Vasoinhibitory action of proteolytic enzyme inhibitors in the capillary bed¹,²

Richard Weiner³ and Benjamin W. Zweifach⁴
Department of Pathology, New York University
Medical Center, New York City

Weiner, Richard, and Benjamin W. Zweifach. Vasoinhibitory action of proteolytic enzyme inhibitors in the capillary bed. Am. J. Physiol. 211(3): 725–729. 1966.—The effect of proteolytic enzyme inhibitors on vascular reactivity to selected vasoactive test agents (epinephrine, norepinephrine, 5-HT, histamine and bradykinin) was studied in the rabbit and rat mesenteric microcirculation. Competitive inhibitors of chymotrypsin, plasmin, and trypsin were found to interfere to varying degrees with the constrictor responses of muscular microvessels to catecholamines and to a lesser extent to 5-HT. In contrast, the vascular responses of histamine and bradykinin were not affected by pretreatment with proteolytic enzyme inhibitors. Evidence is presented to show that the actions of proteolytic enzyme inhibitors on vascular reactivity are not mediated indirectly through an effect on arterial blood pressure, nor as a result of nonspecific injury of vascular smooth muscle. The protective action of inhibitors of proteolytic enzymes in tissue injury may be due in part to an antagonism of the vascular response to catecholamines and serotonin.

Although numerous investigators have shown that proteolytic enzyme inhibitors protect against tissue injury (10, 13, 15, 19, 28), the exact mechanism(s) by which protection is conferred is not known. One possibility is that these agents suppress the formation of vasoactive mediators of the injury reaction (15, 19, 24). Another equally plausible explanation is that such inhibitors may have an effect on the smooth muscle elements of the terminal vascular bed, although there is no evidence in the literature to support this possibility.

In the present report, direct microscopic observations were made on the living vessels of the terminal vascular bed of the rabbit and rat to determine whether competitive inhibitors of proteolytic enzymes affect vascular reactivity.

Materials and Methods

The mesecum was prepared for microscopic observation according to the method of Zweifach and Metz (26). Female rats of the Wistar and Sprague-Dawley strains, weighing 130–200 g, were anesthetized with sodium pentobarbital (Nembutal), 30 mg/kg body wt, intramuscularly. Supplemental anesthesia was given as required to maintain surgical anesthesia. The preparation was kept warm and moist by means of a Ringer-gelatin solution adjusted to pH 7.2 with sodium bicarbonate and thermostatically set at 37.5 ± 0.5 C. Microscopic observations were made at a magnification of 60 X. In selected instances, similar observations were also made on the mesenteries of anesthetized rabbits (sodium pentobarbital, 30 mg/kg body wt, iv).

The functional state of the mesenteric preparation was established by examining the constrictor responses of the muscular microvessels to topically applied epinephrine hydrochloride (0.05 µg/0.1 ml), as well as to the dilator responses to topical administration of histamine diphosphate (3.0 µg/0.1 ml). Only those animals which responded to this concentration of epinephrine in from 10 to 20 sec and to the standard concentration of histamine in 5–10 sec were utilized for further experimentation. Approximately 70% of the 500 animals examined in different studies met these requirements.

Test procedure. All drugs were freshly prepared and made up in 0.1 m phosphate buffer solution, pH 7.4. The phosphate buffer itself does not affect the microcirculation. Test responses were determined by diverting the Ringer-gelatin irrigating solution temporarily to one side and applying the test drug (e.g., epinephrine) to the mesentery. Drugs were delivered locally to the surface of the mesentery in 0.1-ml volumes from a calibrated...
TABLE 1. Standard test stimuli for rat mesenteric microvessels

<table>
<thead>
<tr>
<th>Vasoactive Drugs</th>
<th>Topical Dose, µg</th>
<th>Vasomotor Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine HCl</td>
<td>0.05</td>
<td>M. art.</td>
</tr>
<tr>
<td>Norepinephrine bitartrate</td>
<td>0.1</td>
<td>CS</td>
</tr>
<tr>
<td>Serotonin (5-HT) creatine</td>
<td>1.0</td>
<td>CS</td>
</tr>
<tr>
<td>Histamine diphosphate</td>
<td>3.0</td>
<td>DIL</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>0.1</td>
<td>DIL</td>
</tr>
</tbody>
</table>

Observations are of 500 test animals. CS = vasoconstriction; DIL = vasodilation; M. art. = metarterioles; precap. = precapillary sphincters. * Primarily on the venous side.

