Heparin-binding protein (HBP/CAP37): A missing link in neutrophil-evoked alteration of vascular permeability

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Polymorphonuclear leukocyte infiltration into tissues in host defense and inflammatory disease causes increased vascular permeability and edema formation through unknown mechanisms. Here, we report the involvement of a paracrine mechanism in neutrophil-evoked alteration in endothelial barrier function. We show that upon neutrophil adhesion to the endothelial lining, leukocytic β_2 integrin signaling triggers the release of neutrophil-borne heparin-binding protein (HBP), also known as CAP37/azurocidin, a member of the serprocidin family of neutrophil cationic proteins. HBP induced Ca^{++}-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial-cell monolayers *in vitro*, and increased macromolecular efflux in microvessels *in vivo*. Moreover, selective inactivation of HBP prevented the neutrophils from inducing endothelial hyperpermeability. Our data suggest a fundamental role of neutrophil-derived HBP in the vascular response to neutrophil trafficking in inflammation. Targeting this molecule in inflammatory disease conditions offers a new strategy for prevention of endothelial barrier dysfunction caused by misdirected leukocyte activation.

The inflammatory response to tissue injury or infection is characterized by changes in the microcirculation leading to impaired endothelial barrier function, plasma protein and fluid efflux, and extravasation of white blood cells. These adjustments are critical in the normal host defense, yet key elements in the pathogenesis of inflammatory disease. Vascular permeability is modified by inflammatory mediators acting directly on the endothelial cells (ECs), and by leukocytes stimulated by chemotactic factors[1]. Polymorphonuclear leukocytes (PMN), predominantly neutrophilic granulocytes, are the first white blood cells recruited to the inflamed tissue area. As shown 20 years ago[2], and later confirmed in numerous reports, the alteration in vascular permeability evoked by chemotactic inflammatory mediators requires the presence of PMN, or more specifically, an intact adhesive function of these cells. Leukocytic β_2 integrins (CD11/CD18) are critical in this respect because inhibition of their receptor function effectively prevents both PMN adhesion to the EC lining and the associated plasma leakage[3,4]. However, the precise mechanisms by which activated PMN may control EC permeability remain elusive[5]. Recently, we demonstrated that chemoattractant-induced PMN activation and engagement of β_2 integrins triggers neutrophil secretion and release of a soluble factor(s), which via paracrine action stimulates active cytoskeletal reorganization in adjacent ECs, leading to intercellular gap formation and increased EC protein permeability[6]. Here, we report on the nature of this neutrophil-derived factor that seemingly is responsible for rapid PMN-induced alterations in vascular permeability and fluid efflux in inflammation. We identify the factor as heparin-binding protein (HBP)[7], also known as CAP37/azurocidin[8,9], an inactive serine protease homologue belonging to the serprocidin family of neutrophilic cationic proteins[10].

Engagement of neutrophil β_2 integrins triggers HBP secretion
We activated human PMN in suspension through antibody-induced cross-linking of the common β_2 integrin subunit CD18. This mode of stimulation mimics adhesion-dependent engagement of β_2 integrins and leads to the secretion of proteins in the molecular range of 25–30 kD, which seem to be involved in PMN-induced alteration in EC permeability[6]. Here, we demonstrate, through western-blot analysis, the presence of HBP in the cell-free postsecretory supernatant obtained after CD18 cross-linking in suspended PMN (Fig. 1). The release of HBP was confirmed with ELISA (data not shown).

Neutrophil-derived HBP increases endothelial permeability
In order to establish the role of HBP in PMN-induced derangement of the endothelial barrier we first examined the ability of HBP to provoke permeability changes in cultured bovine aorta EC monolayers. Administration of recombinant human HBP (rHBP) (25–75 µg/ml) to the luminal side of the EC monolayer elicited a prompt and dose-dependent decrease in transendothelial electrical resistance, reaching lowest value within 15 min after stimulation (30 ± 3% of control at 75 µg/ml) and then leveling off (Fig. 2). Concomitant with the decrease in electrical resistance was an increase in macromolecular permeability of the monolayer. The activity profile for rHBP on EC permeability was similar to that previously reported for stimulation with cell-free
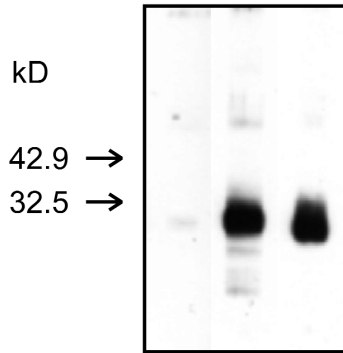
Fig. 1 Immunorecognition of HBP released from activated PMN. The presence of HBP in post-secretory supernatant of PMN subjected to CD18 cross-linking (middle lane) was assessed by western blotting. Incubation with control rabbit IgG did not recognize the protein (left). Recombinant human HBP served as positive control (right). The apparent difference in molecular weight is explained by less glycosylation in insect cells in which recombinant HBP was expressed.

supernatant from PMN activated through CD18 cross-linking[6], and verified also in experiments using EC monolayers of human origin (HUVEC; data not shown).

