin does not cause vasoconstriction (Figure 2). These contradictory results might be caused by differences in species, vessel size, use of a precontractor, and methods of administration. A previous study<sup>16</sup> indicated that thrombin causes vasoconstriction via its catalytic activity, but the current study found that not only its catalytic activity but also PAR-1 were involved with vasoconstriction

(Figure 3).

Intraluminal administration of thrombin did not cause vasoconstriction at 5 mU/ml (Figure 2). In the endothelium-denuded retinal arterioles, intraluminal administration of thrombin (5 mU/ml) showed vasoconstriction similar to the extraluminal administration of thrombin (5 mU/ml). Extraluminal administration of thrombin could cause the vascular responses via both the smooth muscles and endothelium. The permeability of the inner BRB to molecules is below 1 kDa.<sup>24</sup> Because the MW of thrombin is 33.6 kDa, intraluminal administration of thrombin would not affect the smooth muscles. In addition, the normal blood concentration of thrombin is  $5.5 \pm 1.1$  mU/ml in healthy subjects,<sup>42</sup> and the current study showed that intraluminal administration of thrombin (5 mU/ml) did not cause vasoconstriction on normal retinal arterioles (Figure 2). These results suggested that thrombin does not cause vasoconstriction on retinal arterioles of healthy subjects. However, thrombin can cause a vascular response on the outer side of the retinal arterioles. The intravitreal concentration of thrombin is elevated in branch retinal vein occlusion ( $1.6 \pm 1.2$  mU/ml), central retinal vein occlusion ( $2.6 \pm 1.2$  mU/ml), proliferative diabetic retinopathy (21 mU/ml), and proliferative vitreoretinopathy ( $39.30 \pm 14.04$  mU/ml).<sup>19-22</sup> Regarding the



current study (Figure 1), thrombin might cause 50% of constriction in central retinal vein occlusion and 30% of constriction in other ocular disorders on the retinal arterioles, and the constriction might be related to circulatory disorders in those diseases.

Thrombin activates G-protein coupled receptors PAR-1-4. PAR-1, -2, and -4, which are expressed in vascular cells, cause various vascular responses depending on the organs.<sup>10,15,32,43-51</sup> PAR-3 works as a cofactor in PAR-4 activation.<sup>46,52</sup> A previous study<sup>16</sup> reported findings before PAR was investigated. The current study showed that the PAR-1 inhibitor RWJ56110 and specific thrombin antagonist hirudin suppressed the thrombin-induced vasoconstriction in the retinal arterioles (Figure 3), suggesting that PAR-1 contributes to the thrombin-induced vasoconstriction of the retinal arterioles. Although PAR-1 is expressed in both the vascular endothelium and smooth muscle,<sup>10,43-49</sup> the current study showed that removing the vascular endothelium did not change the thrombin-induced vasoconstriction (Figure 4A), suggesting that thrombin causes vasoconstriction via PAR-1 in the smooth muscle of the retinal arterioles.

In addition to either vasorelaxation or vasoconstriction, thrombin also causes both in a concentration-dependent manner in one blood vessel.<sup>9,53-55</sup> In the current study,

extraluminal administration of thrombin also caused vasoconstriction at a low concentration ( $\leq 5$  mU/ml) and vasorelaxation at a high concentration ( $\geq 5$  mU/ml).

Moreover, Ku and Zaleski detected differences in the thresholds at which thrombin receptor agonist peptide induced vasorelaxation and vasoconstriction, i.e., 0.03 and 0.3  $\mu$ M on canine coronary arteries, respectively.<sup>56</sup> Due to the different thresholds, thrombin might cause a biphasic response in the retinal arterioles as in the coronary artery.

