Thrombin Contracts Isolated Bovine Retinal Small Arteries In Vitro
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The effect of thrombin was tested in vitro on rings of bovine retinal small arteries (internal diameter approximately 200 μm). Cumulative addition of thrombin (0.001–10 units/ml) induced a variable concentration-dependent contraction of the retinal arteries. The contractions were slow in onset and reached a plateau after 5–8 minutes when thrombin was added cumulatively to the organ bath. Two vessels remained contracted > 1 hour after wash-out of thrombin. Maximum vessel contraction induced by thrombin was equal to 43% of the vessel Emax (1.36 N/m), with an effective concentration at the 50% level of 0.04 units/ml. Vessel contraction induced by 1 unit/ml of thrombin was, in contrast, transient, reaching a maximum within 2–4 minutes. Thereafter the vessel tension declined again almost back to baseline within the next 10–20 minutes. Contractions to thrombin could not be repeated nor could it be elicited with thrombin inactivated with heat or antithrombin-III. Treatment of vessels with 10−6 M phenoxybenzamine had no effect on the thrombin-induced vessel response. These findings indicate that the contractile effect of thrombin depends on its catalytic activity. Indomethacin at a concentration of 10−5 M did not affect the thrombin-induced vessel response. Methylene blue at a concentration of 3 × 10−6 M potentiated the thrombin-induced response in the larger, >200-μm diameter retinal arteries. The ensuing relaxation of the arteries, after maximal tone was reached, was slower than in the control. Thrombin did not induce any relaxation of prostaglandin F2α-precontracted retinal arteries, even though acetylcholine did relax these vessels, suggesting a negligible contribution of endothelium-derived relaxing factor in the vessel response to thrombin. Threshold-concentrations of thrombin augmented contractions induced by 5-hydroxytryptamine and noradrenaline. Thrombin had no effect on the potassium concentration-response characteristics of the retinal vessels, excluding the possibility that the potentiating effect of thrombin was mediated via partial membrane depolarization. These results indicate that intraocular thrombin infusion normally has a short-lived and therefore negligible direct contractile effect on retinal arteries. The persistent contraction of some arteries, especially after inhibition of the soluble guanylate cyclase enzyme, calls for caution when thrombin is infused into eyes with an already compromised retinal circulation. Invest Ophthalmol Vis Sci 31:2307–2313, 1990

Thrombin, a proteolytic enzyme (activated factor II) which makes a crucial contribution to the normal blood coagulation process by cleaving fibrinogen to fibrin,1 has recently been used as an additional tool to control bleeding during intraocular surgery.2–4 Thrombin activates platelet aggregation1 and has also been shown to induce contraction of some cerebral arteries,5,6 cause dilation of coronary and other extracerebral arteries through release of endothelium-derived relaxing factor (NO),7,8 and increase the production of prostacyclin, at least in endothelial cell culture.9

Thrombin contracts isolated bovine retinal small arteries in vitro. The mechanism by which thrombin exerts its direct contractile vascular effect is not clear. Some studies indicate that both the contracting and relaxing effect of thrombin depends on the enzyme's active catalytic activity.10,11 The remaining available data conflict and suggest activation of α-adrenoceptors5,12 and prostaglandin synthesis5 or exclude these factors13,14 in the contractile effect of thrombin.

The concentration of thrombin in vivo may be as high as 160 units/ml15 if all prothrombin is activated in plasma, but the free concentration of thrombin does normally not exceed 10 units/ml due to plasma inhibitors of thrombin.15 We, therefore, although thrombin is infused into the eye in concentrations up to 100 units/ml2–4 did not exceed concentrations of 10 units/ml in these studies.

We studied the direct contractile effect of thrombin on isolated bovine retinal resistance arteries and its interaction with the vascular endothelium and 5-hydroxytryptamine, noradrenaline, histamine, and potassium-induced vessel responses.
Materials and Methods

Eyes from calves and cows (weighing 300–500 kg) were obtained from the local slaughterhouse and transported to the laboratory in ice-cold oxygenated physiologic saline solution (PSS) with the following composition: NaCl 119 mM; NaHCO$_3$ 25 mM; KCl 4.7 mM; CaCl$_2$ 1.5 mM; MgSO$_4$ 1.18 mM; KH$_2$PO$_4$ 1.17 mM; ethylenediaminetetraacetic acid 0.026 mM, and glucose 11 mM.

