Helicobacter pylori Neutrophil-Activating Protein Stimulates Tissue Factor and Plasminogen Activator Inhibitor-2 Production by Human Blood Mononuclear Cells

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Helicobacter pylori neutrophil-activating protein (HP-NAP) is a virulence factor that activates phagocytic NADPH-oxidase. The effect of HP-NAP on the production of tissue factor (TF), plasminogen activator inhibitor-2 (PAI-2), and urokinase-type plasminogen activator (u-PA) by human blood mononuclear cells (MNC) was evaluated by using functional and immunological assays and mRNA analysis. HP-NAP induced time- and dose-dependent increases in TF and PAI-2, with a maximal effect at 300 nmol/L (>15-fold increase in antigens). No changes in u-PA were observed. When whole bacteria were used, an H. pylori mutant lacking HP-NAP was significantly less active than the wild-type strain. MNC from a patient with chronic granulomatous disease behaved as do normal cells, which indicates that HP-NAP effects can occur independently of NADPH-oxidase. HP-NAP, by inducing the coordinate expression of cell procoagulant and antifibrinolytic activities, might favor fibrin deposition and contribute to the inflammatory reaction of gastric mucosa elicited by H. pylori.

Colonization of the human stomach by Helicobacter pylori leads to chronic gastritis, a condition complicated by peptic ulcer and atrophic gastritis [1, 2]. H. pylori infection also is associated with an increased incidence of gastric adenocarcinomas and lymphomas [3, 4]. In addition, recent clinical evidence suggests that it might increase the incidence of ischemic heart and cerebrovascular diseases [5, 6]. Chronic gastric inflammation is characterized by the infiltration of polymorphonuclear granulocytes (PMNG), lymphocytes, and abundant mononuclear phagocytes in the mucosa [2]. The interaction of several H. pylori products with these cells is likely to determine the extent of tissue damage and to create conditions that favor cancer development [2, 7]. To date, no clear hypothesis for the increased risk of extragastric vascular diseases has been formulated. Some H. pylori virulence factors are encoded within the 40-kb pathogenicity island cag (cytotoxin-associated gene) [8] and are required for epithelial colonization by H. pylori [9, 10] and for the production of interleukin (IL)–8 [11–14] and cyclooxygenase-2 synthase, a phenomenon that is believed to favor neoplastic progression [15]. Other virulence factors are encoded outside the pathogenicity island. Among them, vacuolating cytotoxin A and H. pylori neutrophil-activating protein (HP-NAP) are linked to pathogenicity and are candidates for a recombinant anti-H. pylori vaccine [16].

HP-NAP is a dodecameric protein consisting of 17,000-Da monomers with a central cavity where iron can be accumulated [17, 18]. This bacterial cytosolic protein, encoded by the napA gene, 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 [19] or to PMNG sphingomyelin [20]. HP-NAP also shows intrinsic proinflammatory activities, is chemotactic for PMNG and monocytes, and induces, in both, surface expression of β2-integrins, which are necessary for endothelial transmigration [21]. Moreover, it triggers the production of reactive oxygen intermediates through the activation of NADPH-oxidase [17, 21]. On the basis of this evidence, it has been postulated that HP-NAP contributes significantly to the exacerbation of tissue inflammatory reactions and, therefore, to the tissue damage that accompanies chronic H. pylori infection [21]. The possibility that H. pylori surface proteins promote inflammation also is suggested by their triggering of IL-1 and tumor necrosis factor–α production by mononuclear cells (MNC) [22].

On activation, monocytes express on their surface the 47,000-Da transmembrane protein tissue factor (TF) that is a potent trigger of the protease cascade that leads to fibrin deposition.
Materials and Methods

**Incubation of isolated MNC with HP-NAP.** MNC were obtained from citrated blood drawn from healthy donors and from a patient with X-linked chronic granulomatous disease (CGD). All subjects had abstained from all medications for >1 week before blood collection. Cells were isolated by centrifugation on a Ficoll-Hypaque (Nycomed Pharma) cushion [34] and were suspended in LPS-free RPMI-1640 (Bio Whittaker) at a concentration of 10^7/mL, unless otherwise specified. Final cell preparations contained >97% MNC and <1 platelet per nucleated cell. Monocytes, identified by nonspecific esterase staining, were 18%–30% (mean, 24%) of the MNC. In some experiments, MNC were further enriched in monocytes by adherence [35]. Cell viability, assessed by the trypan blue test, always exceeded 96%. All reagents were free of endotoxin, as assessed by the lymulus amebocyte lysate assay (Sigma).