It has been reported that NO, PGI<sub>2</sub>, and EDHF may contribute to vasodilation caused by thrombin.<sup>3,57-59</sup> In addition, hydrogen peroxide also causes endothelium-dependent vasorelaxation.<sup>34</sup> In the current study, because thrombin-induced vasorelaxation (20 mU/ml) was abolished after denudation, the vasorelaxation depended on endothelium-derived relaxing factor. Moreover, blockade of NO and hydrogen peroxide by L-NAME also abolished the thrombin-induced vasorelaxation (20 mU/ml) but not indomethacin and the combination of apamin and ChTX (Figure 4A, B). Thrombin-induced vasorelaxation also was suppressed by L-NAME.<sup>60</sup> Both L-NAME<sup>34</sup> and indomethacin<sup>36</sup> block hydrogen peroxide and only L-NAME abolished thrombin-induced vasorelaxation. Overall, these results indicated that NO but not PGI<sub>2</sub>, EDHF, and hydrogen peroxide are

produced in the endothelium, resulting in vasorelaxation of the retinal arterioles.

Endothelin-1 (ET-1), a potent vasoconstrictor of the retinal arterioles, is produced by thrombin in the endothelial cells.<sup>61,62</sup> In addition, long-term treatment (~24 hours) with thrombin up-regulates endothelin-converting enzyme-1, which is processed to bioactive ET-1,<sup>63</sup> in human umbilical vein endothelial cells.<sup>64</sup> In the current study, we found that endothelial denudation did not affect the thrombin-induced vasoconstriction (Figure 4A). ET-1 also caused constriction of the retinal arterioles in 20 minutes,<sup>41,65</sup> whereas thrombin-induced vasoconstriction occurred within 2 to 4 minutes in the current study. Overall, thrombin might elicit vasoconstriction via PAR-1 in an endothelium-independent manner in the smooth muscles in the retinal arterioles without any contribution of ET-1 to vasoconstriction caused by thrombin.

PKC activation might be involved in thrombin-induced vasoconstriction.<sup>66</sup> In the current study, the PKC inhibitor Gö-6983 significantly suppressed the thrombin-induced vasoconstriction, whereas the combination pretreatment with both Gö-6983 and RWJ56110 did not intensify the suppression of the thrombin-induced vasoconstriction (Figure 5), which agreed with the previous finding.<sup>66</sup> Therefore, we speculated that PKC may play a

critical role in thrombin-induced vasoconstriction via PAR-1.

Previous studies have reported that activation of ROCK may be involved in vascular smooth muscle contraction in various vasculatures.<sup>67-69</sup> To determine the association between thrombin-induced vasoconstriction and ROCK, thrombin (5 mU/ml) was administered to the retinal arterioles after extraluminal administration of H1152, SNP, or Ca<sup>2+</sup>-free PSS. Thrombin-induced vasoconstriction after H1152 and Ca<sup>2+</sup>-free PSS was suppressed significantly compared with thrombin-induced vasoconstriction after SNP (Figure 6A). In addition, pretreatment with H1152 or Ca<sup>2+</sup>-free PSS reversed the thrombin-induced vasoconstriction (Figure 6B). Our data indicated that ROCK and extracellular Ca<sup>2+</sup> were involved in thrombin-induced vasoconstriction. Although our data did not show an association between PKC and ROCK in the thrombin-induced responses, a previous study reported that PKC and ROCK interact with each other.<sup>70</sup>

No agents abolished the thrombin-induced vasoconstriction in the current study. We also performed additional experiments after pretreatment with higher concentrations of RWJ56110 (20  $\mu$ M) and hirudin (100 mATU/ml) to exclude the possibility that the concentrations of RWJ56110 and hirudin were not sufficiently high to abolish the

thrombin-induced vasoconstriction. We did not find any difference in the vascular responses at higher concentrations. We also performed an additional study to investigate the possibility that thrombin causes responses to retinal arterioles before hirudin suppresses the effect of thrombin. In that study, pretreatment with hirudin in the microtubes containing thrombin did not cause any difference in the thrombin-induced vascular response. Unsuppressed vasoconstriction might be caused by catalytic activity as reported previously.<sup>16</sup>

The current study had two limitations. First, extraluminal administration of thrombin causes vasorelaxation after vasoconstriction in a dose-dependent manner. If the thrombin-induced vasoconstriction is suppressed with inhibitors, i.e., RWJ56110 and hirudin, it is difficult to evaluate precisely the mechanism of vasorelaxation because thrombin caused vasorelaxation at a concentration higher than 5 mU/ml and the vessel diameter in the groups of thrombin with inhibitors differed from the control at 5 mU/ml. Second, our method evaluated the vessel responses to thrombin only based on changes in the vessel diameter. Thus, the current study can describe the responses of the retinal arterioles to thrombin as a phenomenon, but the mechanical evaluation is difficult.