We confirmed the permeability-increasing activity of rHBP *in vivo* through intravital microscopy of microcirculation in the hamster cheek pouch. Topical application of rHBP (100 µg/ml) to the exposed tissue provoked prompt macromolecular leakage from postcapillary and small venules (Fig. 3*a*). Leaky spots were seen within two minutes and a maximum occurred after 7–10 minutes. After the tissue was washed with buffer, the leakage slowly subsided and disappeared completely within 30 min.

HBP induces Ca++-dependent rearrangement of EC cytoskeleton

We used laser-scanning confocal microscopy to determine if HBP alters EC barrier function by inducing Ca++-dependent reorganization of the EC cytoskeleton. Administration of rHBP (75 µg/ml) to confluent EC monolayers (loaded with the Ca++-sensitive fluorophore fluo-3/AM) provoked a rapid increase in fluorescence intensity, indicating a rise in intracellular free [Ca++], which peaked within 1–2 min (Fig. 3*b*) and then gradually declined toward baseline levels. The Ca++ mobilization stimulated rearrangement of the EC cytoskeleton as indicated by the formation of actin-stress fibers spanning the cells (Fig. 3*c*). The coupling between the Ca++-dependent structural change in EC and impaired EC barrier function was evident because pretreatment with the cell-permeant calcium-chelator BAPTA/AM abolished the HBP-evoked EC responses and increase in EC permeability (data not shown).

Anti-HBP attenuates PMN-evoked increases in EC permeability

We administered purified antibodies against HBP or non-specific rabbit IgG (50 µg/ml) to the EC monolayer before stimulation with rHBP (75 µg/ml). Anti-HBP, but not control rabbit IgG, attenuated the HBP-induced decline in electrical resistance (Fig. 4) as well as the increase in macromolecular permeability (net clearance volume was reduced by ~60% from 20.4 ± 3.4 µl in control to 8.6 ± 1.7 µl over a 60-min period).

In order to examine whether activated PMN exert effects on EC barrier function via the release of HBP, we sought to antagonize the cell-mediated response with anti-HBP antibodies. Stimulation of ECs either with cell-free postsecretory supernatant obtained after CD18 cross-linking in suspended PMN or through transendothelial chemotactic activation of PMN layered on the EC monolayer (1×10^{-8} M LTB$_4$ or 1×10^{-7} M fMLP) both evoked changes in EC permeability similar to that seen after challenge with rHBP (75 µg/ml) (Fig. 4). When stimulation

was induced in the presence of antibodies against HBP, the increase in permeability was markedly suppressed (Fig. 4). In fact, the ability of anti-HBP to antagonize PMN-induced permeability changes exactly matched their potency in inhibiting the rHBP-induced increase in permeability.

Though human HBP is devoid of catalytic function, it is still capable of binding Kunitz type protease inhibitors (for example, aprotinin) with high affinity[11,12]. We therefore investigated whether aprotinin could interfere with the activity of HBP. Preincubation of rHBP (75 µg/ml) with aprotinin (20 µg/ml) almost negated the HBP-evoked decrease in EC electrical resistance; in the presence of aprotinin the decline was only 10 ± 2% as compared with 70 ± 3% after stimulation with HBP alone ($P < 0.01$; $n = 6$). We further demonstrated that aprotinin antagonized the effect of HBP by binding specifically to the inactive 'catalytic' site of the HBP molecule. A mutant variant of HBP ([G175Q]HBP), which does not bind aprotinin because of the introduction of a bulky amino acid (replacement of glycine by glutamine) in the dead catalytic cleft[12], possessed permeability increasing activity similar to that of native HBP. However, aprotinin was unable to reverse its effect (67 ± 6% and 64 ± 5% decrease in electrical resistance with aprotinin absent and present, respectively; $n = 6$).

