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Helicobacter pylori neutrophil-activating protein promotes myeloperoxidase release from human neutrophils

Chung-An Wang [a,1], Yen-Chun Liu [a,1], Shin-Yi Du [a], Chio-Wen Lin [a], Hua-Wen Fu [a,b,*]

[a] *Institute of Molecular and Cellular Biology, National Tsing Hua University, 101 Section 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, ROC*
[b] *Department of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan, ROC*

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ABSTRACT

Helicobacter pylori infection induces acute and chronic inflammation and plays a key role in gastric mucosal diseases. *H. pylori* neutrophil-activating protein (HP-NAP), one of its virulence factors, induces not only chemotactic but also oxidative burst responses of neutrophils. Activated neutrophils use myeloperoxidase (MPO) to generate many cytotoxic oxidants, which might result in gastric mucosal injury. In this study, we evaluated whether HP-NAP could promote MPO release from human neutrophils. Recombinant HP-NAP expressed in *Escherichia coli* was purified by two sequential gel filtration chromatographies and then subjected to syringe filtration for endotoxin removal. The purified recombinant HP-NAP assembles into a multimer with a secondary structure of the typical α-helix. In addition to stimulating the production of reactive oxygen species, HP-NAP is able to induce the secretion of MPO in human neutrophils. The increased MPO release from neutrophils induced by HP-NAP may be related to the pathogenesis of *H. pylori*-associated gastritis.

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Helicobacter pylori infects over half of the human population [1]. Infection with *H. pylori* is associated with chronic gastritis, peptic ulcer disease and gastric cancer [2]. *H. pylori* infection elicits an inflammatory cell response, and the severity of mucosal injury appears to be directly correlated with the extent of neutrophil infiltration [3,4]. The water extract, sonicate, and culture supernatant of *H. pylori* were able to induce neutrophil activation and chemotaxis [5–8]. Thus, the presence of bacterial factors of *H. pylori* to attract and activate neutrophils might contribute to *H. pylori*-induced gastritis.

H. pylori neutrophil-activating protein (HP-NAP), a 150 kDa protein first isolated from water extract of *H. pylori*, was identified to be able to stimulate the production of reactive oxygen species (ROS) in neutrophils and promote adhesion of neutrophils to endothelial cells [9]. HP-NAP is a spherical dodecamer formed by twelve identical monomers [10,11]. Each monomer is a four-helix bundle protein with a molecular weight of ~17 kDa [10]. The presence of a large number of positively charged residues on the surface of HP-NAP might account for its unique ability in activating human leukocytes [11].

HP-NAP induces dose-dependent ROS production and chemotaxis in neutrophils and monocytes [12]. HP-NAP also induces the release of IL-8 and CCL4 in neutrophils [13], the production of tissue factor, tumor necrosis factor alpha (TNF-α), IL-6, and IL-8 in monocytes [14,15], and the release of β-hexosaminidase and IL-6 in mast cells [16]. TNF-α and IFN-γ are able to prime human neutrophils to potentiate the effect of HP-NAP on ROS production [12]. Such priming effects may be relevant to the *in vivo* situation, where a synergistic effect between HP-NAP and cytokines could amplify the pathogenic effect of ROS. The production of ROS and those cytokines may also act as pro-inflammatory signals to induce inflammation and degeneration of the stomach mucosa. Thus, HP-NAP might play a role in immune pathogenesis of *H. pylori* infection.

The hallmark of chronic gastritis caused by *H. pylori* infection is infiltration of neutrophils into gastric mucosa [17]. Mucosal biopsy specimens from patients with *H. pylori* infection showed increased amounts of ROS and myeloperoxidase (MPO) [3,7,18,19]. In addition, *H. pylori* density and neutrophils infiltration were correlated with MPO level in gastric antral mucosa [19]. MPO possesses potent proinflammatory properties and may contribute directly to tissue injury. The secretion of MPO from neutrophils can be induced directly by bacterial factors. Indeed, *H. pylori* sonicate proteins stimulate neutrophil degranulation with MPO release in a concentration-dependent manner [7]. *H. pylori* water extract also increases the secretion of MPO from human neutorphils [19]. However, the protein component of *H. pylori* stimulating MPO release from neutrophils has not been identified. In this study, we have characterized the recombinant HP-NAP expressed in *Escherichia coli* by examining its physical characteristics and ability to induce

* Corresponding author. Address: Institute of Molecular and Cellular Biology, National Tsing Hua University, 101 Section 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, ROC. Fax: +886 3 5715934.
E-mail address: hwfu@life.nthu.edu.tw (H.-W. Fu).
[1] These authors contributed equally to this work.