To the best of our knowledge, this is the first study to report that thrombin causes constriction of the retinal arterioles via PAR-1 and to report the different responses between intraluminal and extraluminal administration of thrombin on the retinal arterioles. We also found that a low concentration ( $\leq 5$  mU/ml) of thrombin caused vasoconstriction via PKC, and ROCK on smooth muscle, and a high concentration ( $>5$  mU/ml) of thrombin caused vasorelaxation via endothelial NO synthesis. This novel finding of thrombin-induced responses and an understanding of the complicated mechanism of thrombin-induced vasoactivity might have important clinical implications in the pathophysiology of ocular diseases.

### **Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## Legends

**Figure 1.** Response of isolated retinal arterioles to extraluminally administered thrombin (0.001 mU/ml to 20 mU/ml) ( $n = 13$ ). † $p < 0.01$  versus 0.001 mU/ml. \*\* $p < 0.05$ ; †† $p < 0.01$  versus 5 mU/ml.

**Figure 2.** Comparison of intraluminal and extraluminal administration of thrombin (5 mU/ml) for 15 minutes. Intraluminal administration of thrombin does not cause significant

( $p < 0.01$ ) vasoconstriction compared with extraluminal administration of thrombin and intraluminal administration of thrombin on endothelial-denuded arteriole. † $p < 0.01$  versus intraluminal 5 mU/ml.

**Figure 3.** Roles of PARs in the vascular response to thrombin. Effects of pretreatment with the PAR-1 antagonist RWJ56110 (10  $\mu$ M), PAR-2 antagonist FSLLRY-NH<sub>2</sub> (10  $\mu$ M), PAR-4 antagonist tcY-NH<sub>2</sub> (1  $\mu$ M), and specific thrombin inhibitor hirudin (50 mATU/ml) for 30 minutes. After incubation with the respective blockers, thrombin was extraluminally administered at concentrations ranging from 0.001 to 20 mU/ml. \* $p < 0.05$ ; † $p < 0.01$  thrombin versus Thr+RWJ 56110.

**Figure 4.** A) Effects of removing endothelium with 0.4% CHAPS and pretreatment with the specific NO synthase inhibitor L-NAME, the specific PGI<sub>2</sub> inhibitor indomethacin or specific K<sub>Ca</sub> channel inhibitor apamin plus ChTX. \* $p < 0.05$  thrombin versus Thr+LNAME and Thr (denu). B) The diameter at the concentration of 20 mU/ml is normalized to the diameter at the concentration at 5 mU/ml with basal tone and maximal vasorelaxation and

expressed as the percentage of the maximal relaxation. † $p < 0.01$  versus thrombin.

**Figure 5.** The effect of pretreatment with the PKC inhibitor Gö-6983 for 30 minutes. After incubation with the blocker, thrombin was administered extraluminally at concentrations ranging from 0.001 to 20 mU/ml. \* $p < 0.05$  thrombin versus Thr+Gö-6983 and Thr+Gö6983+RWJ56110.

**Figure 6.** A) Effects of thrombin (5 mU/ml) on the retinal arterioles pretreated with H1152 (10  $\mu$  M) and SNP (30  $\mu$  M) for 15 minutes. † $p < 0.01$  H1152 versus SNP. B) Effects of ROCK inhibitor H1152 on the retinal arterioles pretreated with thrombin (5 mU/ml) for 15 minutes. † $p < 0.01$  Control versus H1152.

**Supplemental material 1.** To determine if hirudin is a specific blocker of thrombin, using the classic vasoconstrictor endothelin-1. As a result, a higher concentration of hirudin (100 mATU/ml) did not suppress the endothelin-1-induced vasoconstriction.



