Cerebral Arterial Contractions Induced by Human and Bovine Thrombin

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SUMMARY Purified human and bovine thrombin produced comparable tonic contractions in isolated canine basilar arteries. The magnitude of the contractions was closely related to the number of thrombin Units studied rather than to the amount of protein added to the isolation bath. Thrombin had a much slower onset of action, but was more potent in generating sustained contractions than either serotonin or prostaglandin F$_2$.

Moreover, in contrast to serotonin and prostaglandin F$_2$, the contractions caused by thrombin were not terminated by equivalent washing. The thrombin-induced contractions were significantly inhibited by prostacyclin, meclofenamic acid, phenoxybenzamine and glycerol. Prostacyclin was the most potent of these inhibitors. The results suggest that thrombin in a “free” form may cause vasoconstriction, in addition to platelet aggregation, in hemostasis and could contribute to the genesis of cerebral vasospasm associated with subarachnoid hemorrhage.

THROMBIN IS ONE OF MANY naturally occurring substances which produce cerebral vasospasm experimentally. Vasospasm persists for hours if thrombin is injected into the subarachnoid space. It might, therefore, be a factor in the genesis of cerebral vasospasm observed clinically. The quantities of thrombin used to produce this experimental phenomenon were approximately equivalent to 0.35 to 4 ml of blood. It was noted that the cerebral vasospasm produced was generally much slower in onset than that induced by many other agents such as serotonin and prostaglandins. This led to the hypothesis that such vasoconstriction was due to an indirect action of thrombin. Subsequent findings supported this view: the intracisternal injection of thrombin markedly elevated the levels of prostaglandins F$_{20}$ and E$_{2}$ in cerebrospinal fluid (CSF) and also caused a pleocytosis. Since the synthesis of prostaglandins by fibroblasts is stimulated by thrombin, it was assumed that such synthesis might account for the elevated CSF levels of prostaglandin and the delayed appearance of vasospasm. Brain, blood vessels and other tissue were considered possible sources of vasoactive substances released in response to thrombin, but the mechanisms responsible for the delayed constriction of the cerebral arteries in vivo was believed likely to be an indirect one.

Recently Linder and Alksne demonstrated that the constriction of isolated cerebral arteries caused by the addition of whole blood to the tissue bath was enhanced by thrombin. They attributed this enhancement to an increase in the synthesis of prostaglandins by platelets — an indirect effect. However, cerebral arteries also synthesize a number of prostaglandins which are known to cause vasoconstriction of these vessels and it is possible that thrombin might produce constriction directly by stimulating this synthesis within the arterial wall.

The present study was performed to 1) determine whether thrombin exerted a direct action on cerebral arteries, 2) obtain information of its potency in relation to other agonists, and 3) to ascertain whether certain drugs would modify this action. The results indicate that thrombin could be involved in pathophysiological phenomena in ways heretofore unsuspected.

Methods

Mongrel adult dogs of either sex weighing 17 to 26 kg were anesthetized with sodium pentobarbital (30
mg/kg) given intravenously and sacrificed by rapid exsanguination via the femoral artery. The brain was removed and the basilar artery was dissected free and placed in a dish containing Krebs-Ringer buffer at 37°C. A 4 mm segment was cut from the middle of the basilar artery and, with the aid of an operating microscope, carefully mounted on rigid parallel prongs of an isolation chamber similar to that described by others, except our bath was designed to hold 25 ml of fluid rather than 10 ml. Previous experience indicated that for best results the procedures from the time of sacrifice to the insertion onto the prongs should not require more than 20-25 min. The chamber was filled with 25 ml of Krebs-Ringer buffer solution with the following composition (mM conc.): NaCl 120, KCl 4.5, CaCl2 2.5, Mg SO4 1.0, NaHCO3 120, KH2PO4 1.0, NaEDTA 0.01, and glucose 10.0, through which a 95% O2 - 5% CO2 gas mixture is bubbled continuously. The bath temperature was maintained at 37 ± 0.5°C and the pH of the bath buffer at 7.4 ± 0.05. The vessel was allowed to stabilize for 2 h during which time resting tone was set by means of a fine positioning device (FTA 1011 Hewlett Packard) at 1 g. Afterward, the tension was set once at 3.0 g for the duration of the experiment. Isometric tension was recorded by means of a Statham strain gauge transducer (11388, G 1-1.5-300) attached to a Grass Model 7 polygraph. Calibration of these recordings was done prior to each experiment with standard gram weights. Shortly after mounting the vessel, the chamber was washed 6 times with 50 ml of 37°C Krebs-Ringer buffer.

