

Activation of Platelets by Eosinophil Granule Proteins

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Summary

Two of the four principal cationic proteins of the eosinophil granule, major basic protein (MBP) and eosinophil peroxidase (EPO), were shown to be platelet agonists. Both MBP and EPO evoked a dose-dependent nonlytic secretion of platelet 5-hydroxytryptamine in unstirred platelet suspensions even in the presence of 10 μ M indomethacin. MBP also evoked secretion of platelet α granule and lysosome components. Secretion by MBP and EPO was inhibited by 1 μ M PGE₁, but the nature of the inhibition differed from that observed with thrombin. Thus, MBP and EPO can be classified as strong platelet agonists with a distinct mechanism of activation.

An association between activated eosinophils and platelets has been described in the pathology of several diseases, most notably asthma (1–3) and hypereosinophilic syndrome (4, 5). Upon activation, the eosinophil releases four main granule proteins; major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO), into the extracellular fluid (6–8), where they are able to exert a variety of biological actions (9, 10). For example, both MBP and ECP are potent helminthotoxins, while EDN is a weaker helminthotoxin (11). MBP is also cytotoxic for mammalian cells and likely acts as a mediator of tissue damage in bronchial asthma (12, 13). EDN and ECP produce the Gordon phenomenon, a neurologic syndrome characterized by stiffness, muscle weakness, and wasting and ataxia, when injected into rabbits or guinea pigs (14, 15). EPO exhibits pronounced cytotoxic activity when combined with hydrogen peroxide and a halide, and has been shown to be an agonist for mast cell degranulation (16, 17). Given the wide range of biological activities of the eosinophil granule proteins, we hypothesized that one or more of these proteins may be a platelet agonist. The results of studies that support this hypothesis are described here.

Materials and Methods

Purification of Eosinophil Granule Basic Proteins. The four proteins, MBP, EPO, ECP, and EDN, that constitute the main basic eosinophil granule basic proteins were purified to physical homogeneity from the eosinophils of patients with hypereosinophilic syndrome as previously described (18–20).

Preparation and [¹⁴C]5-Hydroxytryptamine Loading of Washed Human Platelets. Freshly drawn platelet-rich plasma from healthy normal adult blood donors was obtained from the Mayo Blood Bank. Platelets were isolated from this platelet-rich plasma as previously described (21). The washed platelets were resuspended in Tyrode's

buffer at a concentration of 2×10^8 cells/ml for all studies except those used in the measurement of the secretion of β -N-acetylglucosaminidase (β -NAGase), where the platelet concentration was 5×10^8 cells/ml. Platelets to be used to assay 5-hydroxytryptamine secretion were loaded with [¹⁴C]5-hydroxytryptamine before resuspension in Tyrode's buffer (21).

Assay of 5-Hydroxytryptamine, β -Thromboglobulin, and β -N-Acetylglucosaminidase Secretion. For all studies involving measurement of 5-HT secretion, unstirred reaction mixtures containing 100 μ l of [¹⁴C]5-HT loaded, washed platelets and 15 μ l of the various eosinophil granule proteins were incubated for 15 min at room temperature. Secretion was terminated by the addition of 15 μ l of 8% paraformaldehyde, and the reaction was centrifuged to pellet the platelets. Aliquots of the supernatant were counted to quantify 5-HT release. Release is reported as a percentage of the total platelet [¹⁴C]5-HT, as determined by treatment of the loaded platelets with 2% Triton X-100. All reactions were performed in duplicate. For studies in which the effect of indomethacin or PGE₁ on 5-HT secretion was examined, the washed, loaded platelets were treated with freshly prepared 10 μ M indomethacin or 1 μ M PGE₁ for 15 min before stimulation with the eosinophil granule proteins.

For studies measuring the secretion of β -thromboglobulin (β -TG) and β -NAGase, the reaction mixtures contained 500 μ l of washed, unloaded platelets and 50 μ l of eosinophil proteins. The reactions were terminated after 15 min at room temperature by centrifugation through a 1:1 mixture of Apezion A oil (Biddle Instruments, Blue Bell, PA) and butyl phthalate, since paraformaldehyde is known to interfere with the assay of these components. Aliquots of the resulting aqueous upper layer were then assayed as previously described (22) for β -TG and β -NAGase using a RIA and a spectrophotometric assay, respectively.

