PHYSIOLOGY

Activation of Rat Mast Cells upon Stimulation of Protease-Activated Receptor (PAR-1)

B. A. Umarova, T. N. Dugina, E. V. Shestakova, E. Gluza,* and S. M. Strukova

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In vivo experiments on the model of wound healing showed that thrombin and thrombin receptor agonist TRAP-6 stimulated heparin secretion by mast cells in rat subcutaneous fat: the saturation of mast cells with heparin decreased, while degranulation and granulolysis increased. *In vitro* studies showed that TRAP-6 caused a dose-dependent release of β-hexosaminidase from peritoneal mast cells. TRAP-6 also induced heparin release from these cells and inhibition of amidase activity of thrombin. Heparin released from mast cells had low anticoagulant activity. These data suggest that activation of mast cells with thrombin is mediated by PAR-1.

Key Words: *mast cells; proteolytically cleaved receptor; thrombin; β-hexosaminidase; heparin*

Thrombin is a polyfunctional enzyme involved in the regulation of some physiological processes (hemostasis, vascular tone, and wound healing) and playing a role in the pathogenesis of thrombosis, atherosclerosis, and Alzheimer's disease. Postcoagulation effects of thrombin are realized via its interaction with various cells, including endotheliocytes, leukocytes, fibroblasts, and smooth muscle, nerve, and mast cells (MC) [3].

Thrombin possesses antiinflammatory properties, activates chemotaxis of leukocytes and their adhesion to the endothelium, stimulates expression of E- and P-selectins by endotheliocytes and platelets, increases endothelial permeability, and modulates vascular tone [7,8]. Thrombin also activates MC [4,11] secreting some potent inflammatory mediators, in particular histamine, platelet-activating factor, cytokines, *etc.*

The mechanisms of interaction between thrombin and MC are poorly understood. It is known that thrombin induces the secretion of histamine and β-hexosaminidase by bone marrow MC in mice [11] and secretion of heparin by peritoneal MC in rats [4]. Binding of thrombin to peritoneal MC membranes is a specific, rapid, saturable, and reversible process independent on the active site of the enzyme [3]. It is believed that there are several types of thrombin receptors on MC [14]. One of these receptors responsible for activation of MC with thrombin is a seven-transmembrane-domain proteolytically activated type I receptor (PAR-1) belonging to the G-protein-dependent receptor superfamily [14].

The goal of the present study was to prove the involvement of PAR-1 in *in vivo* and *in vitro* activation of rat peritoneal MC with thrombin.

MATERIALS AND METHODS

α-Thrombin obtained by the method described elsewhere [2], peptide SFLLRN, thrombin receptor agonist (TRAP-6, Jena Institute of Molecular Biology), antithrombin III (Imtek), heparin (Serva), substrate of

Department of Human and Animal Physiology, M. V. Lomonosov Moscow State University; *Center of Vascular Surgery and Medicine, F. Schiller Jena University, Erfurt, Germany

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β-hexosaminidase (*p*-nitrophenyl N-acetyl-β-D-glucopyranoside, Sigma), synthetic chromogenic thrombin substrate H-D-Phe-Pip-Arg-pNA (S-2238, Kabi Diagnostica), substance 48/80, and bradykinin (Sigma) were used.

Experiments were performed on male and female outbred albino rats weighing 200-250 g.

The ability of TRAP-6 to simulate *in vivo* effects of thrombin on MC was studied on rat model of wound healing.

Nembutal-anaesthetized rats (40 mg/kg) were fixed on a table, dorsal skin was treated with 70% ethanol, and 1-cm skin fragment was dissected. Thrombin (30 μl, 2×10^{-6} M) or MTRAP-6 (30 μl, 2×10^{-3} M) were then applied to the wound. Control rats were treated with equivalent volumes of 0.85% NaCl.

On day 3 after skin damage, the rats were decapitated. Morphometry of MC population in the wound was conducted using film preparations of subcutaneous fat [1]. The analysis was performed at this period, because phase I of wound healing (inflammation) lasting for 3-5 days after tissue damage is characterized by the involvement of MC releasing various inflammatory mediators, including histamine, chemoattractants, proteases with mitogenic activity, and heparin (cofactor of basic fibroblast growth factor).