The time of onset of action (constriction or dilation), degree of action (number and types of microvessels participating), and the duration of action were recorded. Observations were recorded for from 2 to 4 min, after which time the mesentery was washed with the irrigating solution for an additional 3-5 min to allow control vasomotor activity to be reestablished. This routine was used with selected test stimuli (epinephrine, norepinephrine, 5-HT, histamine, and bradykinin). The vasomotor responses to these test drugs were then studied after local or intravenous administration of selected proteolytic enzyme inhibitors. A double-blind study was utilized to prevent subjective interaction between experimental procedure and results.

**Local application.** Each experimental compound (e.g., tosylarginine methyl ester (TAME)) was examined first for its intrinsic vasomotor properties and was washed out. The extent to which the enzyme inhibitors modified the contractile responses of the muscular microvessels to test stimuli were examined in the following manner: the experimental agent was applied on the mesentery and left in place for 1 min, then the test drug was applied on the mesentery. The mesentery was then washed for 3.5 min. This two-step procedure was then repeated for each of the standard stimuli. Particular experimental agents were examined in separate groups of animals.

**Systemic injections.** The proteolytic enzyme inhibitors and related compounds were administered intravenously to the animal via a catheter in the femoral vein and the contractile responses of the mesenteric vascular bed to local application of the usual test drugs were analyzed as above. None of the experimental agents were administered more than once to any individual animal.

**Criteria for grading changes of vascular reactivity.** Three categories were recognized: 1) unaffected or normal response—the agent did not alter the time of onset of constrictor or dilator action, the intensity of vasomotor activity (numbers and types of microvessels participating) and the duration of the constrictor or dilator action of the test stimulus; 2) partial block—the agent delayed the time of onset, decreased the intensity and/or diminished the duration of the vasomotor response to the test stimulus; 3) complete block—the agent abolished the musculotropic action of the test stimulus.

**Blood pressure studies.** Blood pressure responses were studied in female rats weighing 160-200 g. Rats were anesthetized with sodium pentobarbital (30 mg/kg, ip) and the carotid artery and femoral vein catheterized. Arterial pressure was monitored via the carotid by a Statham pressure transducer and recorded on a Grass polygraph. Intravenous injections were made via the femoral vein catheter.

**Vasoactive test stimuli.** 1-Epinephrine hydrochloride (Adrenalin Chloride—Parke, Davis); 2-norepinephrine bitartrate (Levophed bitartrate—Winthrop), serotonin (5-HT) creatine sulfate, histamine diphosphate (Nutritional Biochemicals) and bradykinin (Sandoz).

**Competitive inhibitors of plasmin and trypsin (1, 14, 22).** N-benzoyl-L-arginine methylester HCl (BAME), epsilon aminocaproic acid (EACA), L-lysine methylester diHCl (LME) and L-tosyl-L-arginine methylester HCl (TAME) (Mann Research Laboratories).

**Competitive inhibitors of chymotrypsin (14).** N-acetyl L-tyrosine ethylester HCl (ATEE) and L-tyrosine ethylester HCl (TEE) (Mann Research Laboratories).

**Compounds without antiproteolytic enzyme activity (7, 18).** L-Arginine monoHCl, L-lysine diHCl, and L-tyrosine (Mann Research Laboratories).

**RESULTS**

**Effects of standard test stimuli on the microcirculation.** Table 1 illustrates the effects of the several test stimuli on the various components of the rat terminal vascular bed. Local application of epinephrine (0.05 µg/0.1 ml) or norepinephrine (0.1 µg/0.1 ml) produced essentially similar reactions—partial constriction of metarterioles, complete closure of many precapillary sphincters, and a partial constriction of the venules. Constriction appeared in 10-20 sec, was maximal within 35-40 sec, began to subside within 70-75 sec, and by the end of 2 min was no longer evident. Topical application of serotonin (40 µg/0.1 ml) produced only a partial narrowing of metarterioles and precapillary sphincters, but resulted in a pronounced constriction of the small venules. The muscular venules could be seen to narrow within 25-35 sec; maximal constriction occurred within 50-55 sec and had disappeared within 2 min. Local administration of histamine (3.0 µg/0.1 ml) dilated all muscular components of the terminal vascular bed. The dilation appeared within 5-10 sec and wore off within 70-80 sec. The polypeptide bradykinin (0.1 µg/0.1 ml) had a generalized dilating action on metarterioles, precapillary sphincters, and venules. The dilation developed within 3-5 sec, reached a maximum within 15-20 sec, and disappeared by the end of 1 min.