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ROS production in neutrophils. Most importantly, the release of MPO from human neutrophils induced by HP-NAP has been investigated.

Materials and methods

Reagents. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from MDBio (Taipei, Taiwan). Ficoll-Paque PLUS and dextran 500 were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Roche (Basel, Switzerland). *N*-α-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phorbol 12-myristate 13-acetate (PMA), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), 3,3′,5,5′-tetramethylbenzidine (TMB), myeloperoxidase (MPO) from human leukocytes, bovine serum albumin (BSA), and lipopolysaccarides (LPS) from *E. coli* O111:B4 were purchased from Sigma–Aldrich (St. Louis, MO). LPS was dissolved in deionized water to a stock concentration of 1 mg/mL and stored at −20 °C until use.

Production of recombinant HP-NAP. Escherichia coli BL21(DE3) cells harboring pET42a-NAP (see Supplemental material) were grown in 5 mL of LB broth containing 50 μg/mL kanamycin at 37 °C. The overnight culture (1 mL) was inoculated into 100 mL of fresh media and cultured at 37 °C until the OD_{600} reached 0.4. The expression of HP-NAP was induced by adding 0.4 mM IPTG and the culture was incubated for 3 h at 37 °C until the OD_{600} reached ~1.7. Then, cells were harvested by centrifugation and washed once with Dulbecco's phosphate-buffered saline (D-PBS), pH 7.2. The cell pellet was resuspended in 10 mL of ice-cold D-PBS, pH 7.2, with a protease inhibitor mixture containing PMSF, TLCK, and TPCK [20]. After disruption of cells by the high pressure homogenizer (Avestin Inc., Ottawa, Canada) at 17,000 psi, soluble proteins were isolated by centrifugation at 30,000*g* at 4 °C for 1 h. Five milliliters of supernatant was subjected to gel filtration using a XK 16/100 column packed with ~180 mL of Sephacryl S-300 high resolution resin (GE Healthcare Bio-Sciences). The column was pre-equilibrated with D-PBS, pH 7.2, at a flow rate of 1 mL/min by ÄKTA Prime. The eluted fractions containing recombinant HP-NAP were further purified through a HiLoad 16/60 Superdex 200 prep grade gel filtration column (GE Healthcare Bio-Sciences) equilibrated with D-PBS, pH 7.2, by ÄKTA FPLC at a flow rate of 1 mL/min. Recombinant HP-NAP was eluted as a single peak. The peak fractions containing HP-NAP were pooled and stored at 4 °C no more than 1.5 months before use. The purity of HP-NAP was higher than 98% as confirmed by SDS–PAGE stained with PhastGel Blue R. To remove endotoxin from purified HP-NAP, pooled fractions were subjected to syringe filtration through the Acrodisc unit with positively-charged, hydrophilic Mustang E membrane (Pall, East Hills, NY). The amount of endotoxin present in recombinant HP-NAP was less than 14 endotoxin unit (EU)/mg protein.

Isolation of human neutrophils. Peripheral venous blood samples from six healthy volunteer donors, who signed informed consents, were drawn in vacuum blood collection tubes containing heparin. Heparinized blood was mixed with an equal volume of 3% dextran in 0.9% saline solution and incubated at room temperature for 20 min to sediment erythrocytes. The leukocyte-rich plasma (upper layer) was centrifuged at 250*g* at 5 °C for 10 min. Then, the pellet was resuspended with 0.9% saline solution and layered onto Ficoll-Paque PLUS. After centrifugation at 400*g* for 40 min at 20 °C with no brake, the pellet was subjected to two 30-s hypotonic treatments to remove contaminated erythrocytes. The final cell pellet was resuspended in Hanks's Balanced Salts Solution (HBSS), kept on ice, and used within 5 h. More than 92% of the leukocytes were neutrophils as judged by light microscopic examination of at least 600 cells on each Wright's stained cytocentrifuged slide. The cell viability exceeded 91% as gauged by trypan blue exclusion test.