All drug solutions were made fresh prior to use and kept refrigerated between trials. All drugs were dissolved in deionized water except thrombin which was dissolved in physiological saline. The drugs studied were prostaglandin F6 (PGF6), prostaglandin I2 (PGI2) (Upjohn Co., Kalamazo, MI), serotonin creatinine sulfate, glycerol, highly purified human (Sigma Chem. Co., St. Louis, MO) and bovine thrombin (ICN Nutritional Biochem., Cleveland, OH), meclofenamate (Parke-Davis, Ann Arbor, MI) and phenoxycbenzamine HCl (Smith Kline and French Labs., Philadelphia, PA). The meclofenamate acid was dissolved by the incremental addition of 0.1 N NaOH. The solutions were added in 25 to 250 microliter quantities to the bath and the total volume added never exceeded 2% of the chamber volume. In these quantities, adding the vehicle for the drugs at comparable pH ranges never influences subsequent responses to the substances studied.

Serotonin (5-HT) and PGF6 were used because each produces reliable contractions in this preparation7-8 by which other vasoactive compounds may be compared and because they are chemically distinct agonists. Human and bovine thrombin were compared because they represent distinct species of thrombin and varied in thrombin activity (human, 3000 Units/mg; bovine, 180 Units/mg). The remaining drugs were selected because they relax isolated cerebral arteries, presumably by different mechanisms. They were also used in an attempt to analyze pharmacologically the nature of the contractions induced by thrombin. The relaxant properties of glycerol were discovered fortuitously and presumably represent a non-specific effect. Prostacyclin (PGI2) is reportedly the only prostaglandin (PG) to relax these arteries in "physiological" doses.6 meclofenamate is an inhibitor of PG synthesis,4 and phenoxycbenzamine reportedly blocks serotonin and adrenergic receptors.8 The agonists 5-HT and PGF6 were added to the bath in quantities sufficient to obtain standard dose-response data4-8 so that the magnitude of these responses could be used for comparison with thrombin. The thrombin was added in a cumulative manner as thrombin Units because it was presumed that any constriction which resulted would be related to these Units rather than to the quantities of protein added. The pharmacological inhibitors were each added 5 min after the thrombin-induced contractions peaked and plateaued (see table 2). To remove the experimental solution from the bath, the tissue was routinely washed 6 times with 50 ml of buffer at 37°C. Further details concerning the use of the drugs is presented in Results. Student's t-test was used for statistical analysis.

Results

As shown in figure 1, thrombin produced graded contractions in doses of 0.1 to 13 Units which compared favorably with those produced by standard doses of 5-HT and PGF6. The contractile activity caused by thrombin was related to thrombin Units of activity rather than to the quantity of protein added to the bath chamber. That is, similar Units of either species of protein produced a similar magnitude of contraction even though human thrombin protein had 16.7 times more thrombin Units per mg than the bovine source (3,000 U/mg versus 180 U/mg). In contrast to the nonprotein agonists, the lag and rise times of the contractile response produced by thrombin were remarkably prolonged (table 1). Increasing the number of thrombin Units (and therefore the amount of protein) 100 times only significantly shortened the rise time (p < 0.05). This suggests that the prolonged lag time might be related to an incubation period required for the response rather than to a slow diffusion or tissue penetration of these proteins. The fact that 5-HT was more potent but less effective than PGF6 as a spasmogen has been previously reported.7-8 Thrombin produced a greater contraction than 5-HT in the doses tested in only 4 experiments. However, among the agonists, human thrombin was the most potent, being approximately 20 times more potent than bovine thrombin and 500 times more potent than serotonin (fig. 1 and table 1).