The anterior segments of the eyes were removed together with the vitreous. One or two segments, 1–2-mm long, of retinal arteries were dissected and transferred to the tissue chamber on an isometric myograph. The vessels were then threaded on two 40-μm diameter stainless steel wires which were fixed to two mounting devices connected to a force transducer (Kistler Morse DSC 6 Kistler Morse Corp., WA) and a micrometer, respectively, as previously described. Thus the myograph allowed direct measurement of the vessel wall force while the internal circumference could be controlled.

The arteries were equilibrated in PSS at 37°C, pH 7.4, and oxygenated with 5% CO$_2$ in O$_2$ for 30 min before each vessel was normalized. During this procedure the passive wall tension—internal circumference characteristics of the vessels—were determined. The internal circumference, $L_0$, was then, on basis of the passive characteristics of the vessels, set to 90% of $L_{100}$, which is the internal circumference the vessels would have had when subjected to a transmural pressure of 100 mm Hg (13.3 kPa) and relaxed. Maximal active tension is developed at this circumference.

The effective lumen diameter, $l_0$, of the vessels was calculated as $L_0/\pi$.

Each experiment was begun by stimulating the retinal resistance vessels repetitively with K-PSS until reproducible responses were obtained (K-PSS is similar to PSS except that NaCl is exchanged with KCl on an equimolar basis). The maximal contractile capacity of the vessels, $E_{max}$, was finally determined by activating the vessels with K-PSS to which $10^{-5}$ M prostaglandin F$_2\alpha$ (PGF$_2\alpha$) and $10^{-5}$ M 5-hydroxytryptamine (serotonin) were added. This cocktail causes maximal contraction of the retinal resistance arteries.

The effect of thrombin was studied in cumulative concentration-response experiments by adding thrombin to the PSS in volumes not exceeding 0.3% to reach required effective concentration. Thrombin was tested in the range of 0.001–10 units/ml or until maximal vessel response was reached before the highest concentration. After washout of thrombin the vessels were allowed to stabilize in normal PSS for 45 min before the thrombin concentration-response curve was repeated. Because of total resistance to the thrombin-mediated contraction after one exposure, all vessels were only contracted once with 1 unit/ml of thrombin.

The interaction between thrombin and “classic” receptor systems was studied using retinal vessels which had been incubated with $10^{-6}$ M phenoxycobenzamine (PBZ) for 15 min and equilibrated in drug-free PSS for 30 min before they were challenged with 1 unit/ml of thrombin.

The effect of thrombin on the retinal endothelium was studied using vessels precontracted with $10^{-5}$ M PGF$_2\alpha$. Thrombin was added cumulatively, in increments of 1 log unit from 0.001–10 units/ml, to the tissue chamber after the vessel response to PGF$_2\alpha$ was stable. The vessels were then challenged with $10^{-5}$ M acetylcholine, which mediates endothelium-dependent relaxation in the retinal arteries to test endothelial function. The vessels were then challenged with $10^{-4}$ M sodium nitroprusside to test the relaxing capability of the soluble guanylate cyclase enzyme through which the endothelium-derived relaxing factor exerts its effect, and finally with $10^{-4}$ M papaverine to determine the maximal relaxation of the vessels.

The interaction between thrombin and the vascular endothelium was further examined using vessels incubated with $10^{-5}$ M indomethacin or $3 \times 10^{-6}$ M methylene blue, which inhibits prostacyclin formation and cyclic guanosine monophosphate (cGMP) production of the soluble guanylate cyclase enzyme, respectively.

Finally, the potentiating effect of thrombin on retinal vessel responses to the biogenic amines, 5-hydroxytryptamine, noradrenaline, and histamine, was tested. Two cumulative concentration-response curves were constructed with the three amines. The second curve was then made in the presence of a concentration of thrombin which was just able to induce a weak contraction. The potentiating effect of thrombin was also tested on potassium-induced vessel responses by making cumulative potassium concentration-response experiments in the presence and absence of 0.1 units/ml of thrombin.