HBP mediates PMN-evoked alterations in EC permeability

The fact that EC hyperpermeability evoked both by PMN activation and rHBP stimulation was inhibited to the same extent by anti-HBP clearly indicates that secreted HBP is involved in PMN-induced alteration in vascular permeability. However, in the PMN secretion, in addition to HBP, we identified the structurally related granule proteins elastase and cathepsin G, previously im-
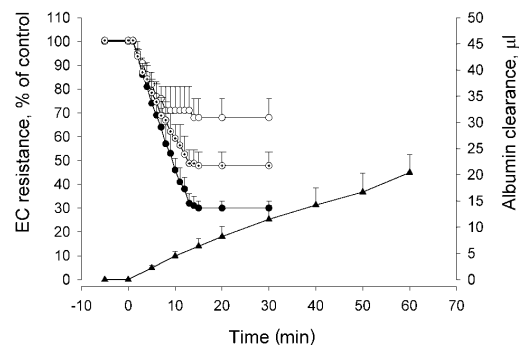

Fig. 2 Kinetics of changes in EC monolayer permeability in response to stimulation with rHBP. The permeability-increasing activity of HBP manifested as a dose-dependent decrease in transendothelial electrical resistance; rHBP 25 (○), 50 (◉) and 75 (●) µg/ml, measured on left *y*-axis. It also manifested as an increase in protein flux across EC monolayer (albumin clearance) as illustrated for rHBP 75 µg/ml (▲), measured on right *y*-axis. Data are mean ± s.d.; $n = 6–8$.
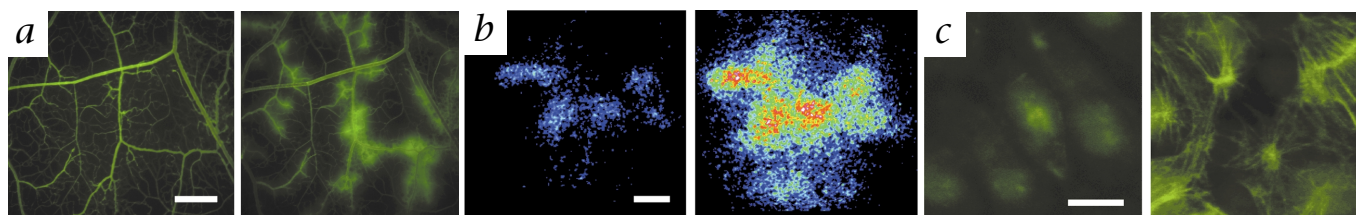
Fig. 3 HBP triggers macromolecular leakage *in vivo* and active cytoskeletal rearrangement in cultured ECs. *a*, *In vivo* micrographs of the hamster cheek pouch microcirculation before (left) and 6 min after (right) topical administration of rHBP 100 µg/ml. Venular macromolecular efflux is visualized through use of intravenously injected FITC-dextran as a plasma tracer. Results are representative of 6 separate experiments. Scale bar, 500 µm. *b*, Change in fluorescence intensity of a fluo-3-loaded EC monolayer in response to stimulation with rHBP (75 µg/ml) depicting a rise in EC cytosolic free Ca^{++}. The images, captured before (left) and 100 s (right) after stimulation, show resting and peak fluorescence intensity of the same grouping of 5 ECs. The response is representative of measurements in 8 separate monolayers. Scale bar, 25 µm. *c*, Staining for actin filaments with FITC-phalloidin reveals the formation of actin stress fibers in ECs stimulated with rHBP 75 µg/ml for 15 min (right) as compared with unstimulated cells (left). Micrographs are representative of >10 separate experiments. Scale bar, 25 µm.

plicated in inducing EC hyperpermeability. In order to find out to what extent these proteins contributed to the permeability increasing activity of the PMN-derived secretion we sought to deplete the postsecretory supernatant of HBP, elastase and cathepsin G, respectively. Specific immunoadsorption could completely and selectively remove each of these three proteins from the supernatant, as verified by sandwich ELISA (data not shown). Removal of HBP rendered the postsecretory supernatant completely inactive with regard to its capacity to induce EC permeability changes, whereas supernatant depleted of elastase and/or cathepsin G was found to induce a fall in electrical resistance of the same magnitude as that evoked by stimulation with native supernatant (Fig. 5). Clearly, these data suggest an exclusive role of HBP in mediating PMN-evoked alteration in vascular permeability.

