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Identifying the functional part of heparin-binding protein (HBP) as a monocyte stimulator and the novel role of monocytes as HBP producers

Morten Schou[1], René Djurup[2], Kjeld Norris[2], Hans Flodgaard[2]

[1]*Bartholin Instituttet XPU, Biocenter Copenhagen, Rigshospitalet, Copenhagen, Denmark*
[2]*Copenhagen, Denmark*

Heparin-binding protein (HBP), an evolutionary ancient and biologically highly important molecule in inflammation, is an inactive serine protease due to mutations in the catalytic triad. The histidine (position 41) in the conserved sequence TAAHC is mutated to serine and this sequence (TAASC) plays a crucial role when HBP binds to monocytes. We synthesized a 20–44 HBP peptide, cyclicized by a sulphur bridge, which encompasses this amino acid and functions as full-length HBP. Using a human monocyte cell line, we have shown that lipopolysaccharide (LPS)-triggered secretion of IL-6 is enhanced up to 10-fold when full-length HBP or the peptide are present in low-to-moderate concentrations. A monoclonal antibody neutralizing HBP also neutralizes the peptide, indicating that the ligand for the HBP receptor is located near serine in position 41 on the HBP surface. A 'back mutated' 20–44 peptide (serine→histidine) has some, but not significant, stimulatory effect on monocytes. Normally, HBP production and release is ascribed to neutrophil granulocytes, but here we find that also monocytes secrete HBP when stimulated with LPS. Furthermore, a small amount of HBP can be demonstrated when monocytes are incubated in medium alone. Our efforts to identify a suggested HBP receptor on monocytes has failed so far.

Keywords: heparin-binding protein, monocytes, HBP, HBP peptide

Introduction

Experiments over the last 20 years have shown that heparin-binding protein (HBP) plays an important role in early inflammation as a signal molecule.[1] We have focused on the structural details of HBP as this could indicate how different parts of the molecule are responsible for the many beneficial functions of this protein during inflammation.

In human monocytes, HBP has been shown to enhance the response to bacterial components leading to significantly increased secretion of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor-necrosis factor (TNF)-α.[2–4] We were interested in finding the functional part of HBP responsible for this stimulated release of cytokines.

The amino acid sequence and the three-dimensional structure of HBP have been determined and show that HBP is a member of the serine protease super-family. However, due to mutations in two of the three essential amino acids in the conserved catalytic triad seen in all serine proteases, HBP is devoid of protease activity and, therefore, serves other functions as mentioned above.[5,6] Two features of the HBP structure attract attention: (1) the protein has a strong dipole moment caused by basic amino acids concentrated on one side of the molecule; and (2) one of the mutated amino acids (in position 41, histidine to serine) is exposed at the surface of the molecule.

Release of HBP from secretory vesicles in neutrophils has been shown both *in vitro* and *in vivo* to be solely responsible for the vascular leakage necessary for plasma efflux and extravasation of neutrophils and monocytes. The HBP-induced leakage was mediated through activation of the contact phase system due to the

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Correspondence to: Morten Schou, Bartholin Instituttet XPU, Biocenter Copenhagen, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark. Tel: +45 35456079; Fax: +45 35456088; E-mail: morten.schou@rh.regionh.dk

unique charge distribution on the surface of the HBP molecule, thus leading to bradykinin generation.[1,7,8]

On the other hand, Pereira *et al.*[9] have shown that a synthetic linear peptide encompassing amino acids 20–44 from the sequence of human HBP has an antimicrobial effect. This peptide includes one of the two mutations that characterises HBP-like molecules, namely the histidine to serine substitution in position 41. Therefore, this peptide could be of significance compared to other peptides derived from the HBP sequence. We, therefore, constructed different peptides from the sequence of HBP and tested them in a whole-blood assay to find one that could mimic the effect of full-length HBP as an enhancer of cytokine release. We chose IL-6 as a measurable output from blood/monocytes because this cytokine plays an important role in inflammation.[10] For further experiments, we used the monocyte cell line Mono Mac 6 in order to make the set-up simpler. A monoclonal anti-HBP antibody was used to verify that the selected peptide was responsible for binding to an HBP receptor, thereby triggering the secretion of IL-6. There is some supportive evidence for the hypothesis that β_2-integrin could be a receptor for HBP coming from an experiment showing that HBP-induced Ca^{2+} mobilization in Mono Mac 6 cells could be prevented by blocking β_2-integrins with a specific monoclonal antibody, IB4.[11] Soehlein *et al.*[12] have presented data also indicating that the β_2-integrin is an HBP receptor on monocytes/macrophages.

Here, we present data supporting the idea that the TAASC motif in a cyclizised 20–44 amino acid peptide is part of the ligand in HBP responsible for activation of the HBP receptor on monocytes. In order to stress the importance of the mutation in position 41, we have investigated a 20–44 peptide containing a TAAHC motif – in fact a 'back mutation'. Finally, we show that monocytes release HBP constitutively in small amounts and even more so when lipopolysaccharide (LPS) is added.