Prostacyclin was the most potent antagonist of the contractions produced by thrombin. It was approximately 100 times more potent than phenoxycbenzamine, meclofenamate or glycerol (table 2). However, in the maximum concentration test (10*4M) prostacyclin always produced contractions which exceeded those induced by thrombin. In the next to the
highest concentration (10^{-4} M) small contractions were evident in 2 experiments and these were eliminated from the data presented in table 2 (N = 6). Such diphasic responses are characteristic of this prostaglandin so that the presence of thrombin did not alter the fundamental effect of this prostaglandin. However, in 10^{-6} M concentrations prostacyclin always caused relaxation which surpassed that produced by the other inhibitors in 10^{-3} M to 5 \times 10^{-4} M concentrations. The only effect obtained with meclofenamate resembled PGI_{2} in that the relaxation produced appeared linearly related to dosage, while with phenoxybenzamine and glycerol the maximum relaxant effect occurred abruptly with the highest concentrations. Drug-induced relaxation was reversible upon washing (table 2, last column).

Meclofenamate and glycerol appear to have a lasting effect on thrombin-induced contractions in that the magnitude of the contractions after wash was significantly less (p < 0.05) than with phenoxybenzamine, prostacyclin, or control experiments (table 2). Additional contractions could be instituted by the application of more thrombin, prostaglandin, or serotonin except that serotonin failed when given after phenoxybenzamine. This latter phenomenon has been reported previously. The discovery that glycerol would relax contracted cerebral arteries was fortuitous in that attempts to preserve the activity of thrombin in a 50% solution of glycerol resulted in a diphasic response of the vessel (relaxation followed by contraction). It was later found that the vehicle alone caused relaxation. The 5 \times 10^{-4} M concentration of glycerol shown in table 2 is almost equivalent to adding 25 \mu l of a 50% solution to the isolated bath. To completely terminate the contractions caused by thrombin required as much as 3 liters of artificial CSF, while the contractions induced by prostaglandins or serotonin only require 300 ml or less of wash.

The thrombin-induced contractions were tonic in nature and persisted, with some decrement, for at least 2 h. The contraction at 20 min was 97%, at 30 min 93%, and at 2 h 63% of peak contraction. This decrement was not apparently due to fatigue because adding more thrombin or one of the other agonists initiated further contractions. It was thought to be due to the autocatalysis by this serine protease but heating thrombin so as to destroy its thrombogenic properties (60^\circ C for 60 min) failed to abolish the contractions induced in 2 experiments. Acidifying to pH 3.0 and precipitating this enzyme, then redissolving in a neutral media, failed to prevent contractions. Heating to 90^\circ C for 15 min, without precipitation, nearly destroyed (92.7% reduction) the contractile property of thrombin in 4 experiments. In these, one-half of a stock solution was heated and the other half refrigerated before use.

To further study the effect of heating on thrombin activity, additional experiments were performed to
verify that heating thrombin to 60°C for 60 minutes would destroy its coagulant property and to determine the effect this treatment would have on platelet aggregation. Thrombin (human) was reconstituted as described; while one aliquot of thrombin was being heated, the other aliquot was stored at refrigerator temperature. Both heated and unheated thrombins were then tested for coagulant activity in a standard thrombin time test. The concentration of thrombin in the final reaction mixture was 1 μg/ml and 0.8 μg/ml and exceeded the amount used to induce arterial contraction. With plasma from 2 different donors it was found that the heated thrombin had lost its coagulant properties. Unheated thrombin clotted the plasmas in 21.5 and 20.1 sec respectively. Heated thrombin clotted the same plasmas in 109.6 and 268 sec respectively. Standard aggregometry was used to study platelet activity. Platelet rich plasma (PRP) was obtained from the same 2 donors, neither of whom had ingested aspirin in the preceding 2 weeks. Aggregation was carried out in the usual manner, using thrombin, heated and unheated, as the aggregating agents. The final concentrations in the aggregometer cuvet were 0.4 μg/ml and 0.5 μg/ml. PRP from both donors failed to show any reaction to the heated thrombin. The unheated thrombin produced a discrete primary wave of 30 and 20%, followed in the first sample by disaggregation and in the second by clotting.