**Direct vasomotor effects of proteolytic enzyme inhibitors.** It was necessary to determine whether the proteolytic enzyme inhibitors had vasoactive properties by themselves. Inhibitors, in a concentration of 2.0 mg/0.1 ml, when applied locally on the mesentery, were found to...
produce only evanescent alterations in microvascular behavior. Three of the six proteolytic enzyme inhibitors tested (BAME, LME, and TEE) produced a short-lived dilation of metarterioles, precapillary sphincters, and venules. The dilation began to develop in 5-15 sec, was maximal by 25-30 sec, and was no longer visible after 1 min. Interestingly, a closely related compound, TAME, produced a transient constriction of precapillary sphincters (50-40 sec). Successively smaller vasomotor responses were elicited when BAME, LME, TAME, and TEE were tested repeatedly on the mesocccal preparation. Two of the enzyme inhibitors (ATEE and EACA) had no visible vasomotor effects when tested on the mesenteric microcirculation.

Vascular responses to test stimuli following local proteolytic enzyme inhibitors. The direct effects of proteolytic enzyme inhibitors on vascular behavior were of short duration and did not interfere with subsequent observations on reactivity to test stimuli. All of the competitive inhibitors, when applied locally (2.0 mg/0.1 ml), with the exception of ATEE (which was poorly soluble), were found to interfere with the constriction of metarterioles, precapillary sphincters, and venules induced by topical epinephrine or norepinephrine (see Tables 2 and 3). The reduced response to catecholamines persisted for 30-40 min after a single application of a proteolytic enzyme inhibitor. This is in direct contrast to control animals where microvascular reactivity remains unchanged for 0-3 hr.

The response to serotonin (5-HT) was affected to a lesser degree by topical administration of inhibitors of proteolytic enzymes. Only two of the inhibitors (EACA and TEE) antagonized the responses to serotonin; topical EACA completely blocked constriction of the venules in one of six animals and partially blocked the response in the remaining five animals. TEE completely abolished serotonin activity in two of five animals and partially blocked the 5-HT reaction in the remaining three animals.

No evidence of tachyphylaxis was observed when proteolytic enzyme inhibitors were tested on the microcirculation; each particular inhibitor was equally effective in suppressing vascular reactivity to catecholamines and serotonin when retested several times on a given preparation.

It was found that local administration of proteolytic enzyme inhibitors did not modify the responses of the peripheral vascular bed to the dilator actions of the diamine histamine (10 animals) or to the polypeptide bradykinin (12 animals).

Effect of intravenous injection of proteolytic enzyme inhibitors on vascular reactivity. Inhibitors of proteolytic enzymes were administered intravenously in a dose range of 10-50 mg/0.1 ml per 100 g body wt. All the agents tested (EACA, 10 animals; LME, 14 animals; TAME, 14 animals; TEE, 19 animals), with the exception of ATEE (5 animals), produced dilation in the terminal vascular bed. Dilation began within 20-30 sec after iv injection and disappeared within 5-10 min. TAME and TEE were most active in this regard. LME produced only a moderate dilation of the small blood vessels, whereas EACA had the least effect.

The effects of iv administration of inhibitors of proteolytic enzymes on vascular reactivity to the standard test stimuli are summarized in Table 4. In the case of ATEE, EACA, and TAME, iv injection produced essentially the same vascular sequelae as seen when given locally. Intravenous EACA and TAME, for example, antagonized the constrictor actions of 5-HT and catecholamines in the terminal vascular bed. The effect persisted up to 2 hr after a single iv injection of the inhibitor. ATEE given iv did not modify the responses of the microvessels to standard test stimuli.

In contrast to their blocking action when applied locally on the mesentery, LME and TEE when administered intravenously did not block the action of either serotonin or catecholamines.