Chemiluminescence assay for ROS production. Isolated human neutrophils adjusted to 2×10^6 cells/mL in HBSS were dispensed as 50 μL into a white 96-well plate (Packard, PerkinElmer, Boston, MA) and incubated at 37 °C. Subsequently, 150 μL of the mixture containing luminol and a stimulus in D-PBS was added into each well to a final volume of 200 μL. Luminol was maintained as a stock solution of 2 mM in dimethyl sulfoxide (DMSO) at room temperature and diluted in D-PBS immediately before use. The final concentration of luminol in the assay mixture was 10 μM. PMA, a positive stimulus, was maintained as a stock solution of 0.16 mM in DMSO at −20 °C until use. The final concentrations of recombinant HP-NAP and PMA were 1 and 0.08 μM, respectively. The emission of chemiluminescence was monitored in triplicate over a period of time by Wallac 1420 Multilabel Counter (PerkinElmer).

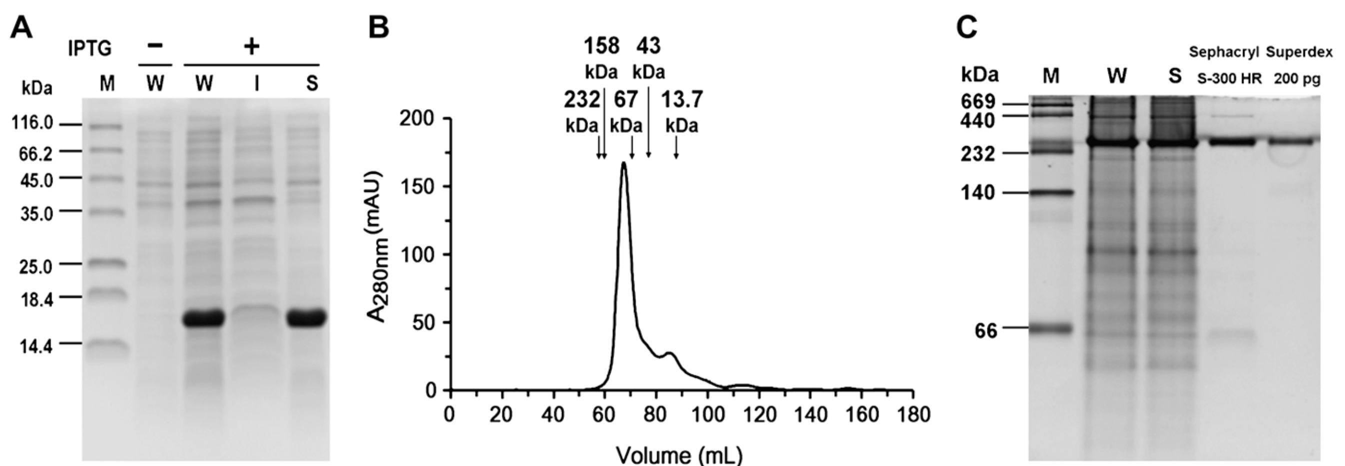

Fig. 1. Expression and purification of recombinant HP-NAP in *E. coli*. (A) Expression of HP-NAP in *E. coli. E. coli* BL21(DE3) harboring pET42a-NAP plasmid was induced in the absence (−) or presence (+) of IPTG. Whole cell lysate (W) was centrifuged to separate insoluble pellet (I) and soluble fraction (S). The samples were analyzed by 18% SDS–PAGE. (B) The UV absorbance profile of HP-NAP purified from a Superdex 200 gel filtration column. The fraction containing HP-NAP eluted from a Sephacryl S-300 HR column was applied to a Superdex 200 prep grade (Superdex 200 pg) column and subsequently eluted with D-PBS, pH 7.2. The eluent was monitored at 280 nm. (C) Native PAGE analysis of recombinant HP-NAP. The protein samples including whole cell lysate (W), soluble fraction (S), and the peak fractions containing the most abundant HP-NAP eluted from Sephacryl S-300 HR and Superdex 200 pg gel filtration columns were analyzed by 10% native PAGE. Molecular weights (M) in kDa are indicated on the left of the stained gels (A,C) or at the top of the chromatogram (B).

