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The Neutrophil-Activating Protein of *Helicobacter pylori* Crosses Endothelia to Promote Neutrophil Adhesion In Vivo¹

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Helicobacter pylori induces an acute inflammatory response followed by a chronic infection of the human gastric mucosa characterized by infiltration of neutrophils/polymorphonuclear cells (PMNs) and mononuclear cells. The *H. pylori* neutrophil-activating protein (HP-NAP) activates PMNs, monocytes, and mast cells, and promotes PMN adherence to the endothelium in vitro. By using intravital microscopy analysis of rat mesenteric venules exposed to HP-NAP, we demonstrated, for the first time in vivo, that HP-NAP efficiently crosses the endothelium and promotes a rapid PMN adhesion. This HP-NAP-induced adhesion depends on the acquisition of a high affinity state of β_2 integrin on the plasma membrane of PMNs, and this conformational change requires a functional p38 MAPK. We also show that HP-NAP stimulates human PMNs to synthesize and release a number of chemokines, including CXCL8, CCL3, and CCL4. Collectively, these data strongly support a central role for HP-NAP in the inflammation process in vivo: indeed, HP-NAP not only recruits leukocytes from the vascular lumen, but also stimulates them to produce messengers that may contribute to the maintenance of the flogosis associated with the *H. pylori* infection. *The Journal of Immunology*, 2007, 178: 1312–1320.

Helicobacter *pylori* is a Gram-negative bacterium, which colonizes the human gastric mucosa and chronically infects more than half of the human population. This infection has been associated with various gastroduodenal diseases and gastric cancers (1–4). *H. pylori* colonization is typically followed by infiltration of the gastric mucosa by polymorphonuclear leukocytes, macrophages, and lymphocytes (5, 6). A strong correlation exists between gastric infiltration by neutrophils (polymorphonuclear cell; PMNs),⁴ mucosal damage, and development of duodenal ulcer disease in *H. pylori* infections (7, 8). However, the mechanism underlying the sustained recruitment of PMNs to the *H. pylori*-infected tissue in vivo still remains to be fully under-

stood. It has been shown that the bacterium activates endothelial cells to express several adhesion molecules and PMNs recruiting CXC chemokines, such as CXCL8 (9), known to trigger integrin activation in PMNs (10). Gastric epithelial cells are also likely to contribute to the generation of a chemoattractant milieu (11). Although it seems that one or more protein(s) encoded by the 30-kb DNA region present in the most virulent bacteria (the *cag* pathogenicity island) has a central role in promoting leukocyte recruitment, by inducing secretion of chemokines (11, 12), other reports suggest that mediators encoded by *cag* pathogenicity island may play a role in strain potency, but are not the only bacterial products involved in endothelial cell activation and in PMN extravasation (9, 13).

A major proinflammatory factor produced by *H. pylori* is *H. pylori* neutrophil-activating protein (HP-NAP) (14). It is a dodecameric protein of 150 kDa with a structure similar to bacterioferritins, including a central cavity for iron accumulation (15, 16). It was originally defined as PMN-activating protein because it stimulates PMNs to produce reactive oxygen radicals (17). In addition, HP-NAP crosses the epithelia to contact inflammatory cells already resident in the tissue (such as macrophages and mast cells) or recruited during the inflammation (18). HP-NAP stimulates the synthesis of tissue factor and the secretion of type 2 plasminogen activator inhibitor by monocytes/macrophages (14, 19). Finally, HP-NAP promotes Th1 responses (20) by inducing the production of cytokines, such as IL-12 and IL-23, in cells of the innate immune system, such as monocytes and macrophages. HP-NAP stimulates mast cells and monocytes/macrophages to produce TNF- α and CXCL8 (19, 20). TNF- α is a pleiotropic cytokine able to stimulate adhesivity of endothelial cells by up-regulating adhesion molecules, such as VCAM-1 and ICAM-1 (21). Moreover, TNF- α can induce activation of integrins on PMNs, directly or by stimulating the secretion of CXCL8 from the endothelium (10, 22).

According to these observations, during *H. pylori* infection it is very likely that HP-NAP, probably together with other bacterial or host-derived factors, may trigger in vivo the PMN accumulation

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⁴ Abbreviations used in this paper: PMN, polymorphonuclear cell; AO, acridine orange; HP-NAP, *H. pylori* neutrophil-activating protein; PAF, platelet-activating factor; PET, polycarbonate; RPA, RNase protection assay; RT, room temperature; VEGF, vascular endothelial growth factor.

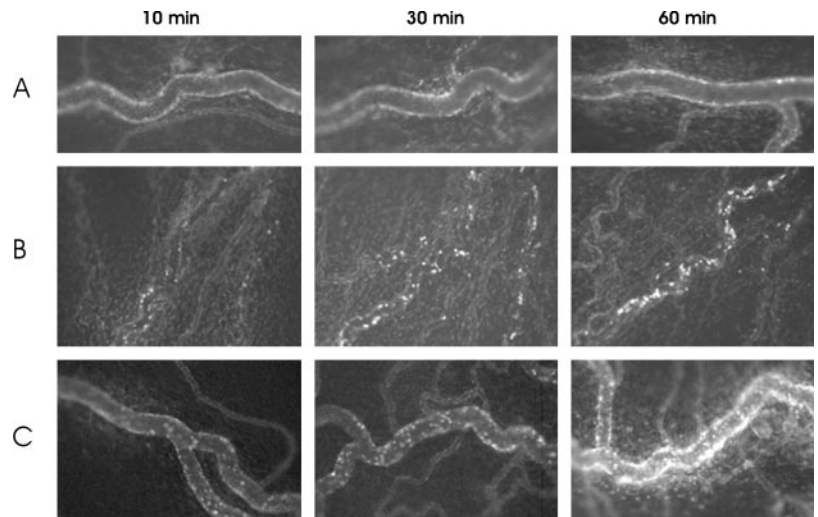


FIGURE 1. Leukocyte adherence to endothelium and extravasation. Video photomicrographs show time-dependent leukocyte adherence to endothelium of rat mesenteric microvessels in response to 1 μM (B), 5 μM HP-NAP (C), or control saline (A) applied to ileal mesentery. Leukocytes labeled *in vivo* with AO were made visible by fluorescence epi-illumination and appeared as bright spheres. Magnifications, $\times 400$ (A and C) and $\times 100$ (B).