HP-NAP was produced in recombinant form from *Bacillus subtilis* [18]. To evaluate its effect on the cell procoagulant and fibrinolytic properties, aliquots of cell preparations in serum-free RPMI medium were incubated at 37°C (30 min at room temperature (RT) before electrophoresis. Gels then were soaked at RT for 60 min in Triton X-100 (2.5% v/v) or in Triton X-100 (2.5% v/v) in H,O, and were placed on 1% (w/v) agarose films containing plasminogen and fibrin, as described elsewhere [37]. After overnight incubation at RT, bands corresponding to free and bound plasminogen activators were visualized by the appearance of lysis areas in the fibrin gel.

**In vitro clot lysis.** The effect of MNC on u-PA-induced clot lysis was studied, using a microplate turbidimetric assay, as described elsewhere [38]. In brief, cells were stimulated overnight with 300 nmol/L HP-NAP or with buffer alone. Aliquots (100 μL) of cell suspensions were added to 95 μL of human fibrinogen (6 mg/mL), 5 μL of citrated human plasma, 10 μL of a mixture of phospholipids (Thrombofax; Instrumentation Laboratory), 10 μL of u-PA (final concentration, 1.25 U/mL), or tissue-type plasminogen activator (t-PA; final concentration, 50 ng/mL, corresponding to ~5 U/mL of u-PA [39]), and 10 μL of 0.5 mol/L CaCl₂ in a 96-well microplate tray and were incubated at 37°C. The decrease in optical density at 405 nm, after the initial increase due to clot formation, was measured every 15 min, and the time necessary to reach a 50% decrease, corresponding to 50% fibrinolysis, was determined. Where indicated, neutralizing rabbit anti-PAI-2 IgG (provided by N. Booth, Dept. of Molecular and Cell Biology, University of Aberdeen, Aberdeen, United Kingdom) or control rabbit IgG was added to the sample before the clot lysis assay, at a final concentration of 20 μg/mL.

**Northern blotting.** RNA was extracted from MNC (10 × 10^6 cells) incubated for 4 h with HP-NAP or solvent, as described elsewhere [40]. Fifteen micrograms of each RNA sample was used for analysis. Detection of TF and PAI-2 mRNA was done by using...
Effect of HP-NAP on TF expression by MNC. Human MNC were treated with different concentrations of purified HP-NAP for 4 h and then were tested for TF activity. Figure 1A shows that HP-NAP increases the TF activity of MNC dose dependently, with a half-maximal effect at the concentration of 100 nmol/L (calculation based on the 17,000-Da monomer). The maximal effect was obtained at the concentration of 300 nmol/L and corresponded to ~1300 TF units, a value comparable to that (~1500 TF units) induced in the same conditions by the powerful TF inducer S. typhimurium LPS (1 μg/mL). When a clotting assay was done with intact HP-NAP-treated MNC, TF activity was 2–5-fold lower than that of corresponding lysed cells at all tested HP-NAP concentrations (data not shown). By using plasma samples deficient in single coagulation factors [36], this activity was found to be totally dependent on factor VII and thus functionally identical to TF (data not shown). Similar results were obtained with monocyte-enriched preparations (85%–95%), which suggests that the effect of HP-NAP is not influenced by the presence of lymphocytes to a significant extent. Enhanced TF expression in HP-NAP-stimulated MNC also was demonstrated by Northern blot analysis and ELISA (figure 1A). After 4 h of incubation with HP-NAP, TF mRNA exhibited a dose-dependent increase, whereas it was undetectable in control cells. Ethidium bromide staining of ribosomal RNA applied to each lane resulted in bands of similar intensity (data not shown), which confirms that equivalent amounts of cellular RNA had been loaded onto each lane. TF antigen increased after stimulation with HP-NAP for 4 h, reaching a maximal value at 300 nmol/L. Time-course experiments with HP-NAP (50 nmol/L) showed a detectable increase in TF activity only after a 1.5-h stimulation (figure 1B), a lag time compatible with activation of gene transcription and pro-
Figure 2. Effect of heat denaturation and of anti-CD14 antibodies (Ab) on Helicobacter pylori neutrophil-activating protein (HP-NAP)-induced tissue factor (TF) expression by mononuclear cells (MNC). MNC were incubated for 4 h at 37°C with HP-NAP, with heat-denatured (by boiling for 10 min) HP-NAP, with HP-NAP in the presence of 10% serum, or with HP-NAP plus serum and anti-CD14 antibodies (5 μg/mL). In these experiments, HP-NAP concentration was 50 nmol/L. Data are of 3 independent experiments.

