Evidence That the Effects of Thrombin on Arachidonate Metabolism in Cultured Human Endothelial Cells Are Not Mediated by a High Affinity Receptor*

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The effect of thrombin and its derivative, diisopropylphosphoryl-thrombin on [3H]arachidonic acid metabolism is studied in cultured umbilical vein endothelial cell monolayers. Thrombin causes a dose-dependent release of radioactivity from endothelial cells fed prostaglandin F1, and prostaglandin [3H]arachidonate. High affinity, active site-independent thrombin binding inhibits thrombin-induced release. We conclude that thrombin, despite causing 98% inhibition of binding of [3H]thrombin to its high affinity binding sites, does not cause release of arachidonic acid or metabolism of esterified arachidonic acid.

Thrombin binds to cultured human endothelial cells in a manner similar to protein ligand-receptor systems (1). Binding is rapid, reversible, and saturable with a linear Scatchard plot yielding a $K_d$ of $1 \times 10^{-10} \text{M}$ and 3000 binding sites/cell. In addition, this binding is active site-independent, i.e. diisopropylphosphoryl-thrombin (DIP-thrombin), which has its active site blocked, binds to endothelium in a manner indistinguishable from active thrombin.

Thrombin has a variety of effects of endothelium: mitogenesis (2-4), inactivation of plasminogen activator (5), synthesis and release of fibronectin (6), release of adenine nucleotides (7), and release of the prostaglandin prostacyclin, a potent inhibitor of platelet aggregation (8, 9). It is reasonable to ask whether the effects of thrombin in these phenomena are mediated via a receptor. To test whether a high affinity receptor is involved in prostacyclin release we used an in vitro system of endothelial cells fed [3H]arachidonate and studied the effects of thrombin on arachidonate metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (essentially fatty acid-free), prostaglandins E1, E2, and A2, arachidonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and indomethacin were obtained from Sigma Chemical Co., St. Louis, MO. Aspirin was from Mallinckrodt Chemical Works. Diisopropylfluorophosphate (DFP) was from Aldrich Chemical Co., Milwaukee, WI, and was diluted to 1 M in dry dimethylformamide before use. Na[17I], $2 \times 10^5 \text{Ci}/\text{mmol}$, and [5,6,6,9,11,12,14,15-3H]arachidonic acid, 112 Ci/mmole, were from Amersham Corp., Arlington Heights, IL. Dr. John Pike, Upjohn Co., Kalamazoo, MI, generously provided 6-keto-PGF$_2\alpha$. Trypsin was from Worthington Biochemicals. All other materials were reagent grade.

**Endothelial Cell Cultures**—Primary cultures of human umbilical vein endothelial cells were provided by the laboratory of Dr. John Hoak as described previously (1) as a modification of the method of Jaffe et al. (10). The experiments were performed on batches of endothelial monolayers derived from pooling several umbilical cords.

**Thrombin Preparation**—Human thrombin and DIP-thrombin were prepared as described (11). The enzyme had $2700 \text{ units/mg}$ based on an extinction coefficient ($E_{190}$) of 19.5 (12). Clotting activity was determined by comparison with a standard curve using National Institutes of Health thrombin (13), and human fibrinogen was purified via the method of Straughn and Wagner (14). Also, in some experiments, thrombin was labeled with $^{125}$I by the lactoperoxidase method of Thorell and Johansson (15), as described previously (1), with the exception that the incubation was carried out in chloride-free sodium acetate buffers. This resulted in a product with a clotting activity which was stable at 4°C for at least 1 week.

**Assay for Release of [3H]Arachidonate and Metabolites by Endothelial Cells**—Three- or four-day-old endothelial cell cultures, consisting of confluent monolayers on 35-mm Petri dishes, were washed three times with Hank's balanced salt solution (Buffer A) and finally diluted with Buffer A to a final concentration of 1 $\mu$g/ml. The monolayers were incubated with 1 ml of [3H]arachidonate in Buffer A. Preliminary experiments indicated that uptake of [3H]arachidonate was most rapid when the dishes were rocked at 37°C. After 1 h, 70 to 80% of the tritium was cell-associated, thus allowing the level reached within the monolayer to remain steady. In experiments described, the [3H]arachidonate was added to rocking dishes for 1 h at 37°C except when aspirin or indomethacin was used (see below).

After incubation with [3H]arachidonate, the cells were washed three times in Buffer A, and then test substances or control solutions were applied in a total volume of 2 ml at room temperature. The dishes were swirled once after addition of test substances and were treated in every other way as binding experiments (see below). At intervals, samples (100 $\mu$l) were taken and pipetted directly into a toluene-based aqueous scintillation fluid containing Triton X-100.