Results and Discussion

When unstirred suspensions of [¹⁴C]5-HT loaded, washed human platelets were exposed to purified preparations of the four eosinophil granule proteins, only MBP and EPO evoked

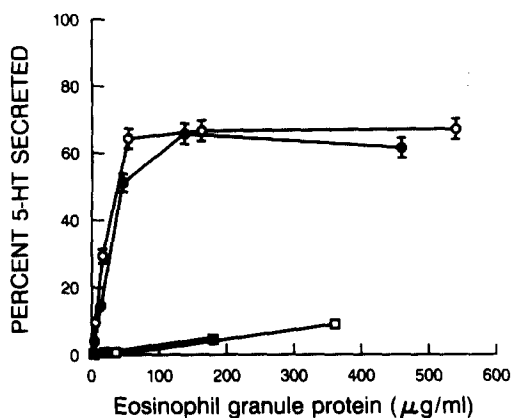


Figure 1. Dose-response curves for the secretion of platelet 5-HT by eosinophil granule proteins. Shown are the dose-dependencies for secretion of [¹⁴C]5-HT from washed human platelets mediated by MBP (●), EPO (○), ECP (■), and EDN (□). The secretion is expressed as a percentage of the total platelet [¹⁴C]5-HT. The values shown represent the mean ± SEM 5-HT secretion determined from seven independent platelet preparations for MBP and EPO, and two independent platelet preparations for ECP and EDN.

the dose-dependent release of 5-HT (Fig. 1). Both proteins released ~60–70% of the total 5-HT with EC₅₀s of 28 and 20 µg/ml for MBP and EPO, respectively. This release was due to secretion rather than to toxic damage (12, 16, 23) to the platelets because MBP and EPO released only 1% and 4% of the total platelet lactate dehydrogenase under conditions where maximal 5-HT release occurred. In contrast to the 5-HT secretion evoked by MBP and EPO, neither ECP nor EDN stimulated appreciable secretion of 5-HT. This observation suggests that the secretion by MBP or EPO is not simply due to the highly cationic nature of the proteins because ECP and MBP have essentially identical pI's (24). Furthermore, several pieces of evidence suggest that the secretion mediated by EPO was not due to its enzymatic activity. First, EPO promoted secretion in the absence of exogenously added substrates, hydrogen peroxide, and bromide or iodide. Second, EPO induced the same amount of 5-HT secretion in the presence of 1,100 U/ml of catalase, a concentration of catalase sufficient to degrade any endogenously produced hydrogen peroxide. Finally, heparin, which had no effect on the catalytic activity of EPO, inhibited EPO-dependent secretion, presumably by binding to the EPO. In this regard, the activation of platelets by EPO differs from the EPO mechanism of mast cell activation. Mast cell secretion by EPO has been shown to be absolutely dependent on the enzymatic activity of EPO and required exogenously added hydrogen peroxide and halide (17).

The secretion of 5-HT induced by MBP or EPO was not dependent upon the production of cyclo-oxygenase metabolites of arachidonic acid. As shown in Fig. 2, the presence of 10 µM freshly prepared indomethacin, a concentration sufficient to inhibit completely platelet thromboxane A₂ formation (25), had no effect on the dose-response curves for 5-HT release mediated by MBP or EPO. Also shown as a control in Fig. 2 is the inefficacy of indomethacin on thrombin-

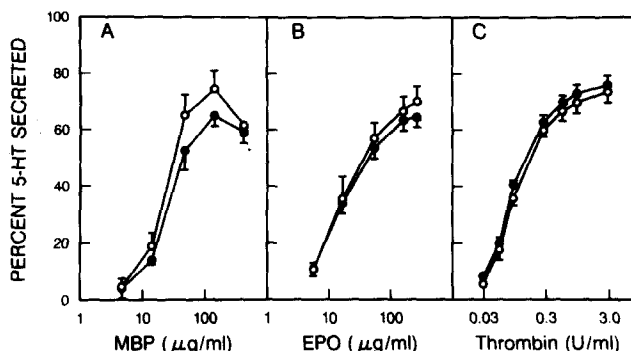


Figure 2. Effect of indomethacin on the MBP and EPO dose-response curves for platelet 5-HT secretion. Shown are the dose dependencies for the secretion of [¹⁴C]5-HT from washed human platelets mediated by MBP (A), EPO (B), and human thrombin (C). In each panel, the dose-response curves for [¹⁴C]5-HT secretion from untreated, control platelets (○) and platelets preincubated for 15 min with 10 µM indomethacin (●) are depicted. The secretion is expressed as a percentage of the total platelet [¹⁴C]5-HT. The values shown are the mean ± SEM secretion from the platelets of four independent donors for MBP and EPO and three independent donors for thrombin.

mediated 5-HT secretion. Detwiler and colleagues (26, 27) have proposed that platelet agonists can be divided into weak and strong categories. Strong agonists are capable of promoting secretion from unstirred platelet suspensions, and the secretion is independent of thromboxane A₂ formation. Based on our observations, both MBP and EPO can be classified as strong platelet agonists.

Although not originally proposed by Detwiler and colleagues as a criterion for strong agonists, several investigators have shown that secretion of lysosomal enzymes is a property restricted to strong agonists (28). As depicted in Fig.