**Supplemental material 2.** The response of PAR-1 selective agonist (TFLLR-NH<sub>2</sub>) on the retinal arterioles.

**Supplemental material 3.** The response of PAR-2 selective agonist (2-Furoyl-Leu-Ile-Gly-Arg-Leu-NH<sub>2</sub>) on the retinal arterioles.

Figure 1

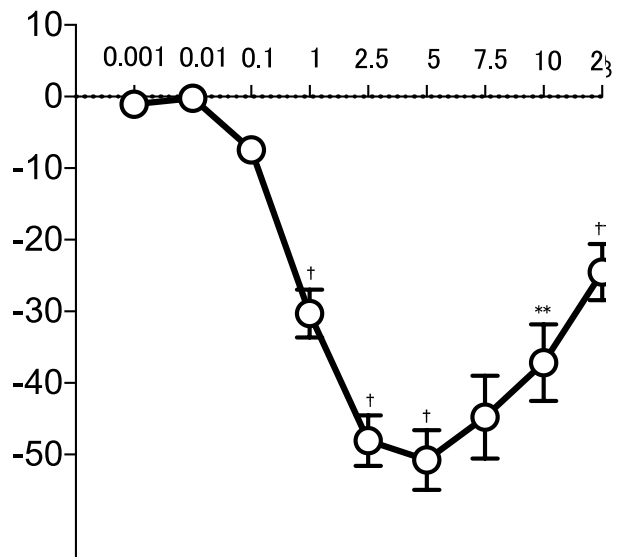


Figure 2

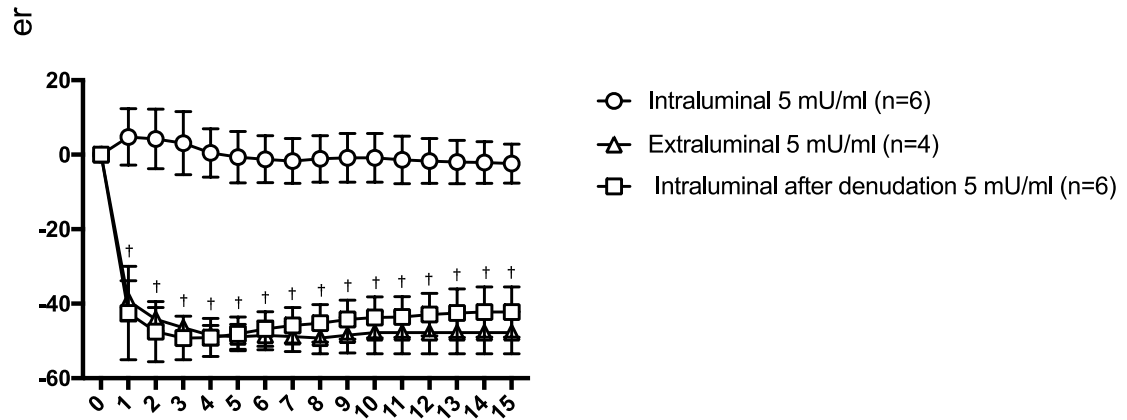


Figure 3.