In experiments where aspirin (1 mM) (16) or indomethacin (20 $\mu$m) (17) were used, the cells, after incubation with [3H]arachidonate, were incubated an additional hour with drug (i.e. total incubation time of 2 h). Indomethacin was dissolved in absolute ethanol prior to addition of buffer to a final ethanol concentration of 0.1% (v/v). In control experiments, this concentration of ethanol had no effect on prostacyclin release.

1 The abbreviations used are: PG, prostaglandin; DFP, diisopropylfluorophosphate; DIP-thrombin, diisopropylphosphoryl-thrombin.

2 The National Institutes of Health unit of thrombin has been redesignated as the United States unit by the Bureau of Biologics, Food and Drug Administration.
Thrombin-induced Arachidonate Release

The addition of thrombin to [3H]arachidonate-fed monolayers resulted in a release of tritiated material into the supernatant (Fig. 1). This release was complete within 8 to 10 min. Both the rate and the amount of radioactivity released were dose-dependent and saturable, reaching a maximum at 1 United States unit of thrombin/ml (≈10^-6 M). The addition of buffer alone also caused a small release, presumably from mechanical stimulation of the cells by the addition of fluid. The satura

Thin Layer Chromatography—After maximal release of tritium was obtained, the endothelial cell supernatants were removed (1 to 1.5 ml) and acidified with 0.1 volume of glacial acetic acid. The supernatants were extracted twice with ethyl acetate and dried under N₂. The samples were then dissolved in a small amount of ethyl acetate, spotted on Eastman Chromagram silica gel thin layer plates and developed in a solvent system containing chloroform/methanol/acetate, and radioactivity was determined. The washing procedure took 9 min. Both the rate and the amount of radioactivity released were dose-dependent and saturable, reaching a maximum at 1 United States unit of thrombin/ml (≈10^-6 M). The addition of buffer alone also caused a small release, presumably from mechanical stimulation of the cells by the addition of fluid (8). The satura

RESULTS

The addition of thrombin to [3H]arachidonate-fed monolayers resulted in a release of tritiated material into the supernatant (Fig. 1). This release was complete within 8 to 10 min. Both the rate and the amount of radioactivity released were dose-dependent and saturable, reaching a maximum at 1 United States unit of thrombin/ml (≈10^-6 M). The addition of buffer alone also caused a small release, presumably from mechanical stimulation of the cells by the addition of fluid (8). The satura

Fig. 1. Thrombin-induced release of tritium by endothelium. Four-day-old endothelial cell monolayers, incubated with [3H]arachidonate as described under "Experimental Procedures," were washed three times with Buffer A and then 2-ml samples of varying concentrations of thrombin in Buffer A were added. At the indicated times, samples (100 μl) were taken and radioactivity was determined. Each point represents the average of values from two dishes. The cell number was constant at 5 x 10⁶/dish. The data are representative of three experiments involving three batches of cells.

of radioactive products released were dependent on the presence or absence of albumin. In the presence of albumin, there was a 2-3 fold increase in amount of radioactivity released. Thin layer radiochromatography revealed that greater than 50% of the radioactivity migrated in the region of arachidonic acid. In the absence of albumin, there was less release of tritium and more than 80% of the product migrated (Rf = 0.6) with 6-keto-PGF₁α and PGF₂α. Similar results have been reported in platelets (20, 21) and fibroblasts (22). As expected, aspirin (1 mM) and indomethacin (20 μM) inhibited the formation of prostaglandins by greater than 95% and had no effect on the release of arachidonate. All further experiments were done in the presence of 0.1% albumin to obtain maximum sensitivity of the release response.

To determine whether the high affinity thrombin receptor on endothelium is involved in arachidonate metabolism, DIP-thrombin was used. This derivative has a diisopropylphosphoryl group covalently bonded to its active site serine with no other alterations in the structure of thrombin. It neither clots fibrinogen nor cleaves synthetic substrates but has been shown to exclude.

Fig. 2. Effect of DIP-thrombin on release of [3H]arachidonate and metabolites. Endothelial cells were prepared as described in the legend to Fig. 1 and either buffer (A), 50 units/ml of DIP-thrombin (C), 1 unit/ml of thrombin (B), or a mixture of 1 unit/ml of thrombin and 50 units/ml of DIP-thrombin (D) were added. Error bars, sample standard errors of quadruplicate determinations (D). The experiment is representative of three experiments involving three batches of cells.