Films were fixed in 4% formaldehyde (pH 7) and stained with 0.1% toluidine blue (pH 4.9). The index of saturation of MC with heparin and indexes of degranulation and granulolysis were calculated to estimate the degree and mechanisms of heparin secretion.

Rat peritoneal MC purified in a Ficoll gradient were obtained as described elsewhere [4].

β-Hexosaminidase secreted by MC was assayed by the method described elsewhere [13]. The cells (2×10^5) were activated with 2×10^{-9}-2×10^{-7} M thrombin or 2×10^{-5}-2×10^{-3} M TRAP-6 for 10 min and then centrifuged at 3000 rpm for 10 min. The supernatant was collected, and an equivalent volume of β-hexosaminidase substrate (0.4 mM in 0.08 M citrate buffer, pH 4.5) was added. The mixture was incubated at 37°C for 2 h. The reaction was stopped by adding an equivalent volume of cold 0.2 M glycine (pH 10.7). Activity of β-hexosaminidase in MC precipitate preliminary destroyed with 0.2% Triton X-100 was estimated by the same method. An equivalent volume of isotonic NaCl (instead of the ligand) was added to cells to analyze spontaneous heparin secretion.

The content of β-hexosaminidase was evaluated from its activity, which was determined spectrophotometrically by the content of colored substrate cleavage product p-nitrophenol. The percentage of released β-hexosaminidase was calculated by the formula: $[S/(S+P)]\times100\%$, where S and P are enzyme activities in the supernatant and cell precipitate, respectively. The content of the enzyme in activated cells was expressed in % of the control (β-hexosaminidase content in unstimulated cells).

Heparin released from MC was assayed by its antithrombin activity (inhibition of amidase activity of thrombin in the presence of antithrombin III excess) and by the reaction with azure [15]. The activity (IU) and content (μg) of heparin were estimated from calibration curves constructed by using commercial heparin with activity of 100 IU/mg. The measurements were performed on a Specord M-40 spectrophotometer. The results were analyzed by Student's t test.

RESULTS

Thrombin and TRAP-6 applied to the wound markedly activated MC (Fig. 1). The content of heparin in MC decreased by 2.2 and 5.4 times, respectively, compared with the control. The indexes of degranulation and especially of granulolysis increased (Fig. 1). These results indicated that granulolysis is the main mechanism of heparin secretion by MC during their stimulation with thrombin and TRAP-6.

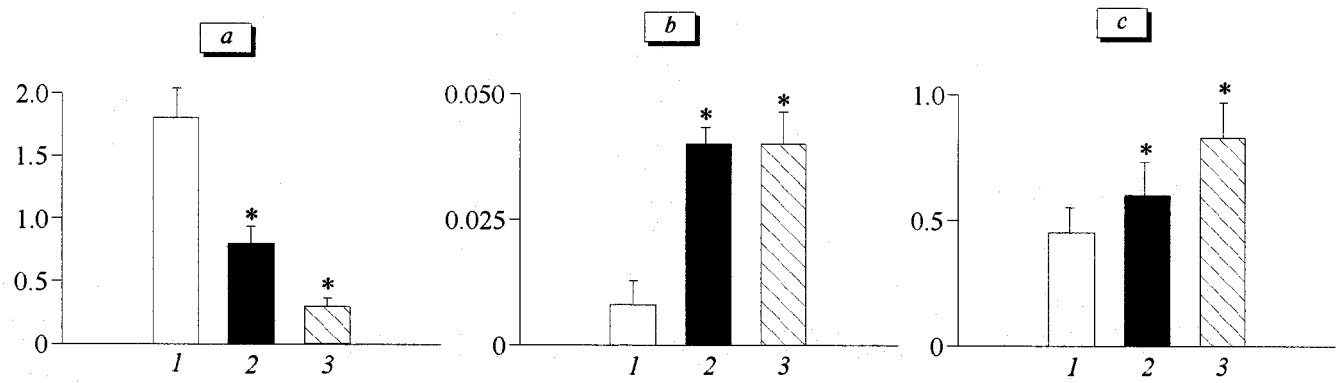

Fig. 1. Indexes of saturation with heparin (*a*), granulolysis (*b*), and degranulation (*c*) of mast cells of rat subcutaneous fat in wound on day 3 after skin damage after application of 0.85% NaCl (*1*), 30 μl 2×10^{-6} M thrombin (*2*), and 30 μl 2×10^{-3} M TRAP-6 (*3*). *$p<0.011$ compared to *1*.