All of the serprocidins display a positive surface charge. In HBP, a large patch of basic amino acid residues is concentrated on one side of the protein, giving the molecule a strong polarity[13]. The surface electrostatic potential and dipole moment in HBP was calculated and rendered using GRASP (ref. 14), and compared with that in elastase. The centering and direction of the monopole vector in HBP and in elastase were found to be nearly identical. However, the dipole moment in HBP was three times larger (1089 Debye units) than that in elastase (358 Debye units) despite the great sequence homology between the molecules. Anionic compounds may interfere with the action of cationic proteins via charge interactions. We therefore examined whether the effect of rHBP on endothelial permeability could be neutralized by the polyanion dextran sulfate, similarly to what we previously have found for the EC response to stimulation with the PMN-derived post-secretory supernatant[6]. Indeed, the permeability change in response to HBP stimulation was completely prevented by simultaneous administration of dextran sulfate (1 mg/ml) (Fig. 4). Again, the effectiveness by which the

EC response to HBP stimulation was antagonized was strikingly similar to that found for the PMN-induced permeability increase.

Discussion

PMN recruitment to sites of injury or infection constitutes the body's first line of defense against noxious stimuli. Stimulated by chemotactic mediators, these cells adhere to the vessel wall and trigger alterations in endothelial barrier function, leading to plasma exudation and edema formation[5]. PMN accumulation in tissues and loss of endothelial integrity has long been implicated in the development of diverse cardiovascular and inflammatory disorders. Accordingly, considerable interest has been directed towards understanding the mechanistic link between activated PMN and alterations in vascular permeability. Numerous PMN-derived factors have been suggested to be involved, including lipid mediators, oxidants and lysosomal enzymes, each of which have potential to induce EC hyperpermeability[15,16]. Here, we provide evidence that the PMN-evoked increase in vascular permeability in inflammation is mediated through a paracrine mechanism via the release of neutrophil-borne heparin-binding protein (HBP/CAP37/azurocidin). We show that in the PMN-EC cellular cross-talk, initiated upon chemoattractant PMN activation, engagement of leukocytic β$_2$ integrins (CD11/CD18) triggers exocytosis and release of HBP, which in turn opens up the endothelial barrier by inducing EC cytoskeletal rearrangement.

Neutrophil-borne HBP is a multi-functional protein with diverse and important activities in host defense. Besides its permeability-increasing activity documented here, HBP has bactericidal capacity[8,17], anti-apoptotic properties[18], and is chemotactic for monocytes and T cells[9,19]. HBP is a 28-kD protein belonging to the serprocidin subgroup of the chymotrypsin-like protease superfamily, which contains another three members, elastase, cathepsin G and proteinase 3 (ref. 20). The greatest similarity is between HBP and elastase, showing 44% sequence homology[7,21]. However, HBP differs from the other serprocidins in
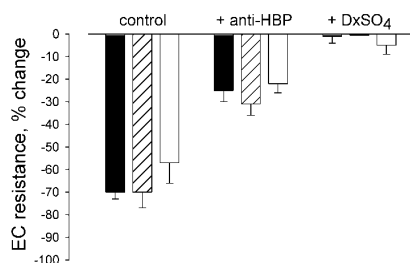

Fig. 4 Antibodies against HBP and dextran sulfate (DxSO$_4$) inhibit HBP- and PMN-evoked increase in EC permeability. Maximum change in transendothelial electrical resistance in response to stimulation with rHBP (■), CD18 cross-linking-induced PMN secretion (▨) or chemoattractant (LTB$_4$)-induced PMN activation (□) in the absence (control) and presence of anti-HBP or dextran sulfate. Similar neutralizing capacity of anti-HBP and dextran sulfate was found when fMLP instead of LTB$_4$ was used as chemoattractant (data not shown). Data are mean ± s.d.; *n* = 6 in each group.
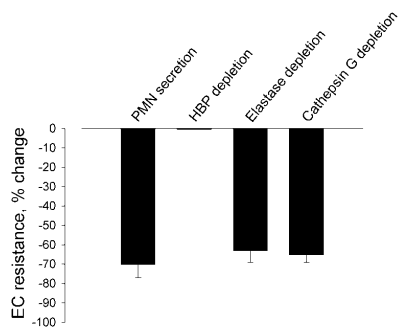
Fig. 5 Involvement of neutrophil-derived granule proteins in PMN-evoked increase in EC permeability. Maximum change in transendothelial electrical resistance was measured after stimulation with intact CD18 cross-linking-induced PMN secretion, and PMN secretion depleted of HBP, elastase and cathepsin G, respectively. The permeability-increasing activity of the PMN-derived secretion is lost after removal of HBP, whereas the activity remains intact after removal of elastase and/or cathepsin G. Data are mean ± s.d.; $n = 6$ in each group.