Materials and Methods

Whole blood experiments

Human whole blood from healthy donors was used in preliminary experiments. Citrated blood (100 µl) was added to 1-ml sterile cryotubes (Greiner Bio-One GmbH, Frickenhausen, Germany). Bacterial products, HBP, HBP-peptides, HBP antibody and 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) were added in different compositions to a final volume of 160 µl. Bacterial products were added last thereby starting the experiment. Each tube was vortexed for 15 s and then incubated (without screw-cap at 37°C and 5% CO_2 in humidified air). Tubes were moved during incubation to keep cells in suspension. After 24 h, 900 µl of 0.1% BSA/PBS was added to each tube, the content was mixed and 0.9 ml was transferred to an Eppendorf tube. Eppendorf tubes were centrifuged and 500 µl supernatant was transferred to a new Eppendorf tube. Supernatants were frozen until measurement of IL-6 by sandwich ELISA, using a commercial kit (R&D Systems Inc., Minneapolis, MN, USA).

Cell line and growth medium

The monocyte cell line Mono Mac 6 purchased from the Department of Human and Animal Cell Cultures (Braunschweig, Germany) was cultured in RPMI 1640 (Cambrex Bio Science Verviers, Verviers, Belgium) with 13.5% fetal bovine serum (FBS; Cambrex Bio Science Verviers) with a low content of endotoxin. In addition, L-glutamine 2 mM (Cambrex Bio Science Verviers), penicillin 200 U/ml, streptomycin 200 µg/ml (Cambrex Bio Science Verviers), non-essential amino acids (NEAA; Sigma-Aldrich, St Louis, MO, USA) and OPI-supplement (10 ml in 1 l) containing oxaloacetic acid, sodium pyruvate and insulin (Sigma-Aldrich) were added according to seller's recommendations (<www.monocytes.de> see culture conditions).

Prestimulation of Mono Mac 6 cells

Before starting a given experiment, cells were established in tissue culture flasks (Greiner Bio-One GmbH) on day 0 at a concentration of 2×10^5 cells/ml. On day 3, they were harvested, the concentration was adjusted to 8×10^5 cells/ml and then prestimulated for the next 24 h with calcitriol (1α,25-dihydroxyvitamin D_3; Sigma-Aldrich) at a concentration of 10 ng/ml of growth medium.[13] Experiments were performed in sterile 24-well plates with 1 ml of growth medium containing cells, LPS, FBS, HBP, peptides and antibodies in different compositions.

Incubation and IL-6 measurement

To each test well, 10^6 cells/ml were added. The cells were added after the addition of LPS, HBP, *etc.*, thereby starting the experiment. Cytokine release was measured as the IL-6 concentration in supernatants after incubation for 3.5 h (37°C and 5% CO_2 in humidified air). During incubation, plates were moved to keep cells in suspension except for the last 30 min when movement was stopped to allow cells to settle. The upper 600 µl of culture medium was carefully removed and supernatants were frozen until IL-6 measurement by sandwich ELISA, using a commercial kit (R&D Systems Inc, Minneapolis, MN, USA).

Because the secretion of IL-6 varies widely in different experiments, we have chosen throughout to represent the data relative to the pure LPS response. This value will, therefore, in all cases be 1.0 (Figs 2A,B, 3, 4A,B and 6).

Heparin-binding protein and HBP-peptides

Recombinant human HBP was produced in HEK293 cells[14] and kept frozen in PBS with 1% BSA at a concentration of 2 mg/ml in order to prevent the molecule from sticking to the walls of the plastic container due to its dipolar nature.[7] For the same reason, all tips used to handle HBP were low retention.

Several HBP peptides were constructed based on the known sequence of human HBP (Schafer-N, Copenhagen, Denmark). One, ser20–44, had a sequence taken from human HBP position 20–44 (see Fig. 1). In position 41 is a serine, and the peptide was cyclizised by a sulphur bridge between two cysteine residues in positions 26 and 42. To avoid electric charges, the ends of the peptide were amidated and acetylated. Another peptide, his20–44 was constructed in the same way, except in position 41 where the serine was replaced by a histidine. The purity of the peptides was checked by mass spectrometry and chromatography.

Antibodies and reagents

A number of monoclonal and polyclonal antibodies raised against porcine HBP were produced at Novo Nordisk A/S (Copenhagen, Denmark), among them an antibody, A5B4, which inhibited IL-6 secretion from blood cells.

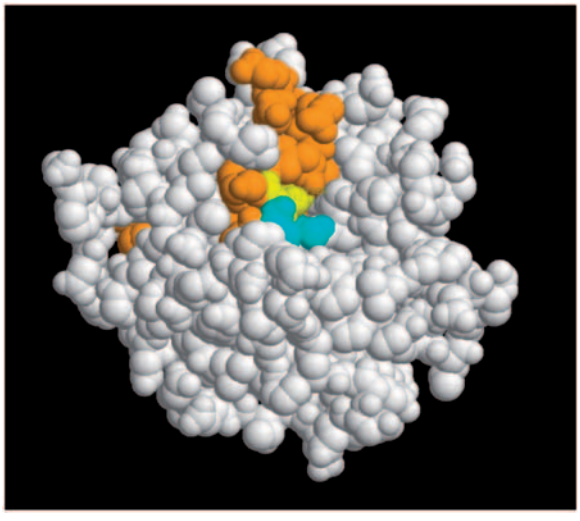

Fig. 1. Space-filling model of HBP. Space-filling structure of ser20-44 (orange) in HBP (white). Cysteine 26 and cysteine 42 are shown in yellow. The inactive 'catalytic' triad H41S, D89 and S175G are indicated in cyan. The catalytic cleft is discernible above the cyan coloured residues.

As a negative control antibody, we used cat. no. MCA929XZ from Abd Serotec (Oxford, UK). The immunogen for this antibody is activated rat T-helper cells.