Vascular effects in the rabbit mesentery. Comparable experiments were performed on the mesenteric circulation of 24 rabbits to rule out species differences. Proteolytic enzyme inhibitors were applied topically in concentrations of 2.0 mg/0.1 ml. The effects on vascular reactivity

**Table 2. Topical proteolytic enzyme inhibitors and epinephrine reactivity**

<table>
<thead>
<tr>
<th>Inhibitor (Dose, μg)</th>
<th>No. of Animals</th>
<th>Effect of Pretreatment on Epinephrine Vasorestriction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blocked completely</td>
</tr>
<tr>
<td>EACA 0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>BAME 0</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>LME 0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>TAME 0</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>ATEE* 0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>TEE 0</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 3. Topical proteolytic enzyme inhibitors and norepinephrine reactivity**

<table>
<thead>
<tr>
<th>Inhibitor (Dose, μg)</th>
<th>No. of Animals</th>
<th>Effect of Pretreatment on Norepinephrine Vasorestriction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blocked completely</td>
</tr>
<tr>
<td>EACA 0</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>BAME 0</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>LME 0</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>TAME 0</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>ATEE* 0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>TEE 0</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations of proteolytic enzyme inhibitors as in Table 2.

*Applied as a suspension.
The proteolytic enzyme inhibitors, EACA, TAME, and TEE, in concentration of
portance only in reference to the systemic injection of
tion. Each of the proteolytic enzyme inhibitors, ATEE, carotid artery blood pressure following iv administra-
response. [10-20 mg/100 g body wt] likewise
had no effect on the vasomotor reactions of the muscular
microvessels (10 animals).
Comparative studies were then made to determine whether the amino acids affected vascular reactivity to the
standard test stimuli. The response to topical epineph-
ne, norepinephrine, serotonin, histamine, and brady-
kinin was not modified by either local administration
(20 animals) of the amino acids (2.0 mg/0.1 ml per 100 g body wt) likewise
had no effect on the vasomotor reactions of the muscular
microvessels (10 animals).
Effect of proteolytic enzyme inhibitors on blood pressure in the
rat. Experiments were carried out to determine whether the effects on vascular reactivity by inhibitors of pro-
etolytic enzymes were initiated through an indirect action on arterial blood pressure. This feature, of im-
portance only in reference to the systemic injection of
inhibitors, was checked by a series of determinations of
carotid artery blood pressure following iv administra-
tion. Each of the proteolytic enzyme inhibitors, ATEE, BAME EACA, TAME, and TEE, in concentration of
10-50 mg/0.1 ml per 100 g body wt, was tested on at least
six rats. Continuous intra-arterial recordings revealed
either no change in the blood pressure or only a transient
fall (10-20 mm Hg). In the few instances where a fall in
pressure was observed, the blood pressure returned to
control levels within 2-3 min. During the subsequent 120
min, corresponding to the period in which the micro-
circulation was examined, blood pressure remained
especially normal.

**TABLE 4. Influence of systemic proteolytic enzyme inhibitors on vascular reactivity**

<table>
<thead>
<tr>
<th>Inhibitor (iv Dose, mg/100 g)</th>
<th>No. of Animals</th>
<th>Response to Test Stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>EACA (50)</td>
<td>10</td>
<td>CB 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 1</td>
</tr>
<tr>
<td>LME (20-50)</td>
<td>14</td>
<td>CB 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 4</td>
</tr>
<tr>
<td>TAME (20-50)</td>
<td>14</td>
<td>CB 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NR 4</td>
</tr>
<tr>
<td>ATFF (10-50)*</td>
<td>5</td>
<td>—Unaffected—</td>
</tr>
<tr>
<td>TEE (10-50)</td>
<td>12</td>
<td>—Unaffected—</td>
</tr>
</tbody>
</table>

Abbreviations of proteolytic enzyme inhibitors as in Table 3.

in the rabbit were found to be qualitatively the same as those described in rats.

**Related compounds without antiproteolytic enzyme action and vascular reactivity.** The question remains whether the suppressive action of competitive inhibitors of proteolytic enzymes on the vascular response to 5-HT and catecholamines is in fact associated with their antiproteolytic properties, or to some other fundamental chemical property. The amino acids, L-arginine, L-lysine, and L-tyrosine, are chemically similar to the proteolytic enzyme inhibitors but do not have antiproteolytic properties themselves. The amino acids tested do not suppress the constrictor actions of serotonin, epinephrine, and norepinephrine on the mesenteric microcirculation, and it is likely that biochemical events related to proteolytic enzyme activation may be involved. Since the amino acids were used in the same concentration range as several of the proteolytic enzyme inhibitors, these findings clearly rule out the possibility that inhibitors of proteolytic enzymes modify vascular reactivity through a nonspecific disturbance of smooth muscle behavior.