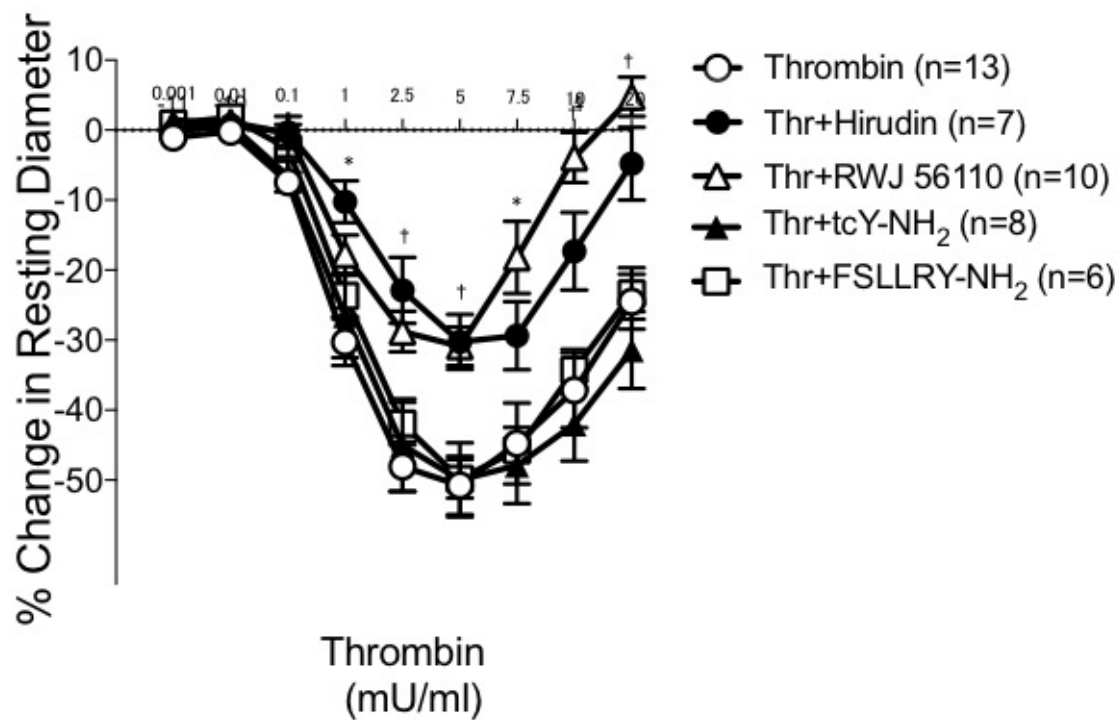
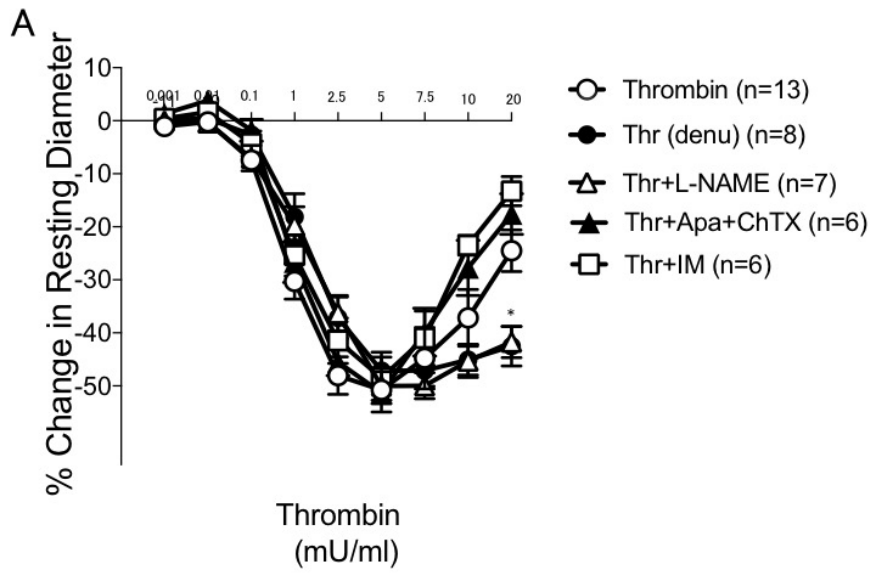


Figure 4



**B**

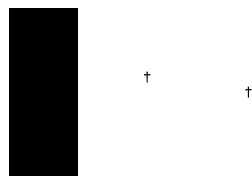


Figure 5.