In experiments where Mono Mac 6 cells were tested for the occurrence of HBP, an anti-human CD64 antibody (Fitzgerald Industries International, Concord, MA, USA) was used to avoid binding of primary polyclonal anti-HBP antibodies to the Fc-receptor. The secondary antibody was FITC-conjugated swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark, code no. F0205).

The monoclonal antibody IB4 binding to CD18 and, thereby, blocking the β_2-integrin was a gift from Lennart Lindbom (Karolinska Institutet, Stockholm, Sweden). In experiments where this antibody was used, cells were also pretreated with anti-CD64 for the same reasons as described above.

The Syk tyrosine kinase inhibitor piceatannol used for blocking signalling through the β_2-integrin was from Sigma-Aldrich (product no. P0453).

Lipopolysaccharide (*Escherichia coli*) and peptidoglycan (PGN, *Staphylococcus aureus*) were purchased from Sigma-Aldrich (product numbers L4391 and 77140). Purified cell wall (PCW, *S. aureus*) was produced at Novo Nordisk A/S.

Immunocytochemistry

After incubation (37°C and 5% CO_2 in humidified air), Mono Mac 6 cells were placed on Superfrost Plus slides (Menzel Gmbh & CoKG, Braunschweig, Germany), dried under cold air and fixed for 7 min in ice-cold methanol. A polyclonal rabbit anti-HBP antibody was used as the first layer; the second was FITC-conjugated swine anti-rabbit antibody. Cells were examined in an Olympus fluorescence microscope.

Statistical analysis

Statistical analysis was performed using Student's *t*-test (two-sided, paired) in Microsoft Excel.

RESULTS

Preliminary whole-blood experiments

A dose-response experiment using human blood stimulated with 15.6 ng/ml of LPS and HBP was carried out showing an increasing release of IL-6 (Fig. 2A).

Screening of a panel of monoclonal antibodies against porcine HBP was performed in human blood stimulated