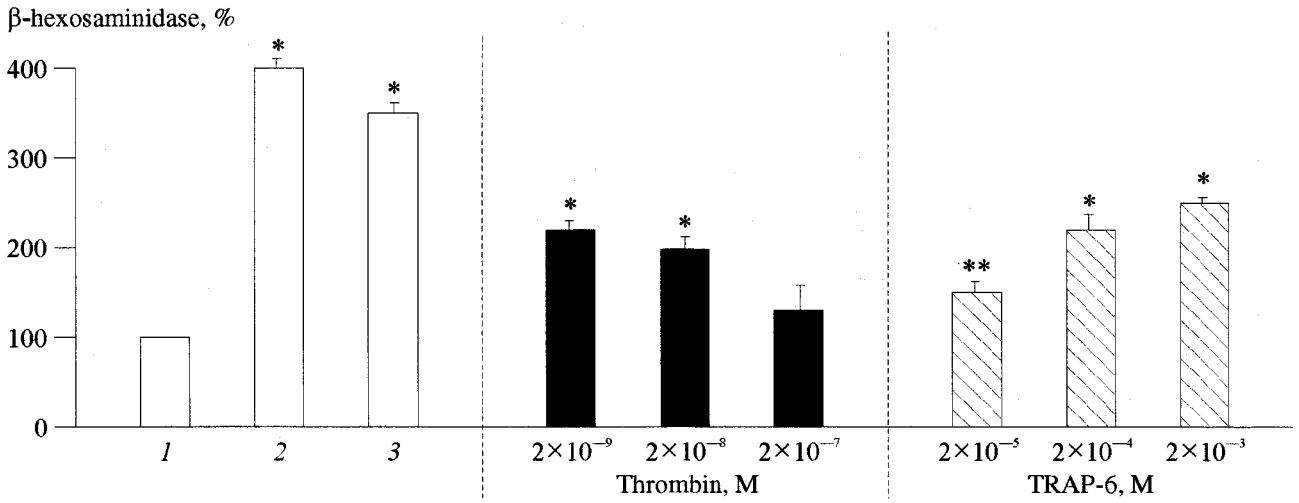

Fig. 2. Release of β-hexosaminidase from 2×10^5 mast cells activated with 2 μg/ml substance 48/80 (*2*), 10^{-4} M bradykinin (*3*), and thrombin or TRAP-6 in various concentrations. Spontaneous release of β-hexosaminidase (*1*). *$p<0.001$ and **$p<0.01$ compared with *1*.

Thus MC intensively secrete heparin during inflammation phase of wound healing. The activation of MC after the application of TRAP-6 to the wound suggests that proteolytically cleaved thrombin receptor PAR-1 is involved in this process.

In vitro effects of TRAP-6 on MC were studied to confirm our assumption on receptor-mediated stimulation of these cells with thrombin. Secretion of β-hexosaminidase by MC paralleled the release of histamine [1] and heparin from MC.

The effects of thrombin and TRAP-6 on MC were compared with those of substance 48/80 and bradykinin (Fig. 2). Thrombin (2×10^{-9} M) and TRAP-6 (2×10^{-5} M) stimulated β-hexosaminidase secretion by peritoneal MC by 109 and 94%, respectively.