Figure 3. Effect of Helicobacter pylori neutrophil-activating protein (HP-NAP) on plasminogen activator inhibitor-2 (PAI-2) and urokinase-type plasminogen activator (u-PA) production by mononuclear cells (MNC). A, PAI-2 and u-PA antigens in the extracellular media of MNC stimulated for 20 h at 37°C with increasing concentrations of HP-NAP. Data are of 3 independent experiments. Inset, mean ± SE of 3 independent experiments. Northern blot analysis of PAI-2 mRNA in MNC incubated for 4 h at 37°C with the indicated concentrations of HP-NAP. B, Representative fibrin autography of extracellular media from MNC stimulated or not with 300 nmol/L HP-NAP, as in A. Samples were preincubated for 30 min with or without u-PA (2.5 U/mL) before processing for fibrin autography, as detailed in Materials and Methods. Lysis zones are visualized as black areas in the fibrin gel. Qualitatively similar results were obtained in 3 additional experiments.

Figure 4. Effect of Helicobacter pylori neutrophil-activating protein (HP-NAP) on plasminogen activator inhibitor-2 (PAI-2) and urokinase-type plasminogen activator (u-PA) production by mononuclear cells (MNC). A, PAI-2 and u-PA antigens in the extracellular media of MNC stimulated for 20 h at 37°C with increasing concentrations of HP-NAP. Data are of 3 independent experiments. Inset, mean ± SE of 3 independent experiments. Northern blot analysis of PAI-2 mRNA in MNC incubated for 4 h at 37°C with the indicated concentrations of HP-NAP. B, Representative fibrin autography of extracellular media from MNC stimulated or not with 300 nmol/L HP-NAP, as in A. Samples were preincubated for 30 min with or without u-PA (2.5 U/mL) before processing for fibrin autography, as detailed in Materials and Methods. Lysis zones are visualized as black areas in the fibrin gel. Qualitatively similar results were obtained in 3 additional experiments.

Figure 5. Effect of HP-NAP–treated MNC on plasma clot lysis. To assess whether the increased production of PAI-2 induced by HP-NAP had functional consequences on fibrinolysis, we evaluated the effect of MNC incubated for 20 h with HP-NAP (300 nmol/L) on the lysis of diluted plasma clots by exogenous u-PA. As shown in figure 4, HP-NAP–treated cells markedly re-

protein synthesis. TF activity augmented rapidly thereafter, reaching a plateau 20 h after the addition of HP-NAP.

Figure 2 shows that the induction of MNC TF activity was totally abolished by heat denaturation of HP-NAP (100°C for 10 min). Moreover, the HP-NAP effect was not influenced by the presence of serum in the culture medium or the inclusion of a blocking anti-CD14 antibody in serum-containing medium, thus ruling out a possible role of contaminating LPS in the HP-NAP action. Indeed, the stimulating activity of S. typhimurium LPS was unaffected by heat-treatment (data not shown) and was potentiated by serum and inhibited by anti-CD14 antibodies [32].

Effect of HP-NAP on PAI-2 and u-PA production by MNC. Incubation of MNC with HP-NAP for 20 h caused a dose-dependent increase of PAI-2 antigen accumulation in the extracellular medium (figure 3A). Also, in this case, the effect of the highest doses of HP-NAP was comparable to that of S. typhimurium LPS (1 μg/mL). Intracellular PAI-2 was also increased by HP-NAP, mean levels in cell extracts being about twice as high as those of the extracellular inhibitor (data not shown). In contrast, extracellular (figure 3A), as well as cell-associated (data not shown), u-PA antigen was not affected by HP-NAP at any concentration. The dose-response curve of HP-NAP–induced increase of PAI-2 antigen and mRNA was very similar to that of TF (figure 3A and figure 1A), with a half-maximal effect at 100 nmol/L.

Fibrin autography of the extracellular medium derived from control and HP-NAP–stimulated MNC showed no visible lysis bands (figure 3B). When extracellular medium was preincu-
Effect of \( \text{Helicobacter pylori} \) neutrophil-activating protein (HP-NAP)–treated mononuclear cells (MNC) on urokinase-type plasminogen activator (u-PA)–induced and tissue plasminogen activator (t-PA)–induced clot lysis. MNC were incubated for 20 h at 37°C, without or with HP-NAP (300 nmol/L), and then were tested in a microplate-diluted plasma clot lysis assay, as described in Materials and Methods. Buffer indicates clot lysis in the absence of cells. Rabbit IgG plasminogen activator inhibitor–2 antibody (PAI-2 Ab) was used at the concentration of 20 \( \mu \text{g/mL} \). Results express the time required for 50% lysis. Data are mean ± SE of 3 independent experiments. * \( P < .01 \), compared with MNC.