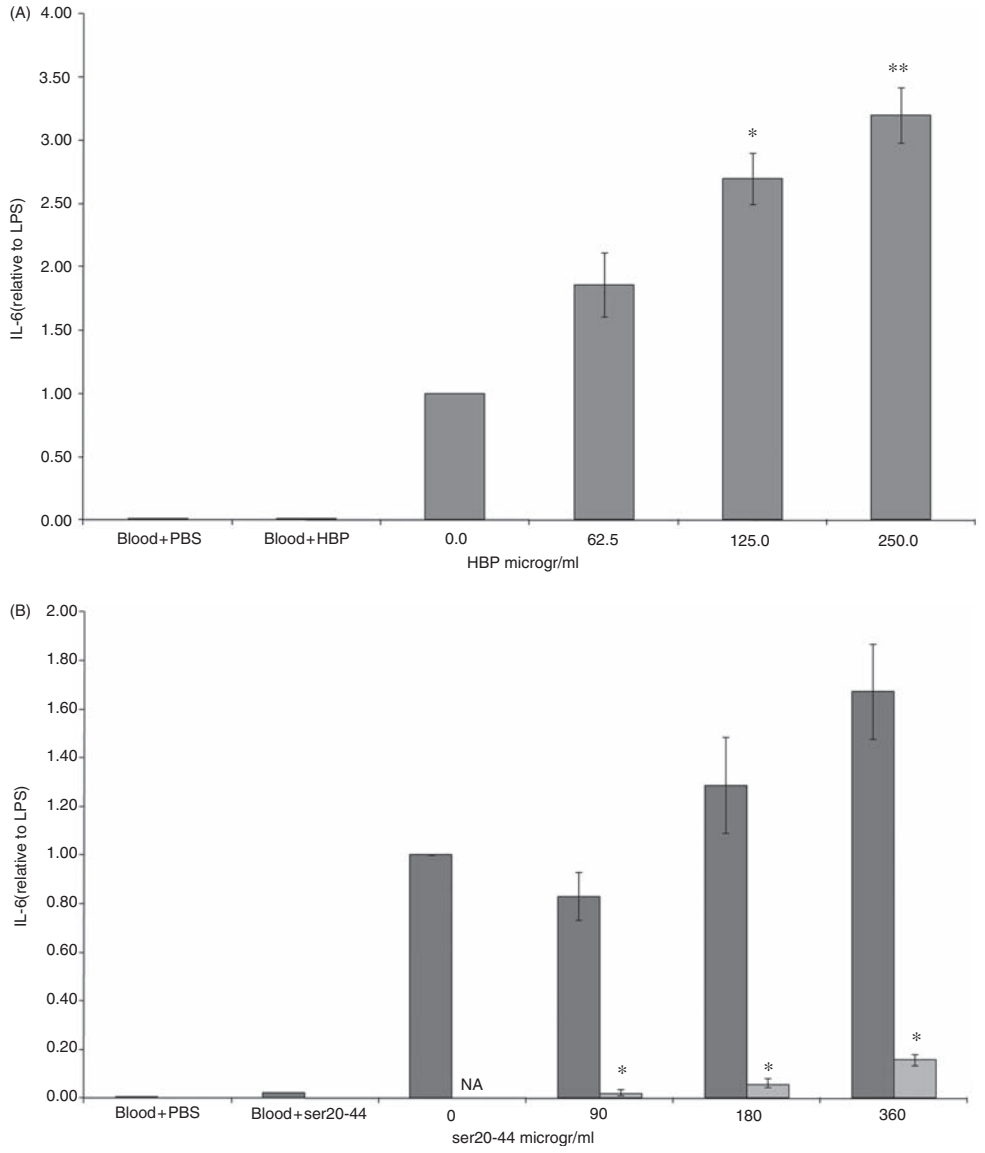

Fig. 2. (A) Standard HBP response in whole blood. Release of IL-6 from whole blood cells stimulated with LPS (15.6 ng/ml) and HBP at various concentrations. Controls (PBS and HBP) showed no IL-6 secretion indicating no contamination with bacterial products. Values are mean ± SEM. Three independent experiments were performed. $*P < 0.05$, $**P < 0.01$ versus LPS alone. Note that LPS alone has no SEM because values are relative to LPS, *i.e*. LPS alone values are 1. (B) Peptide and anti-HBP antibody test in whole blood. Release of IL-6 from whole blood cells stimulated with PGN (31.25 μg/ml) and ser20–44 at various concentrations. Control with only PBS added showed no IL-6 secretion indicating no contamination with bacterial products. Ser20–44 alone gave a negligible release of IL-6. The A5B4 monoclonal antibody, raised against porcine HBP, inhibits IL-6 secretion significantly in all cases. Values are mean ± SEM. Three independent experiments were performed. $*P < 0.05$ versus the different concentrations of ser20–44 used in the experiments. NA = not available. Note that PGN alone has no SEM because values are relative to PGN, *i.e*. PGN alone values are 1.

with PCW (11.25 μg/ml) and HBP (3.125 μg/ml); a typical release of IL-6 was around 1500 pg/ml. Addition of 24 μg/ml of the monoclonal anti-HBP antibody A5B4 inhibited this release totally, the IL-6 values now being the same as in unstimulated blood. Controls where LPS was not added showed no cytokine release.

Experiments were also performed using the ser20–44 peptide. A dose-response curve was established using PGN (31.25 μg/ml) as a bacterial stimulus (Fig. 2B). The A5B4 antibody again lead to a significant reduction in release of IL-6 (Fig. 2B). Using the peptide without PGN stimulation resulted in a minimal release of IL-6. Blood alone gave no release.

Standard HBP response and anti-HBP mAb

The LPS and FBS added to Mono Mac 6 cells gave rise to IL-6 secretion, for both additives, in a dose-dependent manner (data not shown). We decided to use a

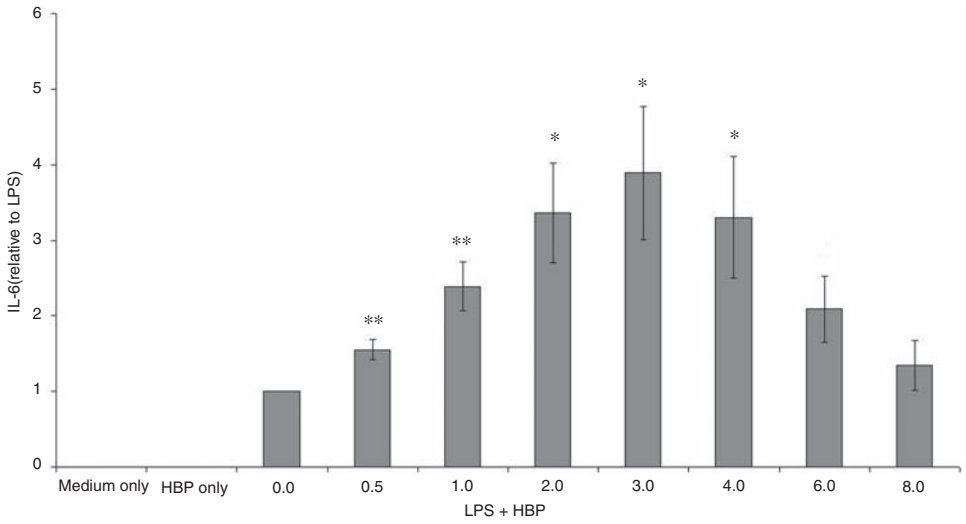

Fig. 3. Standard HBP response in Mono Mac 6 cells. Release of IL-6 from Mono Mac 6 cells stimulated with LPS (3 ng/ml) and HBP at various concentrations. Controls (medium and HBP) showed no IL-6 secretion indicating no LPS contamination. Values are mean ± SEM. Eight independent experiments were performed. $*P<0.05$, $**P<0.01$ versus LPS alone. Note that LPS alone has no SEM because values are relative to LPS, *i.e*. LPS alone values are 1.

combination of LPS and FBS at concentrations of 3 ng/ml and 0.006%, respectively. This combination gave a low release of IL-6. When adding HBP at different concentrations to LPS-stimulated Mono Mac 6 cells, a characteristic response emerged (Fig. 3). The HBP potentiates the effect of LPS in terms of IL-6 release with a maximum at 3 μg/ml of HBP. Although the absolute level of IL-6 secretion varied widely from experiment to experiment (up to 10-fold), there was a significant ($P=0.006$, 0.005, 0.012, 0.018, and 0.032) HBP-dependent enhancement of the IL-6 secretion compared to the control except at HBP levels of 6 μg/ml and 8 μg/ml ($P=0.052$ and $P=0.363$). Growth medium alone and HBP alone at a concentration of 3 μg/ml never gave rise to any IL-6 generation. One well with HBP (3 μg/ml) added alone to Mono Mac 6 cells was used in all experiments as a control to ensure that the HBP solution was not contaminated with LPS.