within the tissue, evoking the adhesive properties of both PMNs and endothelium. The possibility that HP-NAP also directly stimulates PMNs to adhere derives from several studies (13, 17); in the former study, however, protein and PMNs were assayed together without evaluating whether HP-NAP was actually capable of crossing the endothelium to come in contact with PMNs. The latter study demonstrated that HP-NAP activates PMNs even when they are separated by an endothelium grown on a Transwell support, but did not consider the underflow conditions that are of great relevance *in vivo*.

In this study, we demonstrate for the first time that HP-NAP is able to cross the endothelium and to stimulate PMNs to adhere *in vivo*, in underflow conditions. This effect is directly mediated by HP-NAP, which induces a high affinity state of integrins on PMNs, by recruiting a signaling cascade that involves p38-MAPK. Moreover, we addressed the question as to whether HP-NAP was able not only to rapidly recruit a first defense line, but also to sustain a prolonged inflammatory state by acting on the recruited cells; indeed, very little is known about the direct effects of HP-NAP on the production of neutrophil-derived proinflammatory mediators. We show in this study that HP-NAP up-regulates the mRNA expression and the protein release of several proinflammatory chemokines, including CXCL8, CCL3, and CCL4.

Materials and Methods

Reagents

HP-NAP was cloned, expressed, and purified from *Bacillus subtilis* to avoid LPS contamination, as described previously (15). SB203580, tetramethyl benzidine, FITC-conjugated BSA, streptavidin-alkaline phosphatase, *p*-nitrophenyl phosphate, nonfat milk powder, acridine orange (AO), platelet-activating factor (PAF), HBSS, and biotin were purchased by Sigma-Aldrich. Na-citrate solution at 3.8% was from Diagnostic Merk. Dextran and Ficoll-Hypaque were from Amersham Biosciences. mAb for the active conformation of CD11/CD18 (anti-24Ag) was a gift from N. Hogg (Imperial Cancer Research Fund, London, U.K.). Goat FITC-F(ab')₂ anti-mouse IgG and an irrelevant mouse IgG1 control were from Caltag Laboratories. Streptavidin-Texas Red was from DakoCytomation, and streptavidin was from Pierce.

All of the procedures were done under sterile conditions, and used reagents were prepared in endotoxin-free water for clinical use.

Intravital microscopy analysis

Male Wistar Kyoto rats, weighing 250–270 g, were anesthetized with sodium thiobarbital (80 mg/kg, *i.p.*) and prepared for direct microscopy analysis following previously described procedures (23). After surgical setup, the ileal mesentery was exposed and the microvascular trafficking of fluorescent labeled leukocytes (AO, *i.v.* infused at a concentration of 0.025

mg/kg per min, at a rate of 0.5 ml/h) was examined. Segments of three to five unbranched postcapillary venules (25- to 40- μm diameter, 200- μm length) were selected for the analysis. Direct digital recordings of the microscopy images were acquired at different phases, and further analyzed off-line. AO-labeled leukocytes were classified as rolling (whenever they became visible, because of moving more slowly than RBCs) or adherent (if they remained stationary, for at least 30 s, upon the endothelial surface).

In each experimental setting, after excluding vascular lesions or unstable hemodynamic pattern, baseline evaluation was acquired. Then, HP-NAP (1 or 5 μM , diluted in 200 μl of sterile saline) was topically applied to the mesentery for 10 min, and image sequences were further recorded intermittently up to 120 min. Control animals were treated with comparable volumes of either sterile saline alone or boiled HP-NAP. All of the experimental procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and approved by the Italian Ministry of University and Research as well as by the Administration of the University Animal House.

Purification of PMNs and endothelial cell culture

Human PMNs were prepared from healthy donors, as previously described (24).

HUVECs were isolated from three to five normal umbilical cords by collagenase digestion and grown in tissue culture plates (Costar) coated with 2% endotoxin-free gelatin (25). HUVECs were used at their first passage. The cells were kept in culture in medium 199 supplemented with 20% newborn calf serum (Invitrogen Life Technologies), 50 $\mu\text{g/ml}$ heparin, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin (Sigma-Aldrich), and 50 $\mu\text{g/ml}$ endothelial growth supplement (26).

Evaluation of the HP-NAP crossing through the endothelium

HP-NAP was biotinylated, according to the manufacturer's instructions. The biological activity of the biotinylated material was checked by evaluating cytosolic calcium increase in PMNs exposed to HP-NAP (27).

HUVECs (7×10^4) were seeded onto 2% gelatin-coated polycarbonate (PET) inserts of a 12-well Transwell system (12-mm diameter, 0.4- μm pores; Costar) and were used 5 days after plating.

The formation of intact monolayer on the insert was evaluated by adding FITC-BSA (1 mg/ml) to the upper chamber and measuring after 5 min the amount of labeled BSA passed into the lower chamber by a Fluostar microplate reader (SLT Labinstruments). Transwells were used only when the intensity of fluorescence in the lower chamber was negligible.

Biotinylated HP-NAP was added to the lower chamber at a final concentration of 1 μM , and the amount of HP-NAP passed in the top well was evaluated by ELISA.