Discussion

It is clear from these findings that thrombin is a potent spasmogen which acts directly on isolated cerebral arteries, and may therefore act directly on cerebral arteries in vivo to produce cerebral vasospasm when given intracisternally rather than act indirectly as previously postulated. The onset of action is slow both in vivo and in vitro. This might be due to a slow penetration of thrombin into the vessel wall and/or to a slow release of mediators which in turn produce contraction. In either case, the finding that the effect of thrombin was persistent and difficult to remove by standard washing suggests that it may form stable complexes within the vessel wall. Since 1 ml of blood generates 300–360 Units of thrombin and as little as 1 Unit produced a substantial contraction (table 1), thrombin must be considered as a naturally occurring vasoactive substance. It is well established that thrombin causes platelets to release a variety of prostaglandins and thromboxane A2, and all of these are vasoconstrictors of cerebral arteries. It is possible, therefore, that thrombin might contribute to the genesis of cerebral vasospasm associated with subarachnoid hemorrhage by several mechanisms. Although blood contains agents which neutralize or absorb thrombin (antithrombin factor, platelets, Alpha2 macroglobulin, etc.), the present findings suggest that even a small amount of "free" thrombin would be spasmogenic and that experiments designed to study the role of blood in the pathogenesis of vasospasm should consider this action of thrombin. It is possible that the sustained contractions of isolated cerebral arteries induced by extracts of arteries exposed to blood, but not from normal arteries, might be due to the presence of thrombin since the effect of the extracts or thrombin are not easily removed by washing the vessel. This persistent effect of thrombin or blood may help explain why cerebral vasospasm is so difficult to treat clinically. Also, the contractions of isolated cerebral arteries caused by the addition of 100 Units of thrombin to 0.4 ml of blood treated with platelet inhibitors (aspirin and phhalazinol) may be due to a direct action of thrombin. The fact that these inhibitors reduced the magnitude and duration of contractions caused by the
blood-thrombin mixtures suggests that thrombin had important indirect effects as postulated by the investigators and/or that the inhibitors were antagonizing the direct action of thrombin. The present study shows that a variety of arterial smooth muscle relaxants will antagonize the direct effect. An important factor in preventing blood clots from spreading in vivo is dilution by the circulation. The CSF circulation may account for the finding that about 100 Units of thrombin is needed to generate cerebral vasospasm in vivo and why 25 Units (mixed with fibrinogen) failed to cause vasospasm when injected into the chiasmatic cistern. In the latter experiment a reaction with fibrinogen might have also reduced the effectiveness of thrombin. Although there are many factors which may interfere with the actions of thrombin, the present study indicates that in the "free" state one previously unrecognized action of thrombin is vasoconstriction. Thrombin, therefore, resembles serotonin, thromboxane A2 and several prostaglandins in producing platelet aggregation and constriction.

It is evident from this study that heating to 60°C for 60 minutes inactivates both the clotting and the platelet aggregating properties of thrombin. The vessel contracting property, however, is not affected by this amount of heating. These findings suggest that the vasoconstricting effect of thrombin is separate and distinct from its fibrinogen-clotting and platelet aggregating activities. Thrombin's aggregating effect is initiated by the reaction of thrombin with the platelet membrane. It is reasonable to assume that thrombin initiates the contractile reaction by reacting with cell membranes in the vessel wall. It appears that these 2 membrane reactions must be mediated by different loci on the thrombin molecule. The finding that the magnitude of the contractions induced by thrombin appear more related to the strength of the thrombin Units than to the amount of protein added to the bath (fig. 1, table 1) suggests a link between clotting and contractile effects of this protein.