The monoclonal antibody A5B4 also completely inhibited the release of IL-6 in our Mono Mac 6 assay. A concentration of 80 μg/ml was found to be sufficient. A control antibody did not change the amount of IL-6 secretion when LPS was added alone, but the LPS + HBP response was lowered (see below). After incubation of cells and removal of supernatant, the viability of the Mono Mac 6 cells was examined with nigrosin. The number of live cells was the same as before the start of the experiment. In Fig. 4A, the results of four independent experiments are shown. Lipopolysaccharide as well as LPS + HBP induced IL-6 secretion is blocked. Again, the A5B4 mAb was added alone to Mono Mac 6 cells as a control for LPS contamination.

Monocytes secrete HBP

As mentioned above, IL-6 secretion due to LPS alone is completely blocked by the A5B4 antibody against HBP (Fig. 4A). We tried to verify this result by blocking β_2-integrin (a suggested HBP receptor) with the monoclonal antibody IB4[11,12] when LPS was added alone and when HBP was added as well. The IL-6 secretion was lowered considerably, but this was also seen using a control antibody (Fig. 4B). We then pre-incubated Mono Mac 6 cells for 30 min with the Syk tyrosine kinase inhibitor piceatannol (30 μg/ml)[15] to stop signalling through the HBP receptor. This resulted in complete blocking of IL-6 secretion with or without HBP added as seen with A5B4 (Fig. 4A), both findings suggesting that the Mono Mac 6 cells themselves produce HBP. This was confirmed by direct measurements of HBP in supernatants from cells stimulated with LPS alone. Lipopolysaccharide (3 ng/ml) resulted in HBP concentrations with a mean value of 114 ng/ml ($n=7$). This is significantly more than in the growth medium itself (6.6 ng/ml), the *P*-value being 0.007 (Fig. 5). The Mono Mac 6 cells incubated with growth medium without LPS also secreted HBP (27.1 ng/ml), which was significantly higher than in acellular growth medium alone ($P=0.011$). The last mentioned finding could also be confirmed by immunocytochemistry, when Mono Mac 6 cells incubated in growth medium alone stained positive with a polyclonal rabbit anti-HBP antibody. Mono Mac 6 cells appeared in the fluorescence microscope as green circles. In some cells, the superficial staining is apparent (Fig. 7). Controls, second layer alone or incubated with PBS two times, were negative. Raising the LPS concentration to 9 ng/ml did not result in a higher secretion of HBP (Fig. 5).