Briefly, a 96-well microtiter maxisorp plate (Nunc) was coated by overnight incubation with streptavidin (10 $\mu\text{g/ml}$) in 0.1 M NaHCO_3 (pH 9.6) at 4°C. After washing with PBS-0.1% Tween 20, the residual-free sites were blocked with PBS-2% nonfat milk for 1 h at 37°C. After extensive washing with PBS-0.1% Tween 20, samples of the medium were put in the coated wells and left for 30 min at 37°C. The amount of biotinylated

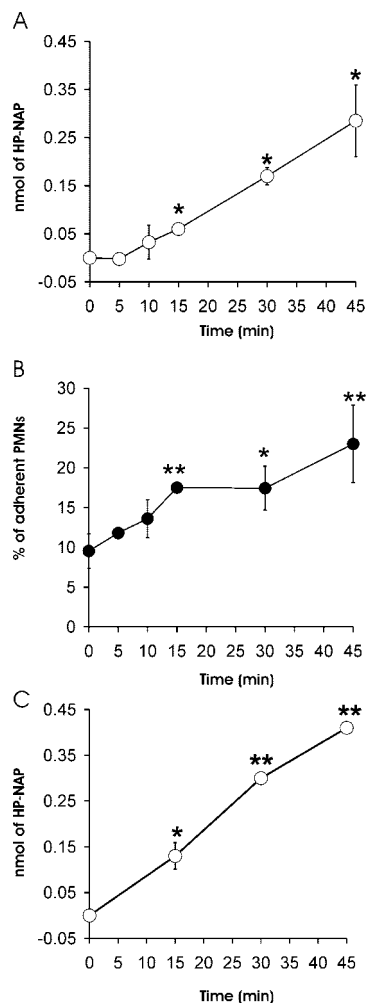


FIGURE 2. Passage of HP-NAP through the endothelium. HUVECs grown to confluence onto the Transwell inserts were exposed to biotinylated HP-NAP (1 μ M) added to the lower chamber. The time-dependent accumulation of HP-NAP after endothelium crossing was measured in the medium, collected in the upper chamber at different time points, by ELISA (A); same samples were tested for the capability of inducing PMN adhesion (B). The amount of crossed HP-NAP bound to the luminal surface of endothelium was evaluated by whole cell ELISA (C). Results represent values \pm SD obtained in a representative of three consecutive experiments. *, $p < 0.05$; **, $p < 0.01$ vs unconditioned medium.

HP-NAP bound to the substrate was revealed incubating with streptavidin-alkaline phosphatase 1/3000, followed by *p*-nitrophenyl phosphate, as reported previously (25). Calibration curves were set up with increasing concentrations of biotinylated HP-NAP directly added to streptavidin-coated wells.

Evaluation of endothelium-bound HP-NAP by whole cell ELISA

Biotinylated HP-NAP was added to the lower chamber of a HUVEC endothelial monolayer at a final concentration of 1 μ M. At different time

points, cells were washed with HBSS containing 1% BSA. The amount of HP-NAP bound to the surface of the endothelium was revealed by whole cell ELISA by incubating the endothelium with streptavidin-alkaline phosphatase 1/3000, followed by *p*-nitrophenyl phosphate, as reported previously (25).

Evaluation of the HP-NAP effect on endothelial permeability

HUVECs were seeded onto PET inserts of a 12-well Transwell system, and each Transwell was checked for the formation of intact monolayer by adding FITC-BSA to the upper chamber, as described above. Stimuli 1 μ M HP-NAP or 1 μ M PAF was added to the lower chamber. FITC-BSA fluorescence was evaluated in the lower chamber at various time intervals. Calibration curves were set up measuring the fluorescence intensity of increasing concentrations of FITC-BSA.

PMN adhesion assay

PMNs (10^5) were added to HUVECs grown to confluence in 96-well tissue culture plates either in medium alone or in 100 μ l of the upper chamber medium collected from the experiment of HP-NAP endothelium crossing. After 30-min incubation at 37°C, unbound leukocytes were removed by washing, whereas the number of adherent cells was evaluated by a colorimetric assay using tetramethylbenzidine as a substrate for myeloperoxidase, as previously described (23). Percentage of PMN adherence was calculated using a calibration curve. For the experiments with p38-MAPK inhibitor, PMNs were preincubated with 30 μ M SB203580 for 30 min at 37°C before exposing to 1 μ M HP-NAP. After a further 30-min incubation, PMNs were washed and added to HUVEC confluent monolayers in 96-well plates. After 30 min at 37°C, adherent PMNs were quantified, as above.

Expression of the 24 active conformational epitope

A total of 2.5×10^5 PMNs suspended in 200 μ l of PBS 0.1% BSA, 1 mM Ca^{2+} , and 1 mM Mg^{2+} was incubated with anti-24Ag or with an irrelevant mouse IgG1 for 10 min at 37°C and subsequently exposed to 1 μ M HP-NAP for 15, 30, and 60 min. Cells were washed in ice-cold PBS, 0.2% BSA, incubated with FITC-F(ab')₂ of goat anti-mouse IgG at 4°C for 30 min, washed again, and then analyzed for fluorescence on a BD Biosciences FACSCalibur flow cytometer. To evaluate whether the differences between the peaks of cells were statistically significant with respect to control, the Kolmogorov-Smirnov test for analysis of histograms was used, according to the CellQuest software guide (BD Biosciences).

When required, PMNs were preincubated with 30 μ M SB203580 for 30 min before adding Abs.