The concentration of drug required to give half-maximal responses ($EC_{50}$) was determined for all concentration-response curves using iterative nonlinear-regression analysis. The responses were fitted to the logistic equation $R/R_{max} = A(M)^n + EC_{50}(M)^n$, with concentrations of agonist A (M) in moles/liter on a logarithmic scale. $R/R_{max}$ is relative vessel response to drug, A, and n is the curve-fitting parameter or “Hill coefficient.”

Drugs used were thrombin (Topostasine; Roche, Switzerland), antithrombin-III (Kabivitrum; Copen-
hagen, Denmark), heparin (DAK, Copenhagen, Denmark), 5-hydroxytryptamine creatinine sulfate complex, noradrenaline HCl, histamine HCl, indomethacin (all Sigma, St. Louis, MO), PGF 2α (Dinoprost; UpJohn, Kalamazoo, MI), PBZ (SKF, Welwyn Garden City, UK), and methylene blue (DAK).

Vessel responses to drugs are either given in terms of effective active wall tension, ΔT₀ (N/m), which was calculated as increase in vessel wall force above resting level divided by twice the vessel segment length or as relative responses with reference to E_max.

Results are given as mean ± standard error of the mean (n = number of vessels). A two-tailed student t-test for paired or unpaired data was used where appropriate. The level of significance for both tests was set at P < 0.05.

Results

Thrombin induced, in the concentration range 0.001–10 units/ml, a concentration-dependent contraction of the bovine retinal small arteries (Fig. 1). The concentration-response characteristics of eight individual vessels are shown in Figure 2. The mean pD₂-value (-log(EC₅₀(M))] was 1.45 ± 0.23 (n = 8) corresponding to 0.04 units/ml. The maximum tension developed during thrombin exposure was 0.70 ± 0.19 N/m (n = 8) or 43 ± 10% (n = 8) of E_max. Two vessels did not relax within 1 hr after washout of thrombin.

A concentration of 1 unit/ml of thrombin induced a transient contraction which reached maximum within 2–4 min before the tension declined to low levels over the next 10–20 min (Fig. 3, left). The thrombin-induced response could not be elicited more than once (Fig. 3, right). Heat-inactivated thrombin and thrombin pretreated with antithrombin-III and heparin did not induce contraction in four vessels tested. Analysis of the vessel response to thrombin showed that the maximal tension development was inversely correlated with the effective vessel lumen diameter (Fig. 4). The equation for the regression line was y = -0.56x + 165.67 (r = -0.77, P < 0.05)

The thrombin-induced vessel response was unaffected by treatment with 10⁻⁶ M PBZ for 10 min. The maximal contraction was 52 ± 8% (n = 8) and 49 ± 13% (n = 8) of E_max in normal and PBZ-treated vessels, respectively. The effective lumen diameter was similar in the two groups of vessels, 201 ± 12 µm and 200 ± 12 µm, respectively. The relative response to 1 unit/ml of thrombin of the PBZ-treated vessels was again inversely correlated with the effective lumen diameter of the retinal arteries, y = -0.95x + 195.23 (r = -0.93, P < 0.001).

Thrombin, 0.001–10 units/ml did not relax retinal small arteries precontracted with 10⁻⁵ M PGF₂α (Fig. 5). The vessels were only modestly relaxed by 10⁻⁵ M acetylcholine, 35 ± 7% (n = 8). Sodium nitroprusside at a concentration of 10⁻⁴ M induced a slightly but not statistically significant greater relaxation: 46 ± 6% (n = 8). Papaverine at a concentration of 10⁻⁴ M induced a complete relaxation of the vessels: 97 ± 3% (n = 4) (Fig. 5, insert). Maximal effects of all drugs are obtained at the concentrations used.
The maximal contraction of the retinal vessels to 1 unit/ml of thrombin was not affected by $10^{-5}$ M indomethacin, being $56 \pm 6\%$ (n = 8) of $E_{\text{max}}$ (1.47 ± 0.17 N/m). A concentration of $3 \times 10^{-6}$ M methylene blue had a pronounced potentiating effect on the thrombin induced contraction of retinal artery segments taken from near the papilla but not in segments from a more distal location in the retinal circulation (Fig. 6). The maximal thrombin-induced contraction in vessels >200 μm increased from 40 ± 9% (n = 5) in control to 69 ± 2% (n = 5) of $E_{\text{max}}$ ($P < 0.02$) in the presence of methylene blue. The response to thrombin became stable in four of eight vessels during an observation period of 20 min.