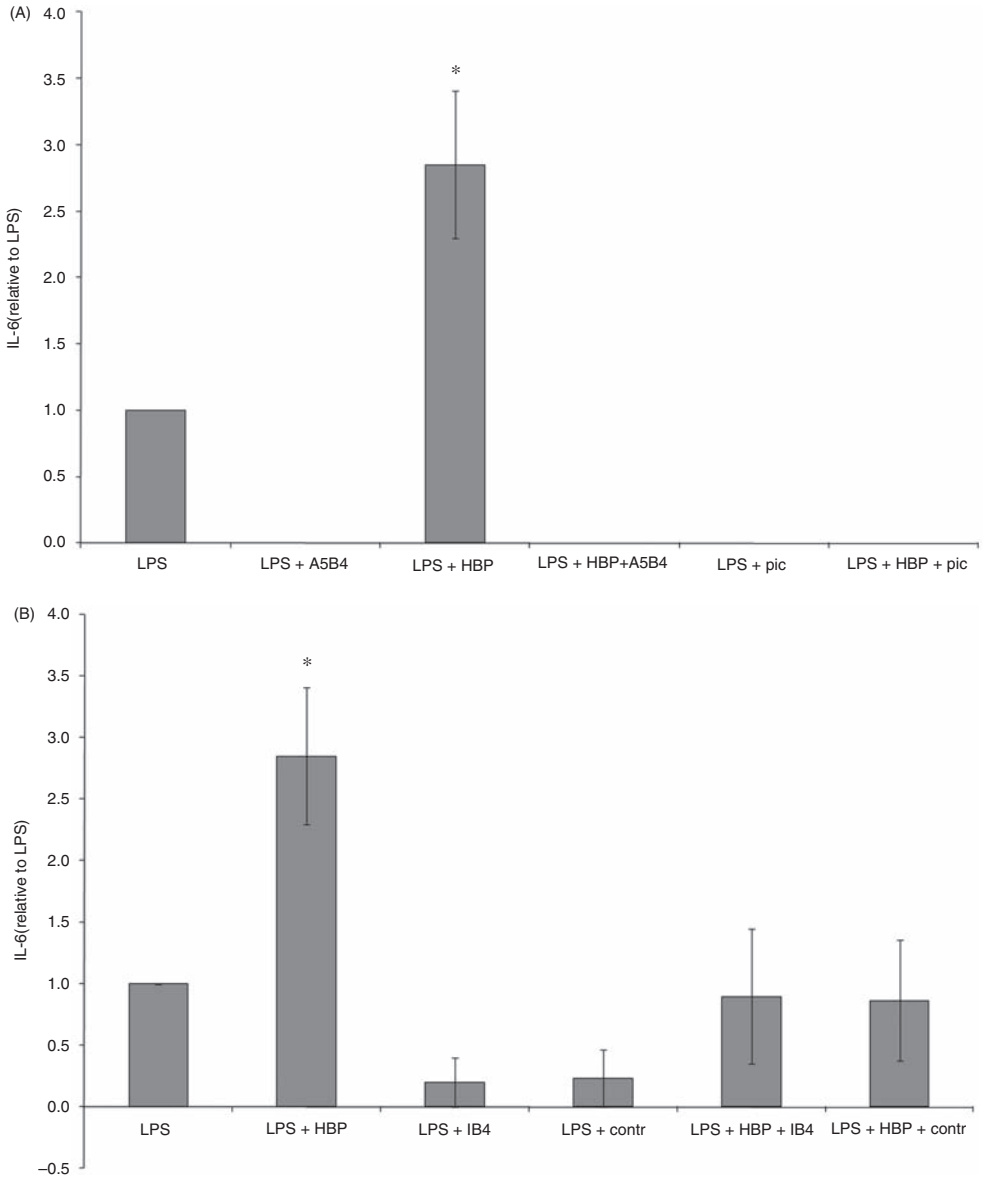

Fig. 4. HBP-signal blocking. (A) The A5B4 monoclonal antibody inhibits IL-6 secretion from Mono Mac 6 cells totally at a concentration of 80 μg/ml when incubated with LPS (3 ng/ml) and HBP (3 μg/ml). This antibody also inhibits IL-6 secretion when cells are stimulated with LPS alone. Values are from four independent experiments. Piceatannol (pic) was used at a concentration of 30 μg/ml. Results are from three independent experiments. (B) In attempts to block the β_2-integrin with IB4 mAb, 10 μg/ml was used and this also applies for the control antibody. Three independent experiments were performed. In all cases, values are mean ± SEM. $*P < 0.05$ versus LPS alone. Note that LPS alone has no SEM because values are relative to LPS, *i.e*. LPS alone values are 1.

Heparin-binding protein peptides

Incubation of the Mono Mac 6 cells with the ser20–44 peptide in the presence of LPS showed a significant potentiation ($P = 0.035$; $n = 12$) of IL-6 release (see Fig. 6) compared to LPS used alone, when a concentration of 50 μg/ml were used. However, the 'back mutated' his20–44 peptide was significantly less potent ($P = 0.028$; $n = 6$) compared to ser20–44 (see Fig. 6). As mentioned earlier, the mAb A5B4 inhibited full-length HBP but was also able to inhibit ser20–44 completely at a concentration of 80 μg/ml (see Fig. 6).

Discussion

Preliminary whole blood experiments

Whole blood was chosen for the preliminary experiments to mimic the *in vivo* situation. The dose-response curve

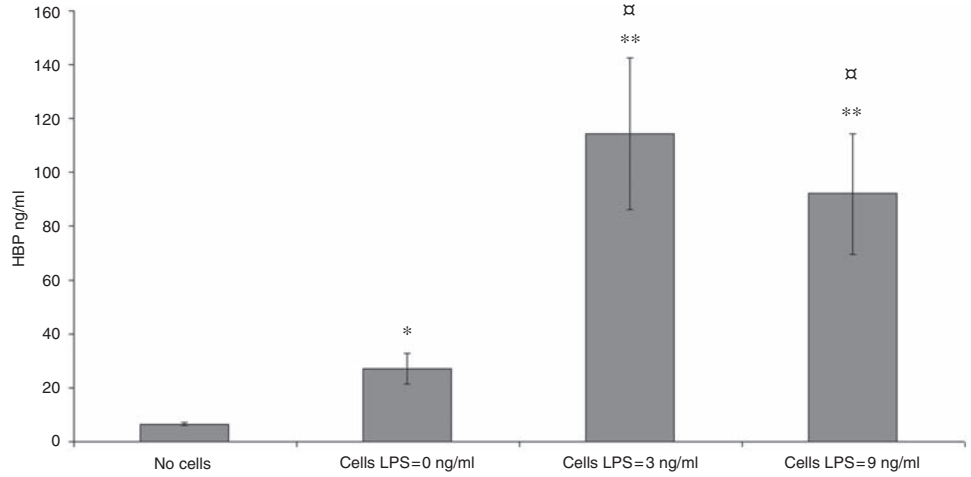

Fig. 5. Secretion of heparin-binding protein from Mono Mac 6 cells. Heparin-binding protein was found in supernatants when Mono Mac 6 cells were incubated with medium alone or when LPS was added to the medium at a concentration of 3 ng/ml or 9 ng/ml. The small amount of HBP seen in medium without cells must originate from the small amount of FBS added to the growth medium indicating that the bovine HBP has some epitopes in common with human HBP. Values are mean ± SEM. Seven independent experiments were performed. $^{*}P<0.05$, $^{**}P<0.01$ versus medium alone. $^{¤}P<0.05$ versus cells without LPS.