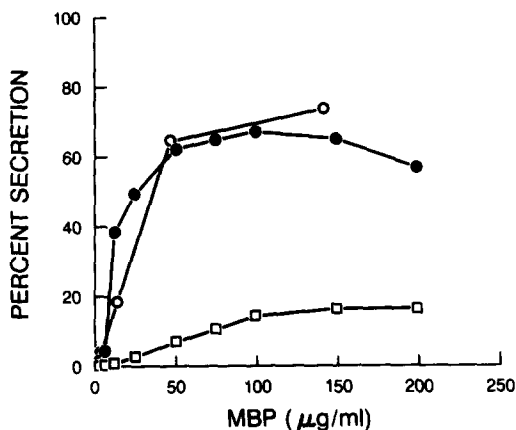


Figure 3. Dose-response curves for the MBP-dependent secretion of platelet 5-HT, β-thromboglobulin, and β-N-acetylglucosaminidase. Shown are the dose-response curves for the secretion of [¹⁴C]5-HT (○) from the platelet dense granules, β-thromboglobulin (●) from the α granules, and β-N-acetylglucosaminidase (□) from the lysosomes of washed, human platelets. The secretion is reported as a percentage of the total platelet content of 5-HT, β-TG, or β-NAGase. The values shown are the mean secretion from the platelets from three independent donors.

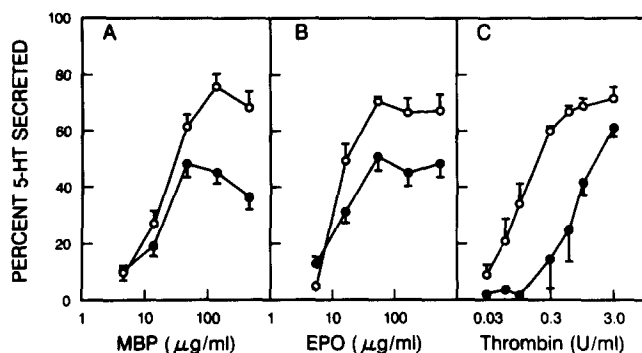


Figure 4. Effect of PGE₁ on the MBP and EPO dose-response curves for platelet 5-HT secretion. Shown are the dose dependencies for the secretion of [¹⁴C]5-HT from washed human platelets mediated by MBP (A), EPO (B), and human thrombin (C). In each panel, the dose-response curves for [¹⁴C]5-HT secretion from untreated, control platelets (○) and platelets preincubated for 15 min with 1 µM PGE₁ (●) are depicted. The secretion is expressed as a percentage of the total platelet [¹⁴C]5-HT. The values shown are the mean ± SEM secretion from the platelets of three independent donors for MBP and thrombin and the mean ± range secretion from the platelets of two independent donors for EPO.

3, MBP promotes the secretion of components of the dense granules, α granules, and lysosomes in a dose-dependent manner. The similarity in extent of secretion and MBP dose requirements for secretion from the dense and α granules, along with the lesser release from lysosomes at higher MBP concentrations, is consistent with the effects reported for other strong platelet agonists (28). As expected from their failure to induce 5-HT secretion, both EDN and ECP also failed to induce secretion of β-TG from the α granules (data not shown).

Although both MBP and EPO can be classified as strong platelet agonists, their mechanism of platelet activation appears to differ from that of other strong platelet agonists based on the effect of PGE₁ on platelet activation. PGE₁ is an inhibitor of platelet activation by all known platelet agonists. It is believed to act by increasing the cellular level of c-AMP, which, in turn, activates a c-AMP protein kinase. Activation of this protein kinase results in the phosphorylation of a platelet

protein thought to regulate cytosolic calcium concentrations with the resulting inhibition of platelet granule secretion (29). As illustrated in Fig. 4, the strong platelet agonist thrombin can overcome this inhibition at sufficiently high agonist levels. The rightward shift in the secretion dose-response curve in the presence of PGE₁ is typical of the effect of PGE₁ on previously described strong platelet agonists (30, 31). The effect of 1 µM PGE₁ on MBP- and EPO-mediated secretion is, however, quite different. PGE₁ had relatively little effect on 5-HT secretion at low levels of these agonists but reduced the maximal secretion by 30–50% without shifting the dose curves. Additional studies indicated that high doses of PGE₁ had no additional effect on secretion (data not shown). These results suggest that the mechanism of platelet activation by MBP and EPO is distinctly different from that of other strong platelet agonists.

Activation of platelets by eosinophil granule proteins may play a role in the pathogenesis of several diseases. The degranulation of eosinophils and subsequent release of granule proteins in the airways may provide a mechanism for the localized activation of extravascular platelets in asthma. Abnormalities of platelet function have been shown in patients with asthma. Also, platelets recovered from the bronchoalveolar lavage fluid from asthmatic patients are often associated with eosinophils and show evidence of degranulation (1, 2). In these patients, MBP has been found deposited on the damaged respiratory epithelium (1, 12, 13) and also recovered in bronchoalveolar lavage fluid (32) and sputum (13) at concentrations consistent with those required for platelet activation found in this study. Platelet activation by MBP and EPO may also be important in other diseases characterized by high levels of eosinophils, such as hypereosinophilia syndrome. This disease is characterized by high levels of blood eosinophils and cardiac involvement associated with the formation of mural thrombi and emboli (4, 5). MBP is deposited on the endocardium and intima of blood vessels at the sites where thrombus formation almost uniformly occurs (33). Platelet activation by the deposited MBP may provide an explanation for the close association between eosinophil degranulation and thrombus formation in this disease.

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