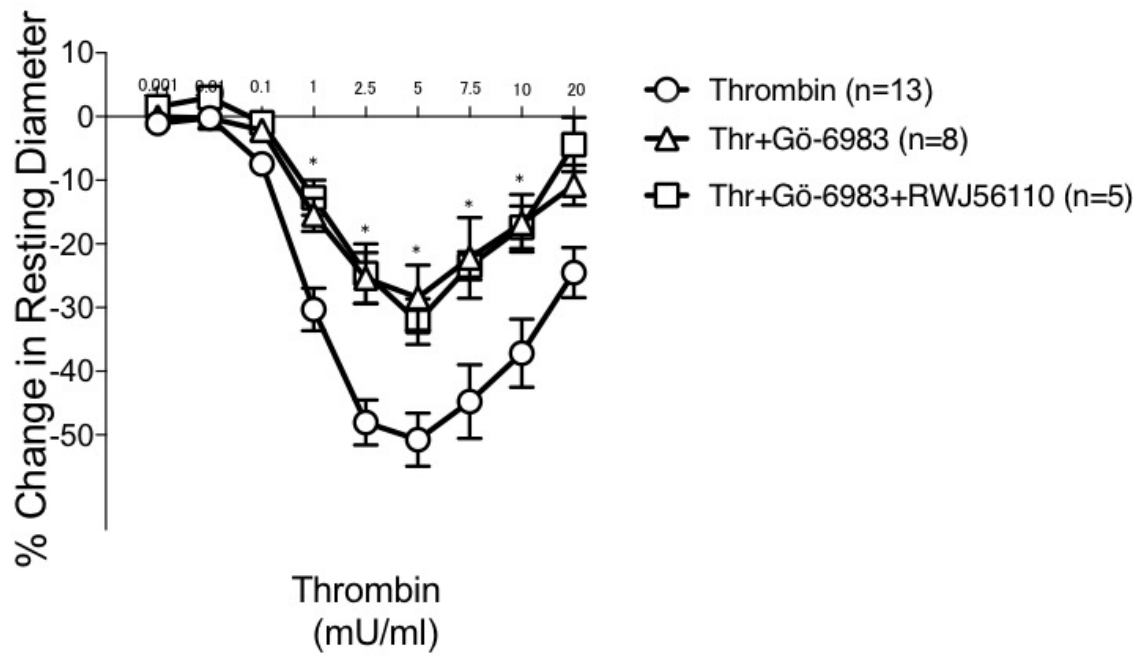
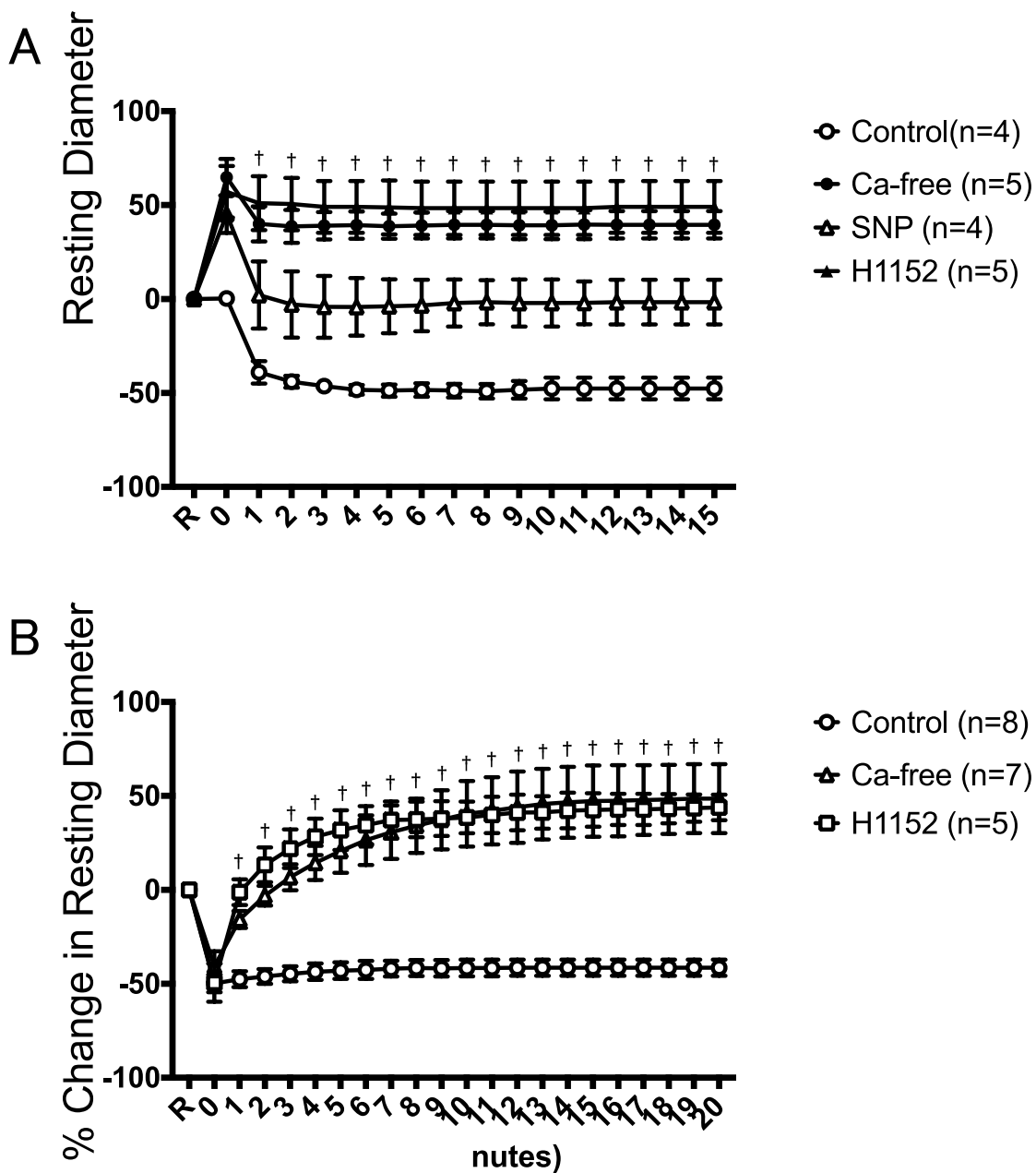
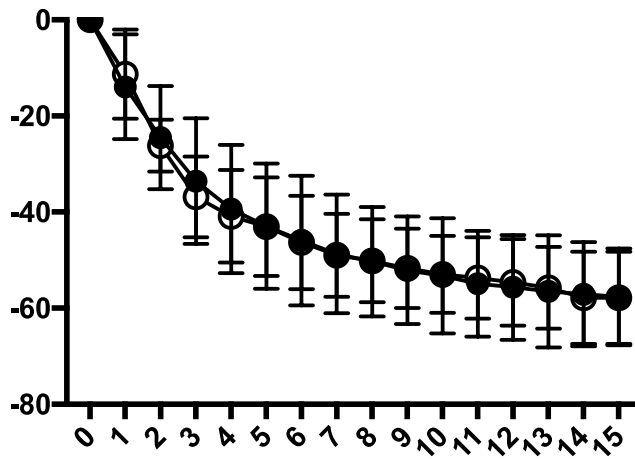