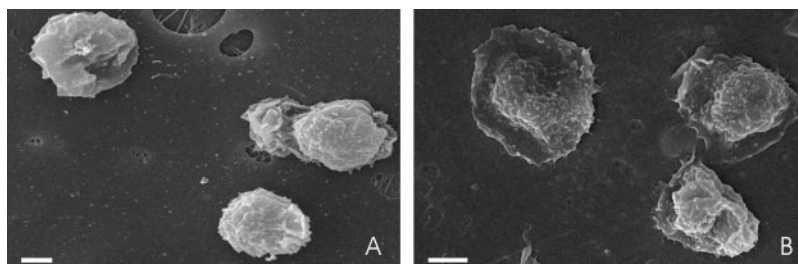
Scanning electron microscopy

An approach similar to that described for the HP-NAP passage across the endothelium was followed, except that HUVECs (2×10^4) were seeded onto inserts of a 24-well Transwell system (6.5-mm diameter, 0.4- μ m pores; Costar). HP-NAP (1 μ M) was added to the lower chamber, and after 45 min of incubation at 37°C, the PMNs were added to the endothelial layer and left for 30 min at 37°C. The adherent PMNs were fixed and processed for scanning microscopy, as described previously (28).

Immunoelectron microscopy

To analyze the intracellular localization of HP-NAP, 1 μ M biotinylated protein was added to an endothelial monolayer grown on a Transwell system, as described above. After 45 min at 37°C, cells were fixed in 0.5% glutaraldehyde dissolved in 0.1 M cacodylate buffer for 5 min at room temperature (RT), and then washed in cacodylate buffer and permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT. Cells were incubated with 20-nm colloidal gold-labeled streptavidin (British Biocell International; dilution 1/100). After several washings in cacodylate buffer, cells were fixed in 2% glutaraldehyde dissolved in PBS and processed, as previously described (29).

FIGURE 3. Scanning electron microscopy of human PMNs adherent to HUVECs. HUVECs grown to confluence onto the Transwell inserts were exposed for 45 min either to medium alone (A) or to 1 μ M HP-NAP (B) added in the lower chamber. PMNs were then added on top of the monolayers. After 30 min of incubation at 37°C, endothelial cells were processed for scanning electron microscopy analysis. Bars, 2 μ m.



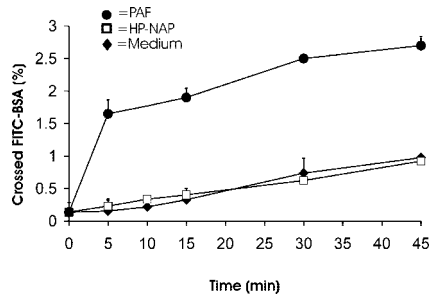


FIGURE 4. Kinetic analysis of BSA leakage through HUVECs. Cells were grown to confluence onto the inserts of Transwell and exposed to 1 μ M HP-NAP, 1 μ M PAF, or medium added to the upper chamber. The passage of BSA into the lower chamber at various time intervals was evaluated by Fluostar. Values as means \pm SD of duplicate determinations of three separate experiments.

Confocal microscopy analysis

HUVECs grown on PET Transwell and treated with biotinylated HP-NAP for 0, 10, and 45 min were washed twice in PBS with 1 mM Ca^{2+} and 1 mM Mg^{2+} and fixed in 1.5% paraformaldehyde for 15 min at RT. The PET membrane supporting the cells was cut and inserted into a multiwell plate, where the cells were permeabilized according to the method reported previously (30). Biotinylated HP-NAP was stained with streptavidin Texas Red conjugated in TBST-3% BSA for 1 h. After three washings with TBST-1% BSA, PET membranes were mounted on glass slides with elvanol mounting medium. Images were obtained with a Confocal Ultraview microscope (PerkinElmer).

Real-time PCR and RNase protection assay (RPA) studies

Real-time PCR was performed, as described (31), using gene-specific primers (purchased from Invitrogen Life Technologies) available in the public database RTPrimerDB (<http://medgen.ugent.be/rtprimerdb/>) under the following entry codes: CXCL8 (3553), CXCL10/IP-1 (3537), CCL2 (3533), CCL3 (3599), CCL4 (3535), IL-6 (3545), vascular endothelial growth factor (VEGF) (3598), IFN- β (3542), TRAIL (3552), β_2 -microglobulin (3534), and GAPDH (3539). The reaction conditions were identical for all primer sets, as follows: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. β_2 -Microglobulin was selected as a normalizing gene, according to its stable expression levels in leukocytes (32). Data were calculated with Q-Gene software (www.BioTechniques.com) and are expressed as *n*-fold of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration (fmol/ μ l)/mRNA β_2 -microglobulin (fmol/ μ l)/mRNA β_2 -microglobulin (fmol/ μ l)). For the RPA experiments, the RiboQuantTM hcK-5 Human MultiProbe Template Sets were used according to the manufacturer's instructions (BD Pharmingen), as already described (33).

Detection of CXCL8 and other cytokines in culture supernatants

Culture supernatants of stimulated PMNs were collected, as indicated, and the amounts of CXCL8, CCL4, CXCL10, and VEGF were measured in the cell supernatants by commercial ELISA kits from BioSource International and R&D Systems.

Statistical analysis

Group means were compared by Student's *t* test, and difference was accepted at $p < 0.05$.

Results

HP-NAP induces in vivo leukocyte adhesion to endothelium

We first sought to determine whether HP-NAP was able to stimulate leukocyte endothelium adhesion in vivo. Intravital microscopy analysis of rat mesenteric venules exposed to HP-NAP for 15 min before image acquisition showed the ability of AO-labeled leukocytes (the bright spheres in Fig. 1, *B* and *C*) to adhere to the endothelium. Leukocyte accumulation, already visible at 10 min, was more evident at 30 min, and reached the maximum after 60 min (Fig. 1). Accumulation remained constant until 120 min (data