![Figure 4. Effect of Helicobacter pylori neutrophil-activating protein (HP-NAP)–treated mononuclear cells (MNC) on urokinase-type plasminogen activator (u-PA)–induced and tissue plasminogen activator (t-PA)–induced clot lysis.](https://academic.oup.com/jid/article-abstract/183/7/1055/859204)
detectable with the limulus assay, was responsible for or contributed to the observed HP-NAP-induced responses is ruled out by the following observations. First, heat treatment of HP-NAP resulted in a complete loss of its stimulating activity. Second, the procoagulant response of MNC to HP-NAP was not influenced by the presence of serum in the culture medium or by the addition of an anti-CD14 antibody in serum-containing medium. This is at variance with the procoagulant response of MNC to LPS, which is greatly potentiated by serum because of the binding of LPS to a serum factor termed LPS-binding protein (LBP) and because of the subsequent engagement of the membrane receptor CD14 by LPS-LBP complex [32, 45].

Cell wall LPS has been proposed as a potential pathogenic factor in *H. pylori* infection, and there is evidence that *H. pylori* LPS induces TF and PAI-2 production in MNC, although it is much less active than other enterobacterial LPSs [32]. To evaluate the relative contribution of HP-NAP in MNC stimulation, we compared the effect of a *ΔnapA* isogenic mutant of *H. pylori* with that of the corresponding wt bacteria. It is interesting that bacteria that lack HP-NAP were significantly less active than were wt bacteria in stimulating wt bacteria in both TF and PAI-2 synthesis by MNC. Considering that whole bacteria may induce TF expression in MNC by complex mechanisms, including bacterial phagocytosis [46], our observation strengthens the prominent role of HP-NAP as an inducer of TF and PAI-2 synthesis.

HP-NAP binds to an unidentified receptor, coupled to trimeric GTPases, and generates intracellular signals [21]. In this study, we show that the transduction pathway(s) triggered by HP-NAP eventually leads to TF and PAI-2 gene activation. In agreement with data previously obtained with other agonists [24, 25], this gene activation requires PTK and PKC activation and NF-κB. In fact, TF expression induced by HP-NAP is fully prevented by genistein and H7, which inhibit PTK and PKC, respectively, as well as by CAPE and HNE, 2 agents that prevent NF-κB release and nuclear translocation [43, 44].

The observation that MNC from a patient with CGD, which lacked NADPH-oxidase, behave as normal cells when stimulated by HP-NAP indicates that activation of NADPH-oxidase, one of the best characterized actions of HP-NAP [21], does not contribute to a significant extent to the induction of TF and PAI-2. Our data also show that the intracellular pathway triggered by HP-NAP that leads to PAI-2 synthesis is partially different from the pathway that triggers TF expression. In fact, PAI-2 induction, like TF induction, requires PKC but is much less sensitive to PTK activity.

Taken together, these results support the hypothesis that HP-NAP is a major virulence factor of *H. pylori* that is capable of increasing the procoagulant potential and of inhibiting the fibrinolytic capacity of MNC, thus tilting the cell coagulation-fibrinolysis balance toward fibrin formation and persistence. This is achieved by the induction of parallel intracellular pathways that lead to activation of TF and PAI-2 gene transcription. Hence, in addition to the factors encoded within the pathogenicity island *cag*, HP-NAP must be included in the category of virulence determinants that alter the gene regulation of host cells, with the possible role of supporting *H. pylori* colonization. The action of HP-NAP described here is expected to favor chronic development of gastritis and tissue disruption by hampering tissue healing, which requires degradation and removal of fibrin and tissue debris. At the same time, fibrin deposition might hinder movement of phagocytes toward *H. pylori* cells and protect them from phagocytosis.

Expression of TF on mononuclear phagocytes also has an
Figure 6. Effect of inhibitors of signal transduction pathways on Helicobacter pylori neutrophil-activating protein (HP-NAP)–induced tissue factor (TF) and plasminogen activator inhibitor–2 (PAI-2) production by mononuclear cells (MNC). MNC were preincubated with the indicated inhibitors or the respective vehicles, as described in Materials and Methods, before challenge with HP-NAP (300 mmol/L). The concentration of inhibitors was as follows: genistein, 100 μmol/L; 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H7), 180 μmol/L; caffeic acid phenethyl ester (CAPE), 90 μmol/L; 4-hydroxynonenal (HNE), 25 μmol/L. TF antigen (white columns) and PAI-2 antigen (hatched columns) were measured after 4- and 20-h incubation, respectively, and are expressed as percentage of values obtained with control MNC incubated with HP-NAP in the absence of inhibitors. Data are mean ± SE of 3 independent experiments. *P < .05, compared with control.

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References