Figure 6

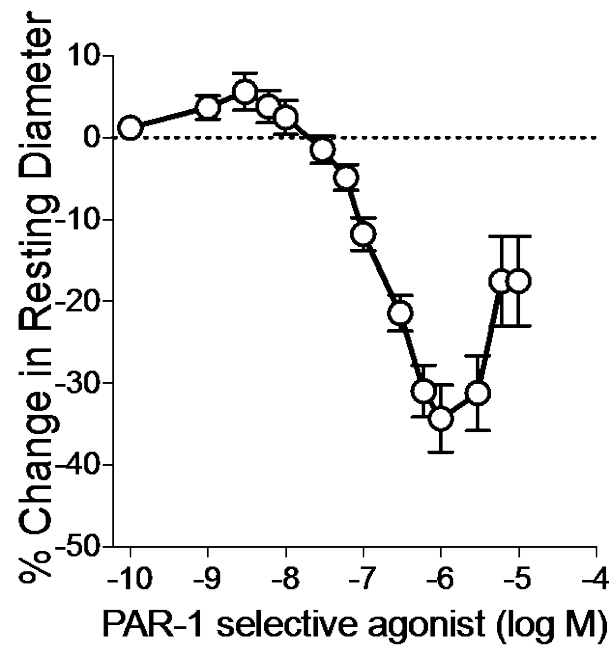


Supplemental material 1





## Supplemental material 2



## Supplemental material 3