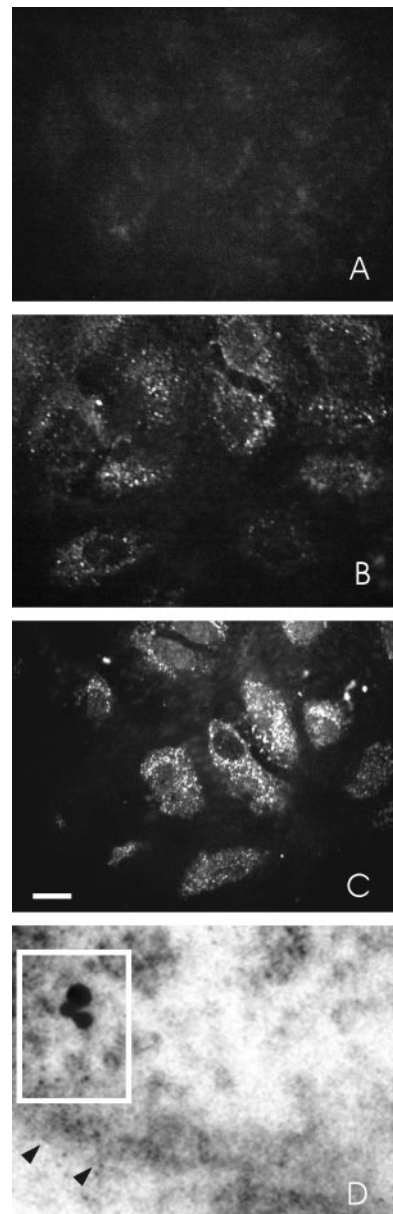


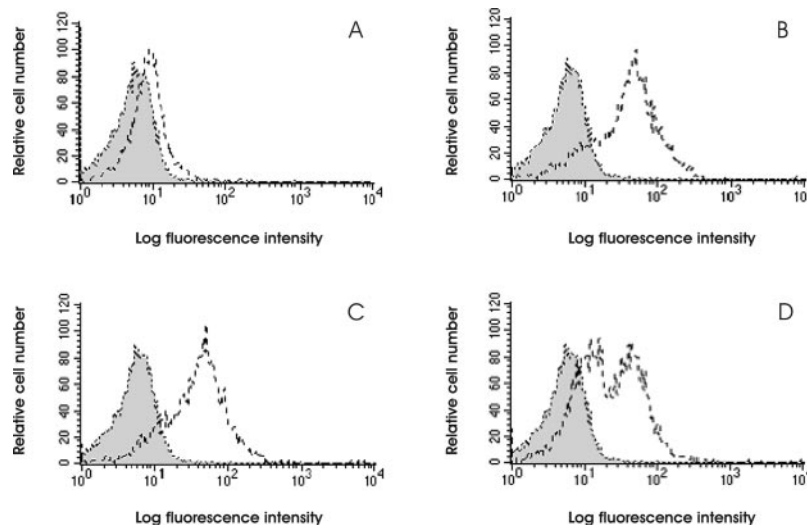
FIGURE 5. Accumulation of HP-NAP inside HUVECs. HUVECs were grown to confluence onto the inserts of Transwell. Biotinylated HP-NAP (1 μ M) was added to the lower chamber, and the inserts were cut immediately (*A*), after 10 min (*B*), and after 45 min (*C*), and then processed for fluorescent image acquisition by confocal microscopy. Bar, 10 μ m. *D*, Shows immunogold labeling of HP-NAP after 45 min of internalization. Accumulation of intracellular HP-NAP is visible in a vesicle (boxed) approaching to the plasma membrane (arrowheads). Magnification, $\times 28,000$.

not shown). No significant cell accumulation was observed in animals treated with saline (Fig. 1*A*) or with heat-inactivated HP-NAP (data not shown). Of note, 1 μ M HP-NAP did not cause significant leukocyte extravasation at any time point, whereas 5 μ M HP-NAP, a concentration that greatly enhances leukocyte adhesion, caused the appearance of a significant proportion of AO-labeled cells in the extravascular space after 60 min of treatment.

HP-NAP crosses the endothelium and induces PMNs to adhere

Considering that addition of HP-NAP to the extravascular side of the endothelium promoted leukocyte adhesion, we next addressed the question as to whether the protein was able to cross the endothelium, exerting a direct effect on leukocyte-endothelium interaction. For this

FIGURE 6. Flow cytometric analysis of the expression of 24Ag conformation epitope in PMNs exposed to HP-NAP. The expression of 24Ag activation epitope on PMNs exposed to HP-NAP (1 μ M) for 15 (B), 30 (C), and 60 min (D) was evaluated by flow cytometry. A, Refers to untreated cells. Dotted lines correspond to HP-NAP-treated PMNs; shaded histograms represent isotopic control. Results of a representative of four consecutive experiments are reported.



purpose, we evaluated the passage of a biotinylated HP-NAP through a HUVEC confluent monolayer grown onto a Transwell insert. HP-NAP was applied to the lower chamber, and the medium of the upper chamber was collected at different time points and the amount of HP-NAP was quantified by an ELISA. As shown in Fig. 2A, HP-NAP was detected in the upper chamber after 15 min, and the amount of the protein progressively increased, reaching the maximum at 45 min. Measurements of monolayer permeability indicated that after this time point the monolayer began to be not selectively permeable to proteins, as monitored by the passage of FITC-BSA. Thus, we decided to limit our analysis of HP-NAP passage to the first 45 min.

To evaluate whether the protein passed into the upper chamber was sufficient to induce leukocyte adhesion, the same medium used for the ELISA was also applied to a naive endothelium in the presence of PMNs for 30 min. As shown in Fig. 2B, adhesion was already observed with the conditioned medium collected in the upper chamber after 15 min. The extent of cell adhesion increased with the progressive accumulation of HP-NAP in the upper chamber (compare with A). We also investigated whether a proportion of HP-NAP remained associated to the endothelium following transcytosis. To this end, the amount of HP-NAP bound to endothelial cells, which had been exposed to the protein in a transcytosis assay, was measured by whole cell ELISA and found to be essentially similar to that of the released protein (Fig. 2, A and C).