Threshold concentrations of thrombin, 0.001–0.03 units/ml, potentiated the 5-hydroxytryptamine concentration-response characteristics of the bovine retinal small arteries. The maximal response increased from $0.36 \pm 0.18$ N/m (n = 8) to $0.91 \pm 0.12$ N/m (n = 8) ($P < 0.01$, paired t-test). The sensitivity, $pD_2$-value ($-\log [EC_{50}(M)]$) increased by a factor of 4 ($\Delta pD_2 = 0.56 \pm 0.19$, n = 6, $P < 0.05$, paired t-test) from $6.52 \pm 0.12$ (n = 6) in control to 7.14 ± 0.17 (n = 8) in presence of thrombin ($P < 0.02$), corresponding to mean $EC_{50}(M)$-concentrations of $3 \times 10^{-7}$ M (range, $1.5 \times 10^{-7}$ M–$1.1 \times 10^{-6}$ M) and $7 \times 10^{-8}$ M (range, $9.0 \times 10^{-9}$ M–$3.0 \times 10^{-7}$ M), respectively. The maximal noradrenaline-induced contraction, 0.5 ± 0.13 N/m (n = 8), was slightly increased by thrombin to 0.79 ± 0.16 (n = 8) ($P < 0.1$). The sensitivity was increased by a factor of 3.5 by thrombin, $pD_2$-values were $6.28 \pm 0.08$ (n = 8) in control and $6.82 \pm 0.25$ (n = 7) in presence of thrombin ($P < 0.01$, unpaired t-test) (mean $EC_{50}(M) = 5 \times 10^{-7}$ M; range, $2.5 \times 10^{-7}$ M–$1.3 \times 10^{-6}$ M; and $1.5 \times 10^{-7}$ M; range, $1.7 \times 10^{-8}$ M–$1.8 \times 10^{-6}$ M, respectively), giving a $\Delta pD_2$-value of $0.56 \pm 0.26$ (n = 7) ($P < 0.05$, paired t-test). Thrombin had no effect on the histamine concentration-response curve (Fig. 7).

The potassium concentration-response characteristics of the retinal small arteries were not affected by thrombin at 0.1 units/ml (Fig. 8). The $pD_2$-value for potassium was $1.30 \pm 0.03$ (n = 10) in control and $1.32 \pm 0.03$ (n = 10) in presence of thrombin, corresponding to $EC_{50}(M)$-concentrations of 50 mM and 48 mM of potassium, respectively. The maximal ac-
tive tension development induced by potassium was, on average, unchanged, being $0.66 \pm 0.10 \text{ N/m (n = 10)}$ in control and $0.66 \pm 0.15 \text{ N/m (n = 10)}$ in presence of 0.1 units/ml of thrombin. The maximal potassium-induced vessel response was equal to $54 \pm 10\% (n = 10)$ of $E_{\text{max}}$ ($1.44 \pm 0.20 \text{ N/m with n = 10}$).

**Discussion**

Thrombin has been used in the recent years to control bleeding during intraocular surgery. Most attention has been paid to a possible secondary toxic effect of thrombin on the corneal endothelium. However, the electroretinogram b-wave-stimulus curve is shifted to the right after thrombin infusion in rabbit eyes which could be due to a secondary effect of thrombin on the retinal vasculature. Our experiments indicate that thrombin, in much lower concentrations than normally used, has a pronounced and normally short-lived contractile effect on the retinal arteries. The smaller peripheral arteries were contracted to a greater extent than those near the papilla. A size-dependent effect of thrombin is also found in cerebral arteries.