Fig. 3. Inhibition of [3H]thrombin binding to endothelium by DIP-thrombin. The binding of [3H]thrombin to endothelial monolayers in the presence of increasing concentrations of DIP-thrombin was studied as described under "Experimental Procedures." The binding represents the amount specifically bound in the absence of DIP-thrombin and B is that bound with the indicated DIP-thrombin concentrations. Nonspecific binding is defined as the amount of [3H]thrombin bound in the presence of a 100-fold excess of thrombin and equaled 60% of total binding. Nonspecific binding was subtracted from total binding to give specific binding. Data represent the averages of duplicate determinations.
shown to bind to endothelium in a manner indistinguishable from thrombin (6). At a concentration of $5 \times 10^{-7}$ M (equivalent to 50 units/ml), DIP-thrombin caused no release of tritium over control (Fig. 2). This concentration is 500 times the amount of active thrombin needed to cause measurable release. However, the presence of 50 units/ml of DIP-thrombin had no effect on the release of tritium by 1 unit/ml of thrombin (Fig. 2), even though it caused 98% inhibition of specific binding of 1 unit/ml of $^{3}H$-thrombin to endothelium (Fig. 3). Likewise (data not shown), lower concentrations of DIP-thrombin had no effect.

**DISCUSSION**

In this paper, we provide evidence that the high affinity active site-independent binding sites for thrombin on endothelial cells are not involved in thrombin-induced release of arachidonate and arachidonate metabolites. If thrombin binding was involved, then an agent which inhibits thrombin binding (i.e. DIP-thrombin) should behave as a competitive antagonist. However, blocking the binding sites with DIP-thrombin has no effect on thrombin-induced changes in arachidonate metabolism, which makes it unlikely that these binding sites are involved. It could be argued that DIP-thrombin blocked less than 100% of the binding sites and occupancy of the remaining small number was responsible for the full effect. This "spare receptors" argument would not be consistent with the dose-response relationship shown in Fig. 1, where doses of less than 1 unit/ml of thrombin (which was used in the experiment shown in Fig. 3) result in progressively less release of tritium.

A similar phenomenon has been reported in platelets (23) where DIP-thrombin binds with equal affinity to the same receptor as active thrombin, but neither causes $[^{3}C] serotonin release nor blocks thrombin-induced $[^{3}C] serotonin release. Although the authors claim that the binding sites they measure are responsible for thrombin-induced aggregation and secretion (24), this argument has been attacked by others (25). In addition, it has been shown recently that ADP binds to platelets with all the characteristics of a high affinity receptor, but this binding is apparently not related to ADP-induced platelet aggregation (26). Similarly, the binding of $[^{14}C]$norepinephrine to a variety of tissues was initially thought to be causally related to its biological effect. This was seriously questioned when a variety of compounds (e.g. d isomers of catecholamines, pyrocatechol, dihydroxymandelic acid) were found to inhibit binding without affecting biological response (27, 28), and it now appears that a different class of binding sites (with higher affinity) is involved in the response (29).

A high affinity thrombin receptor has been proposed to be responsible for other effects of thrombin on endothelial cells, which include mitogenesis (2, 3), inactivation of plasminogen activator (5), and release of fibronectin (6), but cause-effect relationships have neither been demonstrated nor ruled out by experiments similar to those described in this paper.

Thus, the initial step on the pathway of thrombin stimulation of prostacyclin production is not known. Since stimulation is active site-dependent, proteolytic cleavage of a plasma membrane substrate may be involved. Presumably, then, this enzymatic process would involve the presence of an enzyme-substrate (Michaelis) complex on the plasma membrane. The cellular response conceivably would depend on the turnover rate of the enzyme. This is in contrast to models of biological response to classical bimolecular ligand-receptor interactions, where the amount of bound receptor in part governs the magnitude of response (30). The amount of enzyme-substrate complex on the membrane might be small relative to amounts of ligand-receptor complexes usually seen (10$^3$ to 10$^5$ ligand molecules bound/cell at saturation). If this were true, then standard radioligand binding assays might not be sensitive enough to detect the presence of enzyme-substrate complexes.

Another possibility is that there is an active site-dependent receptor for thrombin which is distinct from the receptor that binds both DIP-thrombin and active thrombin. The model for an active site-dependent receptor would differ from that for the membrane-associated proteolytic substrate in that cellular response would not involve proteolysis of the receptor. If the affinity of thrombin for this receptor were low or the number of receptors smaller than the active site-independent binding sites, then binding of thrombin to the receptor would be difficult to distinguish from "nonspecific" binding seen in all receptor assay systems.

In conclusion, the interaction of thrombin with cell membranes is more complex than previously assumed. Caution should be exercised when ascribing cause and effect relationships to the interaction of thrombin with cell surfaces and biological response.

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