Analysis of adherent PMNs by scanning electron microscopy (Fig. 3) showed that PMNs bound to HUVECs exposed to HP-NAP (Fig. 3B) had the morphologic appearance of flattened cells with large protrusions extending on the surfaces of endothelial cells, whereas PMNs added to untreated endothelial monolayer maintained a rounded shape (Fig. 3A).

HP-NAP does not alter endothelial permeability and does not cross the endothelium via the paracellular route

We assessed the possibility that the exposure of endothelium to HP-NAP may cause an increase in permeability that could permit the movement of the protein via the paracellular route. For this purpose, we monitored the passage of FITC-BSA, applied in the upper chamber, through endothelial monolayers in the presence of HP-NAP. The transport of native albumin across continuous endothelium occurs via noncoated plasmalemmal vesicles or caveolae rather than via the paracellular route (34, 35), making BSA a good marker for the evaluation of endothelial integrity (29). As shown in Fig. 4, in the presence of HP-NAP, the amount of FITC-

BSA retrieved in the lower chamber was comparable to that of the control experiment. We noticed a slight time-dependent increase, compatible with the transcellular transport of BSA. On the contrary, PAF, a known permeabilizing agent, added to the endothelium caused a significant passage of BSA in the lower chamber. These observations suggest that unlike PAF, HP-NAP does not alter the endothelial barrier. Moreover, it is unlikely that the dodecameric HP-NAP, which is much larger than BSA, moves through the interendothelial clefts. In other words, these data suggest that HP-NAP crosses the endothelium layer via an intracellular route.

HP-NAP is transported within the endothelial cells

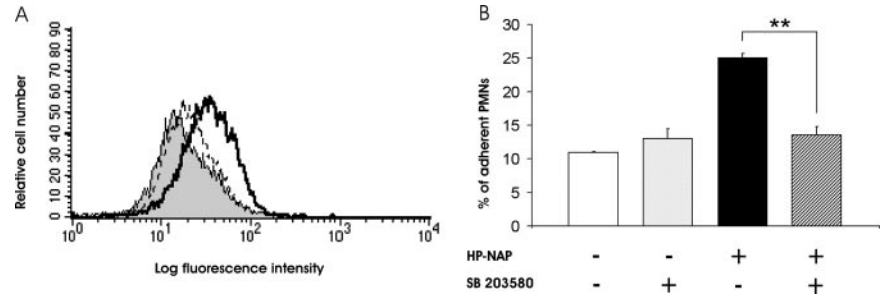
To obtain further evidence for an intracellular route of HP-NAP movement through the endothelium, we performed a kinetic analysis of internalization of biotinylated HP-NAP by HUVECs grown onto the insert of Transwells. Cells were labeled with Texas Red-conjugated streptavidin at different time points, and confocal microscopy analysis clearly demonstrated that HP-NAP was internalized as early as 10 min following its administration and that this process increased with time, reaching its maximum after 45 min (Fig. 5, A–C). These data are kinetically compatible with the time-dependent accumulation of HP-NAP in the apical medium (see Fig. 2) and further substantiate the transport of HP-NAP within the endothelial cells. To further analyze the intracellular distribution of HP-NAP, HUVECs exposed to the biotinylated protein for 45 min were incubated with gold-labeled streptavidin and examined by electron microscopy. The microphotograph shown in Fig. 5D reveals that HP-NAP localizes into structures resembling vesicles.

HP-NAP induces a conformational change of β_2 integrins

Previous reports suggested that HP-NAP may directly stimulate PMNs to adhere to the endothelial cells (13, 17). We therefore evaluated the ability of HP-NAP to modulate the adhesion of PMNs mediated by β_2 integrin, the molecule responsible for the arrest of rolling cells on the endothelial surface (10). It has been demonstrated that one modality of activation of β_2 integrin is the acquisition of a conformation with high affinity for the ligand (10). This structural change can be monitored following the exposure of new epitopes such as the 24Ag (36).

To investigate whether the increased adhesion of PMNs stimulated by HP-NAP was due to the induction of an integrin high affinity state, PMNs were incubated with HP-NAP, and the expression of the 24Ag was evaluated by flow cytometry at different

FIGURE 7. Effects of p38-MAPK inhibition in HP-NAP-treated PMNs. Expression of 24Ag was evaluated in PMNs treated with 30 μ M SB203580 before exposing to HP-NAP (A). Solid lines correspond to HP-NAP-stimulated neutrophils; dotted lines to HP-NAP-stimulated neutrophils pretreated with SB203580; and shaded histograms represent untreated cells. Effect of p38-MAPK inhibition on HP-NAP-neutrophil adhesion was determined (B). Results of a representative of four consecutive experiments are reported. **, $p < 0.01$.



time points. As illustrated in Fig. 6, PMNs express significant levels of the epitope after 15 min of treatment (Fig. 6B) as compared with basal levels (Fig. 6A). Exposure of this epitope remained constant at 30 min (Fig. 6C) and then started to decrease after 1 h of treatment, as shown by the appearance of a cellular population with a fluorescence comparable to that of untreated cells (Fig. 6D). No increase in 24Ag expression was revealed on untreated PMNs over all the time points considered (data not shown).