that the molecule lacks protease activity owing to mutations in the catalytic-site triad[20,22]. Comparisons of cDNAs for the serprocidins from different species indicate that these proteins are evolutionary well conserved[23], and it is obvious from the present and previous[19] findings that human HBP is effective across species barriers. Reportedly, HBP is localized, together with the other serprocidins, to the azurophil (primary) granule of the neutrophil[10]. However, the notion that this should be the sole storage compartment does not seem compatible with the documented instantaneous response of ECs to PMN activation[6,24], inasmuch as this granule pool is only slowly mobilized upon cell activation. Thus, localization of HBP to a more readily mobilized intracellular compartment (H. Tapper *et al.*, manuscript submitted) likely accounts for the fast kinetics of the HBP-induced EC response.

The lack of proteolytic activity of HBP suggests a non-lytic mechanism behind its permeability increasing capacity. This is supported by the fact that the rise in EC permeability is reversible and associated with Ca^{++}-dependent rearrangement of the EC cytoskeleton. HBP carries a large number of positively charged amino acid residues concentrated to one side of the protein[13], creating a strong basic patch that favors binding to negatively charged molecules. Electrostatic interactions between the basic patch and negatively charged compounds serve to explain the neutralizing capacity of dextran sulfate, previously documented with respect to polycation-induced plasma leakage *in vivo*[25]. Affinity purifications have shown that HBP binds to EC surface proteoglycans[18]. We postulate that this interaction, because of the strong dipolar nature of HBP, stimulates EC contraction through an as yet unidentified mechanism. Notably, the structurally similar elastase and cathepsin G also display positive surface charge, and have been suggested to exert effects on EC permeability by virtue of their cationic nature rather than proteolytic activity[25,26]. However, in contrast to HBP, the positively charged residues in these molecules are not concentrated predominantly to one side of the protein. Accordingly, the dipole moment in HBP is three times larger than that calculated for elastase, which may explain the unique character of HBP as mediator of PMN-evoked EC permeability changes. The fact that native HBP and the mutant variant were equally potent in inducing EC hyperpermeability indicates that the inactive 'cat-

alytic' site does not participate in this reaction. Yet, we found that the serine protease inhibitor aprotinin was capable of interfering with the permeability-increasing activity of HBP. The effect of mutant HBP, on the other hand, remained unaltered, likely because access of aprotinin to its binding site is eliminated by the mutation introduced in the dead catalytic cleft[12]. Presumably, binding of aprotinin to HBP will affect the dipole moment of the molecule, thereby diminishing the activity of HBP. Moreover, it is possible that aprotinin binding may result also in steric hindrance. In the clinic, protective effects are attributed to the use of aprotinin in patients undergoing extensive surgery and/or cardiopulmonary bypass[27]—conditions characterized by leukocyte sequestration in organs and fluid loss from the vasculature. However, the mechanisms of action of the protease inhibitor in these situations are not well defined. Here we propose a novel mechanism by which aprotinin may exert anti-inflammatory action and prevent organ failure during systemic inflammatory reactions irrespective of its function as protease inhibitor.

We have defined a paracrine mechanism in the cellular cross-talk between activated PMN and endothelial cells that regulates macromolecular efflux in conjunction with leukocyte trafficking in inflammation. Leukocyte-induced derangement of the endothelial barrier contributes to vascular dysfunction and plasma fluid leakage in diverse conditions such as infectious and allergic disease, ischemia-reperfusion, endotoxemia and extracorporeal circulation. Our findings suggest an imperative role of neutrophil-derived HBP in this process. Targeting this molecule introduces a new prospect to interfere with detrimental effects of leukocyte activation and may signify a new therapeutic strategy for intervention in inflammatory disease conditions.

Methods

EC monolayers. Bovine aorta ECs were isolated and cultured as previously described[24]. The cells were seeded onto 3-µm pore size polycarbonate filters (Tissue Culture inserts, 10 mm; NUNC, Roskilde, Denmark) at a density of 2×10^5 cells/filter and grown to confluent monolayers. The filter insert was placed in a two-compartment diffusion chamber with the monolayer separating an upper (luminal) and a lower (abluminal) fluid-filled well. Altered transendothelial electrical resistance and efflux of Evans blue dye-conjugated albumin were used as indices of stimulus-induced changes in EC barrier capacity[24].