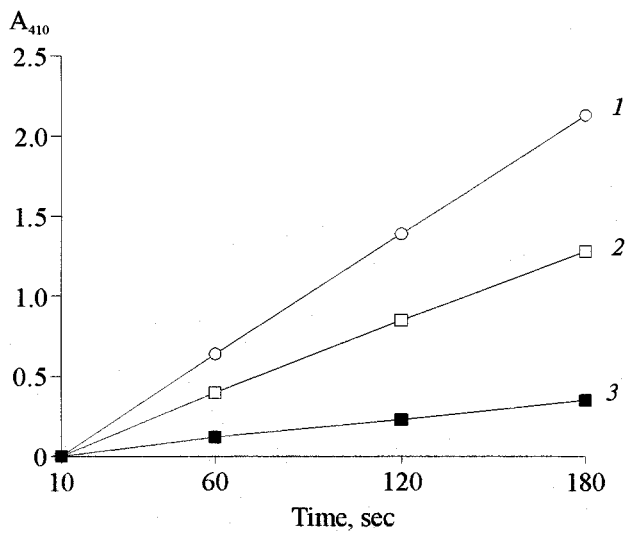

Fig. 3. Amidase activity of thrombin in the control (*1*) and in the presence of supernatants of 5×10^6 (*2*) and 10^7 mast cells (*3*) activated with TRAP-6 (2×10^{-4} M).

It should be emphasized that the effects of TRAP-6 and thrombin were comparable only in the case, when the concentration of TRAP-6 4-fold surpassed that of thrombin, which agrees with previous data [8] and can be due to the presence of several types of thrombin receptors on cell membranes and lower affinity of the synthetic peptide agonist for PAR-1 [5]. The absence of the phase of proteolytic cleavage and unchanged conformation of the receptor during its activation with the peptide agonist probably hinder their interaction, which is manifested in decreased affinity for TRAP-6.

TRAP-6 induced a dose-dependent release of β-hexosaminidase from MC (Fig. 2). At the same time, TRAP-6 in high concentrations (2×10^{-7} M) slightly decreased the secretion of β-hexosaminidase (Fig. 2). This was probably due to the presence of several types of thrombin receptors on MC differing in their affinity for the enzyme and triggering some mechanisms activating or suppressing its secretion, as well as due to inactivation of thrombin with MC-derived chymase [10]. In addition, the negative feedback regulation of β-hexosaminidase secretion related to the release of NO can also be involved [12].

Heparin released from MC plays a role in tissue repair. Heparin is a cofactor of fibroblast growth factor enhancing angiogenesis. Previous studies revealed that heparin is released from rat peritoneal MC after their activation with thrombin [4]. It was shown that intravenous injection of thrombin [2] or intramuscular administration of TRAP-6 (into the plantar muscle) [6] induces degranulation of MC detected morphometrically. However, we found no direct evidence for heparin secretion by MC after their stimulation with TRAP-6.

Supernatant of MC (5×10^6) activated with TRAP-6 sharply inhibited cleavage of the chromogenic sub-

strate with thrombin. The supernatant of 10^7 MC activated with TRAP-6 completely blocked thrombin amidase activity (Fig. 3). This antithrombin activity is related to the release of heparin from MC. The antithrombin activity of secreted heparin evaluated by this method was 0.24 IU/10^7 cells.

The data on anticoagulant activity of heparin secreted by MC are scanty and ambiguous. Some authors reported that MC-derived heparin displays low anticoagulant activity [9].

In our experiments, 10^7 MC activated with TRAP-6 secreted 48.7±4.9 μg heparin, which corresponded to 4.9 IU. A comparison of calculated antithrombin activity of heparin with activity estimated by the amidolytic method indicated that specific antithrombin activity of MC-derived heparin is lower than that of commercial unfractionated heparin.

Hence, thrombin *in vivo* and *in vitro* stimulates the secretion of mediators by MC. The secretion marker β-hexosaminidase is released together with histamine [11] and heparin displaying low anticoagulant activity. These results and previous data [4,11] indicate that thrombin is a nonimmune activator of MC, which can stimulate (similarly to other regulatory peptides substance P, bradykinin, and neurotensin) the release of inflammatory mediators from MC. At the same time, the effects of thrombin on MC are realized via its binding to PAR-1, which is confirmed by activation of MC not only with thrombin, but also with TRAP-6 simulating the effect of thrombin on MC.

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