HP-NAP-induced β_2 integrin activation involves p38-MAPK

Several studies suggest that HP-NAP may interact with at least two receptors on the plasma membrane of leukocytes. The engagement of TLR-2 (20) seems to be related to the production of cytokines by monocytes, whereas the interaction with a G protein-coupled receptor is linked to burst activation, adhesion, and chemotaxis of PMNs (27). These latter effects are abrogated by inhibition of p38-MAPK, suggesting a role for this kinase in the signaling cascade (37). As p38-MAPK triggers β_2 integrin activation in TNF- α -stimulated PMNs (38), we decided to ascertain whether expression of 24Ag induced by HP-NAP also required p38-MAPK. PMNs were pretreated with the specific p38-MAPK inhibitor SB203580 before being exposed to HP-NAP. Evaluation of the 24Ag expression after 30 min is illustrated in Fig. 7A. As compared with the values of PMNs exposed to HP-NAP alone (solid line), inhibition of p38-MAPK completely abolished the exposure of the 24Ag epitope (compare the dotted line with the shadowed histogram representing the basal level). To definitely confirm that the integrin high affinity state was the main one responsible for the adhesion of PMNs exposed to HP-NAP, we examined the effect of the p38-MAPK inhibition in an adhesion assay. Fig. 7B shows that when p38-MAPK was inhibited, the extent of adhesion was reduced to values comparable with those of untreated cells, despite stimulation of PMNs with HP-NAP. Of note, the inhibitor SB203580 did not affect adhesivity per se, further confirming that p38-MAPK is positioned downstream of HP-NAP in the promotion of the adhesivity state of PMNs.

HP-NAP activate PMNs to synthesize and release chemokines

Several studies reported that infection by *H. pylori* is associated with an increased expression of chemokines, and that endothelial and gastric epithelial cells are believed to contribute to this increase (9, 11, 39). Although it has been demonstrated that factors other than HP-NAP stimulate endothelial cells to produce CXCL8 (13), the release of CXCL8 or other chemokines from PMNs stimulated by HP-NAP has not been investigated yet. To shed light upon this aspect, total RNA was extracted from PMNs incubated with HP-NAP and examined by RPA and real-time PCR. As shown in Fig. 8A, treatment of PMNs with HP-NAP resulted in a rapid induction of CXCL8, CCL3, and CCL4 mRNAs that reached maximum levels at 3 h for all the chemokines and slowly declined thereafter. These observations were substantially confirmed by

real-time PCR studies (Fig. 8B), which also revealed that the mRNAs for the CXCL10 and CCL2 chemokines, or for other cytokines, such as VEGF, TRAIL, and IFN- β , were not induced

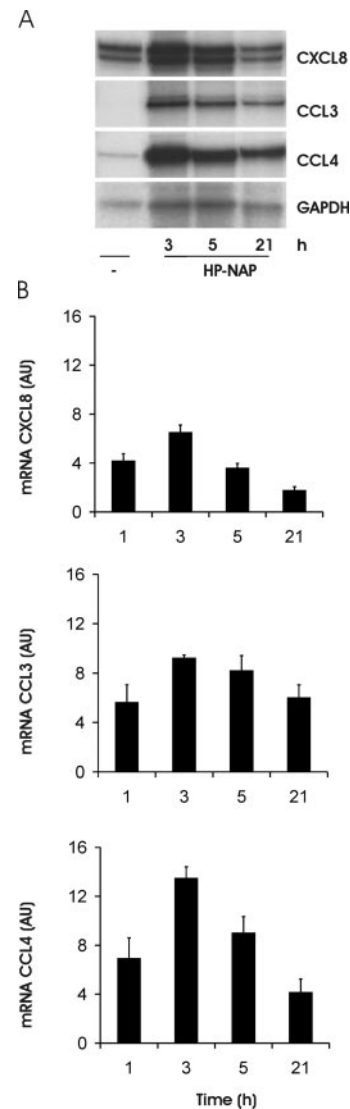


FIGURE 8. Time-dependent mRNA expression for various chemokines in HP-NAP-treated neutrophils. Total RNA was extracted from neutrophils cultured with HP-NAP and analyzed by either RPA (A) or real-time PCR (B) for CXCL8, CCL3, and CCL4 mRNA expression. B, Expression levels are depicted as n -folds of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration (fmol/ μ l)/mRNA β_2 -microglobulin (fmol/ μ l)/mRNA β_2 -microglobulin (fmol/ μ l) of triplicate reactions for each sample. Data show representative experiments of three performed for each panel.

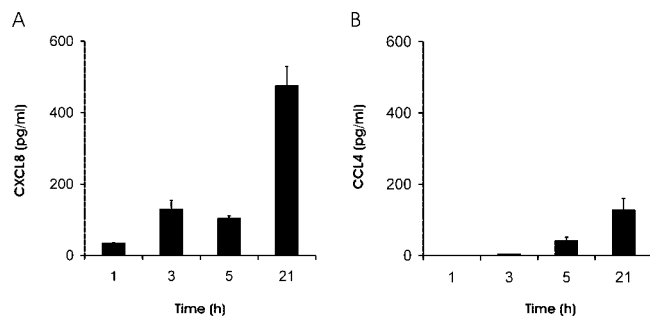


FIGURE 9. Kinetics of CXCL8 and CCL4 extracellular production by HP-NAP-treated neutrophils. PMNs were stimulated with 1 μ M HP-NAP for the times indicated, and CXCL8 (A) and CCL4 (B) levels were then determined in the cell supernatants by ELISA. For each cytokine, the figure shows the mean value \pm the SD of duplicate assays for each time point, each obtained from three experiments performed under the same conditions.

in HP-NAP-treated PMNs (data not shown). In line with the gene expression data, culture supernatants of PMNs stimulated with HP-NAP showed a remarkable extracellular production of both CXCL8 and CCL4, which began, respectively, as early as after 3 and 5 h ($p < 0.001$, $n = 5$), and continued to progressively increase up to 21 h ($p < 0.005$, $n = 6$) (Fig. 9). Under the same conditions, neither CXCL10 nor VEGF was found to be released by activated neutrophils, even if PMNs were costimulated with IFN- γ (data not shown).

Discussion

The infiltration of neutrophils and mononuclear inflammatory cells within the *H. pylori*-infected stomach mucosa is a common finding, and the degree of mucosal damage is correlated with neutrophil infiltration. Several protein components in *H. pylori* extracts were found to attract and activate neutrophils and other inflammatory cells (17, 40–49).