CD18 cross-linking-induced PMN activation and secretion. PMN activation and secretion was induced through antibody cross-linking of the common β chain (CD18) of $β_2$ integrins[6]. Human PMN, isolated from leukocyte-rich plasma, were incubated with monoclonal antibody IB4 against CD18 (3 µg per 1×10^6 PMN) for 30 min, washed to remove unbound antibody, and subjected to CD18 cross-linking through addition of goat F(ab')$_2$ against mouse IgG (diluted 1:20; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). The PMN were sedimented by centrifugation at 300g for 15 min and the post-secretory cell-free supernatant was analyzed for its content of HBP through western-blot analysis. Proteins were separated by SDS–PAGE, and then transferred to nitrocellulose and incubated with rabbit antibody against human HBP (ref. 28) or control rabbit IgG (Sigma) diluted in blocking buffer. Immunoreactivity was detected using horseradish peroxidase-conjugated goat antibody against rabbit IgG (Bio-Rad) and visualized by chemiluminescence (ECL, Bio-Rad).

The PMN post-secretory supernatant was incubated under gentle rotation for 15 min with each of anti-HBP (ref. 28), anti-cathepsin G (Athens Research and Technology, Athens, Georgia) and anti-elastase (The Binding Site, Birmingham, UK) antibodies (400 µg IgG/ml supernatant) coupled to Protein A Sepharose (CL-4B, Amersham Pharmacia Biotech, Uppsala, Sweden) for selective removal of each respective protein. The Sepharose beads were spun down, and the efficacy of immunoadsorption was verified with sandwich ELISA (ref. 6).

Stimulation of EC monolayers. ECs were stimulated with recombinant human HBP (ref. 29) or the PMN post-secretory supernatant. Alternatively, PMN (2×10^6) were added to the luminal compartment (PMN to EC ratio = 10:1) and allowed to sediment for 10 min onto the EC monolayer. Activation of PMN was induced either with LTB_4 (1×10^{-8} M) or fMLP (1×10^{-7} M) added to the abluminal compartment. Changes in transendothelial electrical resistance and albumin efflux were measured as described[6]. EC monolayers were in some experiments pre-treated with the cell-permeant Ca^{++} chelator BAPTA/AM (5 μM; Molecular Probes Europe BV, Leiden, the Netherlands) for 30 min at 37 °C and washed twice. Antibody against HBP (50 μg/ml) or control rabbit IgG (50 μg/ml), or dextran sulfate (1 mg/ml; molecular weight: 500,000; Pharmacia, Uppsala, Sweden) was added to the luminal compartment prior to stimulation in some experiments. In a different set of experiments, rHBP or the mutant variant [G175Q]HBP (ref. 12) (75 μg/ml) was preincubated with the serine protease inhibitor aprotinin (20 μg/ml, Novo Nordisk, Bagsvaerd, Denmark) for 5 min prior to use.

Intracellular [Ca^{++}] and F-actin distribution. Confluent EC monolayers were incubated for 30 min at 37 °C with the Ca^{++} sensitive fluorescent probe fluo-3/AM (Molecular Probes) according to manufacturer's instructions. Changes in EC [Ca^{++}]$_i$ following stimulation with rHBP (75 mg/ml) were measured through continuous registration of fluorescence intensity using a laser-scanning confocal imaging system (Insight Plus; Meridian Instruments, Okemon, Michigan).

Confluent EC monolayers grown on Biomatrix-coated (Biomedical Technologies, Stoughton, Massachusetts) coverslips and incubated with rHBP or vehicle alone for 15 min at 37 °C were used for analysis of EC F-actin distribution. After fixation, the cells were permeabilized and stained for F-actin filaments with FITC-conjugated phalloidin (Sigma).

In vivo experiments. The left cheek pouch of anesthetized Syrian golden hamsters was everted and prepared for microscopic observation as described[30]. FITC-conjugated dextran (Mw: 150,000; Sigma) injected i.v. (250 mg/kg body weight) was used as plasma tracer to permit visualization in fluorescent light of changes in vascular macromolecular permeability. HBP (100 μg/ml) in bicarbonate buffer at 37 °C was topically applied to the cheek pouch and responses were recorded on video.

Dipole moment of HBP. The surface electrostatic potentials and dipole moment of HBP and elastase were calculated as described[13]. All calculations were made using GRASP (ref. 14). Charges and atom radii were assigned by use of the full.crg and default.siz files within the GRASP package.

Statistical analysis. Data are presented as mean ± s.d. Statistical significances were calculated using Mann–Whitney Rank Sum test for independent samples.

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