Unlike other vasoactive substances it is not well understood how thrombin acts on the vascular smooth muscle. Some data indicate that the catalytic site on thrombin has to be functional. The remaining data on the contractile effect of thrombin conflict, suggesting involvement of $\alpha$-adrenoceptors and prostaglandin synthesis or excluding these factors. Experiments on endothelial cell culture seem to indicate that thrombin transduces its “signal” intracellularly via membrane phosphoinositol breakdown. The thrombin-induced contraction in the bovine retinal arteries appears to be dependent on the catalytic activity of the enzyme and not through activation of the “classic receptor systems,” such as the adrenergic, histaminergic, serotoninergic, and cholinergic receptors.

Thrombin has been reported previously to induce long-lasting sustained contraction of dog basilar arteries, but others showed rapid development of tachyphylaxis and transient responses to thrombin in
the same vessel preparation from humans. We could only induce a single transient contraction of the retinal arteries with thrombin. The tachyphylaxis or desensitization of the retinal vascular smooth muscle may be due to either a complete cleavage of some structure at the "activating" site on the smooth muscle cell membrane by the enzymatic activity of thrombin or to a shortage of phosphoinositol in the membrane.22

Thrombin has attracted attention in connection with angina pectoris based on coronary artery spasm25,26 because of its ability to activate the endothelial cells25,26 which release possibly two vasodilatory substances: endothelium-derived relaxing factor (EDRF),7,8 possibly NO,27 and prostacyclin.9 Thrombin induced neither prostacyclin nor EDRF formation in the retinal arteries. The endothelium was well preserved in the arteries as indicated by equal relaxations induced by acetylcholine, which is endothelium dependent (results not shown), and sodium nitroprusside. Both agents were rather ineffective in relaxing the retinal vessels compared with other arteries where almost complete relaxation can be achieved.7 It may be difficult to demonstrate thrombin-induced EDRF release if thrombin is less potent than acetylcholine and at the same time has a contractile effect on the retinal smooth muscle.

The observation that methylene blue augmented the thrombin-induced contraction of the larger retinal arteries might otherwise indicate that thrombin generates EDRF release in these arteries. The effect of methylene blue may also be explained by an inhibition of either basal EDRF release or endogenous cGMP production in the retinal artery smooth muscle cells.28 Some arteries were completely contracted for 15–20 min in the presence of methylene blue. This may be significant during thrombin infusion in vivo where the retinal circulation might be compromised after intraocular hemorrhage followed by release of hemoglobin, which also inhibits the action of EDRF20 from degrading erythrocytes.

One agonist in subthreshold-to-threshold concentration may augment the vessel response to another agonist, eg, neuropeptide Y-mediated potentiation of noradrenaline-induced contraction of femoral, gastroepiploic, and pulmonary arteries from the rabbit.29 Thrombin potentiated the contractile effect of 5-hydroxytryptamine in the retinal arteries and increased their sensitivity to this amine and also to noradrenaline. The mechanisms behind potentiation of one agonist by another are complex and may involve lack of expression of one receptor type without the other agonist,30 alteration in calcium sequestering mechanisms (for example, inhibition of the "subsarcolemmal calcium sink"),31 smooth muscle membrane depolarization by interference with the Na+/K+-pump or other electrogenic ion transport systems in the cell membrane, or a combination of these. Subsequently, these changes may increase receptor affinity and, in particular, increase calcium influx via the so-called membrane potential-operated calcium channels.32,33 The potentiating effect of thrombin seems to be membrane-receptor specific either because thrombin alters calcium sequestration selectively for such responses or in some way alters the serotonergic and adrenergic receptor function, perhaps through its inherent proteolytic activity.

In conclusion, we showed that thrombin has a potent but short-lived contractile effect on bovine retinal small arteries. The persistent contraction of some arteries indicates that caution should be taken during intraocular infusion of thrombin into an eye with an already compromised retinal circulation.

Key words: thrombin, retinal arteries, bovine, noradrenaline, 5-hydroxytryptamine, histamine, potassium, endothelium

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