Since thrombin causes platelets and fibroblasts to synthesize prostaglandins, and these are known to produce vasoconstriction, it is possible that such synthesis is involved in the phenomenon studied. The enzyme which initiates this synthesis (phospholipase A2) is present in the membranes of cells and may act as a universal receptor. It is activated by collagen, thrombin, norepinephrine, bradykinin, hormones, neurotransmitters, hypoxia, and trauma, among other stimuli. The enzyme cleaves phospholipids to release the precursors of prostaglandins and thromboxane A2. Many of these are considered calcium ionophores which regulate intracellular calcium levels, although the endoperoxide prostaglandins, which form intracellularly, appear to be the best candidates for this effect, and thereby could alter smooth muscle tone. The type of prostaglandin predominantly formed would determine the direction of change. An activation of phospholipase A2 by thrombin would explain why meclofenamate, which is known to significantly inhibit prostaglandin synthesis by cerebral arteries, inhibited the vasoconstriction. This prostaglandin synthetase inhibitor also markedly decreases or abolishes constriction of isolated cerebral arteries induced by arachidonic acid (a precursor of prostaglandins), PGE2, and 5-HT. Hence, it would appear that drugs which interfere with prostaglandin synthesis by arteries can inhibit contractile mechanisms. Moreover, these inhibitors are known to decrease calcium flux. The relaxation obtained with prostacyclin (PGI2) may be caused by another mechanism. This prostaglandin is formed by endothelium, is a vasodilator of cerebral arteries and of vessels generally, and inhibits platelet aggregation by increasing levels of cyclic AMP which in turn inhibits phospholipase A2. Prostacyclin might, therefore, cause relaxation by increasing the intracellular level of cyclic AMP, which some consider to be the intracellular messenger for relaxation of vascular smooth muscle. However, the fact that glycerol and phenoxybenzamine also relaxed the vessels contracted by thrombin (fig. 2) suggests either that no specific contractile mechanisms were blocked or that different mechanisms were involved in the relaxation induced by these drugs. The latter is likely because relaxation of vascular smooth muscle by drugs has been associated with a rise in cyclic AMP, an increase in cyclic GMP, the oxidation of sulphydryl groups, the inhibition of calcium uptake and is attributed to inhibition of PG synthesis. Moreover, phenoxybenzamine (10-4M) not only blocks contractions induced by norepinephrine but also those of 5-HT and it

**Figure 2.** Schematic of events which might account for the contractile effect of thrombin showing the drugs studied and possible intracellular messengers which are reportedly associated with relaxation of vascular smooth muscle. Bold arrows represent influence of thrombin on prostaglandin synthesis, thin arrows indicate changes in intracellular activity. Previous reports indicate that PGI2 might elevate cAMP levels and that meclofenamate would reduce PGs synthesis in this paradigm. Explanations concerning the mode of action for phenoxybenzamine and glycerol would be entirely speculative.
reduced 100 times the effectiveness of PGF$_{2a}$ to produce responses in isolated basilar arteries. Hence, our effort to demonstrate a specific mechanism of action for thrombin may have failed because of the multiple actions of inhibitors of vascular smooth muscle. In this regard, a review of the literature indicates that the control of vascular smooth muscle tone depends upon the algebraic summation of multiple factors which influence vasomotion intracellularly. Although it is not possible to conclude from these experiments that a specific mechanism was responsible for the contractions induced by thrombin, the schematic presented in figure 2 lists some of the factors which could be involved. If thrombin activates phospholipase A$_2$ to produce contraction, specific inhibitors of this enzyme should be effective blockers. However, such inhibitors are not presently available, but the fact that meclofenamate, which inhibits cyclooxygenase, significantly inhibits the synthesis of prostaglandins by incubated cerebral arteries, and inhibited the contractions studied, suggests that prostaglandins may be involved in these contractile responses.

The present study shows that thrombin is a potent direct constrictor of cerebral arteries. Thrombin is formed during blood coagulation and is apparently released during fibrinolysis. It may play an important role in the pathogenesis of cerebral vasospasm associated with clots in the subarachnoid space.

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References

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