Furthermore, related compounds without proteolytic enzyme inhibitory activity, L-arginine, L-lysine, and L-tyrosine, do not suppress the constrictor actions of serotonin, epinephrine, and norepinephrine on the mesenteric microcirculation, and it is likely that biochemical events related to proteolytic enzyme activation may be involved. Since the amino acids were used in the same concentration range as several of the proteolytic enzyme inhibitors, these findings clearly rule out the possibility that inhibitors of proteolytic enzymes modify vascular reactivity through a nonspecific disturbance of smooth muscle behavior.

**DISCUSSION**

Proteolytic enzyme inhibitors have a vasoinhibitory effect on the microcirculation which persists for some 2 hr after a single intravenous injection. The phenomenon is clearly not the result of a concurrent fall in blood pressure. Inasmuch as proteolytic enzyme inhibitors of the type studied do not interfere with the dilator action of either histamine or the polypeptide bradykinin on the muscular microvessels, their actions cannot be the result of a nonspecific disturbance of smooth muscle behavior.

Furthermore, related compounds without proteolytic enzyme inhibitory activity, L-arginine, L-lysine, and L-tyrosine, do not suppress the constrictor actions of serotonin, epinephrine, and norepinephrine on the mesenteric microcirculation, and it is likely that biochemical events related to proteolytic enzyme activation may be involved. Since the amino acids were used in the same concentration range as several of the proteolytic enzyme inhibitors, these findings clearly rule out the possibility that inhibitors of proteolytic enzymes modify vascular reactivity through an osmotic effect.

Substantial evidence exists that injury phenomena in general are associated with the activation of proteolytic mechanisms (12, 23) and, as an end result, vasoactive materials may be released (6, 11, 16). The ability of inhibitors of proteolytic enzymes to suppress the injury reaction is perhaps the most convincing argument for this point of view (10, 13, 15, 19, 28). It has been sug-
ggested that inhibitors of proteolytic enzymes protect
animals against tissue damage primarily by preventing the formation of vasoactive end products which arise through enzymatic cleavage of tissue or blood constituents (15, 19, 24).

The present data focus attention on another possible action of inhibitors of this type, namely an antagonism of the constrictor action of catecholamines (epinephrine and norepinephrine) and serotonin on vascular smooth muscle. This point of view is supported by reports that proteolytic enzyme inhibitors protect against the development of local hemorrhagic necrosis produced by bacterial endotoxins (3, 8, 28), a form of tissue injury in which
This phenomenon has been implicated (9). It is of in-
terest to note that Thomas (21) has demonstrated that conventional adrenergic blocking agents such as
phenox ybenzamine and chlorpromazine have a similar pro-
tective action in this form of tissue injury.

The proteolytic enzyme inhibitors, EACA, TAME, and soybean trypsin inhibitor, have been shown to pro-
tect rabbits and rats against the tissue necrotizing effects of combinations of epinephrine and serotonin, bacterial endotoxin, or blood globulin (27).

On a systemic level, serotonin is thought to play an
PROTEOLYTIC INHIBITORS AND VASCULAR REACTIVITY

important role in the anaphylactoid and anaphylaxis reactions in rodents (17, 25). In this regard, Meyers and Burdon reported (14) that the proteolytic enzyme inhibitors, tyrosine ethylester and lysine ethylester, protect mice against anaphylactic shock. Zweifach and coworkers (28) have shown that the proteolytic enzyme inhibitor EACA prevented the lethal consequences of systemic anaphylaxis in mice but not in guinea pigs. With regard to the latter, it should be noted that serototin is not involved in the anaphylactic reaction in the guinea pig (9, 26).

Epinephrine and other biogenic amines have metabolic, ionic, and other actions (4, 5, 20) which may contribute to the genesis of the injury reaction. The extent to which proteolytic enzyme inhibitors act on such biological systems is unknown but remains a possible mode of action in conjunction with or independent of the effects on vascular smooth muscle.

REFERENCES