Among these molecules, a 150-kDa oligomeric protein isolated from *H. pylori* water extracts was found to promote neutrophil adhesion to endothelial cells (17, 50), and the protein was named HP-NAP. HP-NAP is released in the medium, most likely after cell lysis, and binds to the bacterial surface, where it can act as an adhesin, mediating binding to mucin (51) or to PMN sphingomyelin (52).

Until recently, all of the experiments illustrating the capacity of HP-NAP of inducing leukocytes to adhere to the endothelium were conducted, putting PMNs and HP-NAP directly in contact, without considering that in vivo the protein has to cross the endothelium to stimulate neutrophils. A recent report, however, showed that HP-NAP promoted transendothelial neutrophil migration also when added into a lower chamber of a Transwell system consisting of a cultured monolayer of human endothelial cells as barrier between two chambers (13). These findings, although strongly suggesting that HP-NAP was able to cross the endothelium to contact leukocytes, were not reinforced by data, providing direct evidence for the transendothelial passage of HP-NAP. Moreover, the authors did not consider that in vivo the underflow conditions may bias the effects observed in vitro.

In this study, we report for the first time that HP-NAP promotes leukocyte adhesion to the endothelium in vivo. Intravital microscopy, used in our study to analyze the mobilization of leukocytes in response to HP-NAP, allowed us to show that leukocytes circulating in the rat mesentery begin to adhere rather rapidly to the endothelium of the postcapillary venules after the topical applica-

tion of HP-NAP. Adhesion is detectable already after 10 min and reaches a maximum at 60 min. With 5 μ M HP-NAP, a significant leukocyte extravasation is observed after 60 min of treatment. These findings are at variance with previous reports showing that lower concentrations of HP-NAP induced PMN chemotaxis in a rapid and efficient way (13, 27, 37). This discrepancy can be explained by the different complexity of the systems used, which emphasizes the need of using integrated models as close as possible to the in vivo situation. Furthermore, it is possible that CXCL8, secreted by HP-NAP-stimulated peritoneal macrophages, contributed to the accumulation of leukocytes in the tissue. In support of this hypothesis, there is a recent finding that HP-NAP stimulated monocytes/macrophages to secrete CXCL8 (20).

In this study, we have also shown that HP-NAP is effectively transported across the endothelium via intracellular route without any major contribution of the paracellular route. The amount of protein recovered after crossing was small, but biologically relevant, because it was sufficient to stimulate PMNs to adhere to endothelial cells. HP-NAP failed to alter the endothelial permeability in such a way to move through interendothelial clefts, but it was transported within endothelial cells through vesicles, as clearly demonstrated by protein immunogold labeling. The internalization process of HP-NAP was found to be very efficient, as suggested by the number of fluorescent spots visible after labeling of biotinylated HP-NAP with streptavidin Texas Red. This was apparently in contrast with the limited amount of protein that was released after endothelial crossing. A possible explanation is that a significant proportion of HP-NAP remained bound to the endothelium after transcytosis, similarly to what was observed for the chemokine CXCL8 (53). This possibility is supported by our finding that a substantial amount of HP-NAP was detected on the endothelium, and this, in turn, promoted a significant adhesion of PMNs (data not shown).

β_2 integrins on the surface of leukocytes need to be activated to acquire their ligand-binding capacity. Modulation of integrin proadhesive activity involves, in some instances, heterodimer lateral mobility leading to accumulation in discrete areas of the plasma membrane, a process called clustering, which has been proposed as an essential event regulating integrin activation (54, 55). Alternatively, or in addition, integrins may potentially increase their affinity for ligand by altering their three-dimensional conformational state (10, 56). In the last few years, the importance of these conformational changes has been highlighted. Conformational changes upon β_2 integrin activation can be evidenced by monitoring the exposure of activation epitopes such as the 24Ag (36). Using an Ab specific for the 24Ag, exposed after the acquisition of the integrin high affinity state (36), we show in this study that HP-NAP induced the conformational change of the adhesion molecule. A parallel evaluation of HP-NAP stimulation of endothelium to become more adhesive showed, in agreement with a recent report (13), that there was no increased expression of ICAM-1, VCAM-2, and endothelial leukocyte adhesion molecule after 4 and 24 h of stimulation (data not shown). These results, together with our previous observation illustrating that HP-NAP up-regulated the expression of β_2 integrins (27), definitively confirm that HP-NAP has a direct role in stimulating neutrophils to adhere. Finally, we demonstrated the involvement of the p38-MAPK along the signaling cascade responsible for the integrin activation, as already reported for TNF- α (38).

The results of this study also establish that human PMNs respond to stimulation with HP-NAP by expressing and producing several chemokines, including CXCL8, CCL3, and CCL4. Expression of other proinflammatory molecules, such as CXCL10,

IFN- β , and TRAIL, was not induced by HP-NAP, which is consistent with the fact that HP-NAP acts via TLR-2, and consequently through the intracellular Myd88-dependent pathway (57). Importantly, neither IL-6 nor CCL2 mRNAs were induced in HP-NAP-treated PMNs, indicating that the effects attributed to HP-NAP were direct and not mediated by contaminating monocytes or eosinophils (58). Because neutrophils rapidly migrate in large numbers at the infection sites, the fact that they also serve as a chemokine source may contribute to the generation of the conditions necessary for both the recruitment and activation not only of additional neutrophils via CXCL8, but also of monocytes, dendritic cells, and lymphocytes through CCL3 and CCL4. The capacity of neutrophils to produce chemokines in response to HP-NAP, as shown in this study, provides new insights for a better understanding of the cellular mechanisms involved in the *H. pylori*-induced inflammation.

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Disclosures

The authors have no financial conflict of interest.

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