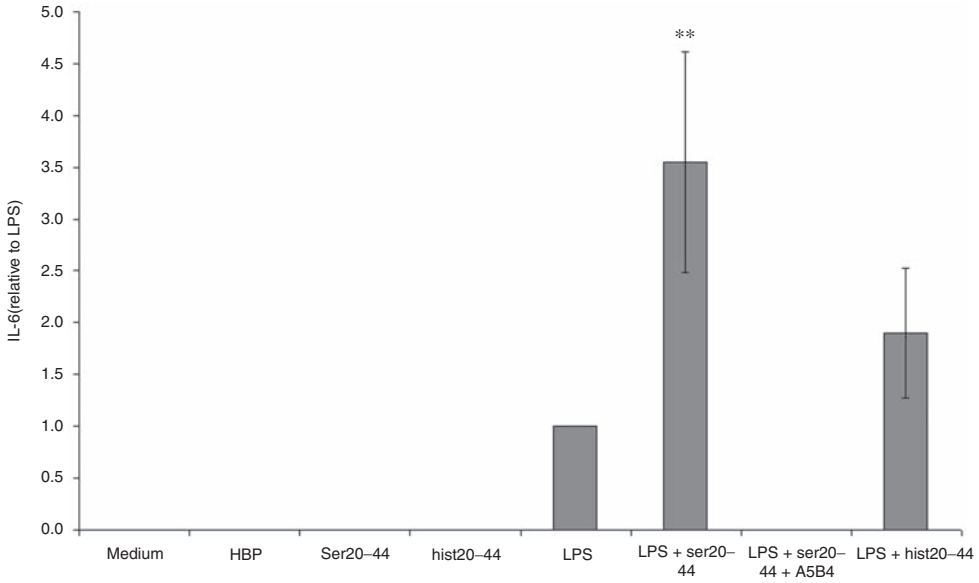

Fig. 6. Stimulation with HBP peptides. Comparison of two peptides for their ability to enhance IL-6 release from LPS stimulated Mono Mac 6 cells. The two peptides ser20–44 and the back-mutated his20–44 were identical, except that in his20–44 the serine in position 41 is substituted by a histidine. For ser20–44, twelve independent experiments were performed, while for his20-44, six independent experiments were carried out. Controls (medium, HBP, ser20–44 and his20–44) showed no IL-6 secretion indicating no LPS contamination. Values are mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ versus LPS alone. Note that LPS alone has no SEM because values are relative to LPS, *i.e*. LPS alone values are 1.

showed an increasing secretion of IL-6 as the concentration of HBP was raised. Using 125 µg/ml and 250 µg/ml HBP caused a significantly higher release of IL-6. Heparin-binding protein used alone did not provoke IL-6 secretion indicating no LPS contamination (see below).

During screening of monoclonal antibodies, when blood was stimulated with PCW, we found that the antibody A5B4 blocked IL-6 secretion totally and this antibody was, therefore, selected for future experiments. The selected peptide ser20–44 was tested in experiments where PGN was used as the bacterial stimulator. Increasing concentrations of this peptide caused higher release of IL-6 (except for 90 µg/ml, where there was a non-significant lowering in IL-6 release). Control with PBS alone showed no cytokine release indicating no contamination. The peptide used alone showed a negligible IL-6 secretion. It could be interpreted as a slight contamination with bacterial products but other

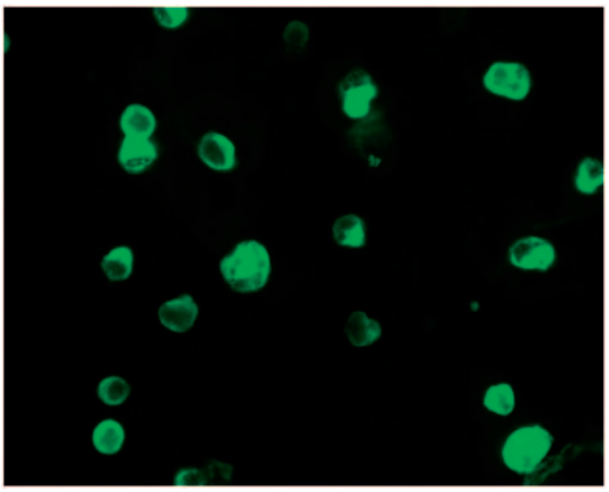

Fig. 7. Detection of HBP in unstimulated Mono Mac 6 cells. Mono Mac 6 cells seen in the fluorescent microscope. Cells were incubated in medium alone. In controls, the second layer was used alone or PBS without antibodies was used twice. In this case, no green cells were observed (not shown), indicating no autofluorescence. Original magnification ×400.

batches of this peptide showed no effect alone. The A5B4 antibody inhibited the peptide significantly at a dose of 24 μg/ml. The interpretation of this result is discussed later.

Standard HBP response and anti-HBP mAb

The cell line Mono Mac 6 was selected because, in several respects, it has the same properties as mature monocytes. These include CD14 antigen expression, phagocytosis and production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α upon stimulation with LPS.[13,16,17] Cells were pre-treated with calcitriol in order to get a higher and more stable release of cytokine from the Mono Mac 6 cells.

In Figure 3, an HBP concentration of 3 μg/ml creates a maximum release of IL-6. Adding more HBP to the test wells resulted in a declining release of cytokine. Adding even more HBP (for example, 20 μg/ml) could inhibit IL-6 release almost completely (data not shown). This is not seen in the whole-blood experiments and other authors[2,4] using naïve human monocytes have not seen this phenomenon. Thus, it may be a property of the Mono Mac 6 cell line. The fact that HBP alone does not cause IL-6 release underlines the fact that, in order to function as an enhancer of cytokine release, bacterial products must be present.[1,4,11] This finding can be used in experiments as a control for LPS contamination in the different additives used in our experiments. It has been done for all additives to the wells (HBP, peptides, antibodies and medium) and the result was that no IL-6 secretion could be detected. The secretion was under the detection limit of the ELISA kit used (2.5 pg/ml) and, therefore, LPS contamination could be excluded in the reagents used. Heparin-binding protein can be released under non-inflammatory conditions and this will consequently not cause symptoms due to cytokine release but HBP may cause severe symptoms like fall in blood pressure (bypass operations) or severe pulmonary damage caused by plasma and blood cells.[18] In these cases, HBP induces leakage of the endothelium.

Monocytes secrete HBP

The reason why we used monoclonal antibody against swine HBP is that Novo Nordisk A/S had planned to make a porcine model of cardiopulmonary bypass after it was shown that patients undergoing open cardiac surgery have dramatic levels of HBP in their blood (results to be published). Several antibodies were tested for their ability to inhibit porcine HBP-mediated leakage.

Looking at the data from wells with only LPS added, where A5B4 blocked the Il-6 response totally, we thought of an error in the experiment (Fig. 4A). We had assumed that the IL-6 secretion would be of the same magnitude as for LPS alone because no HBP was added. But it was repeated several times. Cells were as viable after the experiment as before, excluding cell death as an explanation. Use of control antibody in wells with only LPS did not lower IL-6 secretion and, therefore, confirmed the unexpected result. A problem was that the control antibody lowered the IL-6 secretion when HBP was present at a concentration of 3 μg/ml. Later, we learned from a paper by Soehnlein *et al*.[12] that macrophages change behaviour in response to treatment with HBP in that they enhance phagocytosis of bacteria. Furthermore, HBP causes a 2–3-fold up-regulation of Fc-receptors (CD32 and CD64). This change in cell behaviour could be the reason that the control antibody showed a lowered IL-6 release. Using piceatannol, Mono Mac 6 cells stimulated with LPS alone did not secrete IL-6. Piceatannol blocks signalling from the β_2-integrin and other receptors (see below). A possible explanation for the blocking effect by A5B4 and piceatannol could be that the Mono Mac 6 cells produce HBP themselves. As shown in Fig. 5, monocytes do indeed secrete HBP, even when LPS is not present but in this case only in small amounts. Using 9 ng/ml LPS instead of 3 ng/ml did not lead to higher secretion of HBP. This could have been expected because IL-6 secretion was higher under these circumstances (data not shown). It is the first time that constitutive and LPS-induced HBP release has been demonstrated in monocytes. It is interesting to note that LPS causes IL-6 release only if HBP from the monocyte is present.

Control experiments were performed showing that LPS did not influence the ELISA method used for detecting HBP (data not shown).

The HBP receptor

The inhibition of IL-6 secretion using the monoclonal IB4 antibody blocking the β_2-integrin could not be confirmed using a control antibody. The control antibody had the same effect on IL-6 secretion. Trying to block the signal through the β_2-integrin with piceatannol[19] was successful, but this compound is not specific for the β_2-integrin and cannot be used alone as a proof for β_2-integrin signalling induced by HBP. With the methods used here, we therefore cannot confirm the hypothesis that β_2-integrin is a receptor for HBP in monocytes/macrophages as suggested by Soehnlein *et al*.[12] where HBP-enhanced phagocytosis in macrophages from mice is abolished when CD18 (part of β_2-integrin) is blocked by IB4 antibody and HBP-enhanced phagocytosis was neither seen in macrophages from CD18-knockout mice. On the other hand, we cannot reject the hypothesis as the experimental evidence from others is rather convincing. Experiments are in progress to clarify this. The IB4 antibody was also used by Påhlman *et al*.,[11] who prevented an intracellular surge of Ca^{2+} in Mono Mac 6 cells with this antibody. They used the same control antibody as we did.

Heparin-binding protein peptides

The ser20–44 peptide is interesting as it contains the essential histidine to serine mutation seen in both human and porcine HBP[5] in the otherwise completely conserved TAAHC present in most active serine proteases. Furthermore, the ser20–44 peptide also encompasses the short cysteine bridge conserved in all serine proteases and in both human and porcine HBP. Moreover, from Fig. 1, it appears that the mutated motif is situated at the mouth of the degenerated catalytic cleft and, therefore, exposed to the surface of the HBP molecule. We, therefore, decided to synthesize the two *N*-acetyl, C-amido 20–44 peptides mentioned earlier.

Adding ser20–44 to Mono Mac 6 cells causes a release of IL-6 comparable to that of full-length HBP. This indicates that it is the part of HBP close to the mouth of the degenerated catalytic cleft that binds to the HBP receptor. This is also indicated by the fact that the A5B4 anti-HBP antibody blocks IL-6 secretion when using HBP as well as ser20–44. The importance of the serine in position 41 is obvious when we add the his20–44 peptide. Incubating Mono Mac 6 cells with this peptide, which differs from ser20–44 by only one amino acid, reduces the cytokine release significantly to a level that is not significantly different from that of LPS alone.

Evolution has, with one amino acid substitution, created a protein that binds to a receptor and, thereby, helps the organism to fight an infection more efficiently. The ser20–44 peptide may have clinical importance as an enhancer of cytokine release during inflammation because it is a small molecule and does not have a strong dipole moment, like native HBP, caused by positively charged amino acid residues around the degenerated catalytic cleft. This positive charge on one side of the molecule may play a role when HBP causes leakiness in vessels.[7]

Monocytes secrete HBP

A novel finding was that Mono Mac 6 cells secrete HBP. Even if incubated with medium alone, there is a slight secretion of HBP. Soehnlein and Lindbom[1] described HBP (calling it azurocidin) as an alarmin (endogenous molecules released rapidly in response to infection or tissue injury and sometimes synthesized *de novo*) because of its rapid release during early infection. The observation that Mono Mac 6 cells secrete HBP when stimulated with LPS supports their argument further.

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