Assessment of MPO activity. Neutrophils (1.5×10^5 cells) were incubated with 1 μM HP-NAP, 1 μg/mL LPS, or 0.08 μM PMA for 30 min at 37 °C in a final volume of 150 μL. The cells were spun down by centrifugation at 1000*g* at 4 °C for 5 min. Then, 50 μL of each supernatant was mixed with 100 μL of TMB liquid substrate. After 30-min incubation at room temperature, the reaction was stopped by the addition of 100 μL of 1 M sulfuric acid. The absorbance of each reaction was measured at 450 nm using a THERMOmax microplate reader (Molecular Devices, Canton, MA). To determine the activity of MPO release from neutrophils, the data were applied to the standard curve, which was obtained by plotting the absorbance versus the corresponding known concentration of MPO diluted in D-PBS. MPO activity was expressed as units of activity per milliliter of supernatant.

Results

Purification and characterization of recombinant HP-NAP expressed in E. coli

HP-NAP was expressed in *E. coli* BL21(DE3) transformed with pET42-NAP plasmid under the induction of IPTG. As shown in Fig. 1A, recombinant HP-NAP was expressed as soluble proteins and revealed a molecular weight of ~17 kDa as analyzed by SDS–PAGE. A Sephacryl S-300 gel filtration column was used to purify recombinant HP-NAP. On the elution profile, the second peak corresponds to HP-NAP as confirmed by SDS–PAGE (Figs. S1 and S2). The fraction of this peak was further separated by a Superdex 200 gel filtration column, and HP-NAP was mainly eluted as a single peak (Fig. 1B and Fig. S2). The purity of HP-NAP was over 98%, and the yield of the protein was ~4.67 mg from 50 mL *E. coli* culture.

From gel filtration analysis, the molecular weight of recombinant HP-NAP was ~150 kDa (Fig. 1B), which is similar to the reported molecular weight of HP-NAP purified from *H. pylori* [9]. From native PAGE analysis, purified HP-NAP was visualized as a single band with apparent molecular weight of ~232 kDa (Fig. 1C). In addition, HP-NAP sedimented as a major peak at 9.38 S as analyzed by analytical ultracentrifugation (Fig. 2B), indicating the multimeric association of recombinant HP-NAP. The circular dichrosim spectrum of recombinant HP-NAP has a line shape that is typical of an α-helix (Fig. 2D), which is consistent with the previous reports [10,11]. Thus, recombinant HP-NAP expressed in *E. coli* assembled into a multimer with high helix content.

Increased production of reactive oxygen species in neutrophils stimulated by recombinant HP-NAP

HP-NAP purified from *H. pylori* was able to stimulate the production of reactive oxygen species (ROS) in neutrophils and monocytes [9,12]. Luminol-dependent chemiluminescence (CL) assay was used to determine whether recombinant HP-NAP expressed in *E. coli* also induces ROS production in human neutrophils. To avoid the possibility of endotoxin (lipopolysaccharide; LPS) contamination, a syringe filter with positively-charged, hydrophilic Mustang E membrane was applied to remove endotoxin from purified HP-NAP. As shown in Fig. 3A, a strong CL-response was evoked from neutrophils stimulated by PMA, a positive control. HP-NAP without endotoxin removal (unfiltered) triggered neutrophils to produce a moderate CL-response in comparison with unstimulated control (Fig. 3A). Similarly, HP-NAP with endotoxin removal (filtered) also had the ability to promote a CL-response from neutrophils (Fig. 3A). The peak of CL profile appeared around 13–16 min after the addition of stimuli (Fig. 3A). In order to determine the total responses of neutrophils stimulated by HP-NAP, the integral value of CL-response was served as a quantitative measure of ROS production. As shown in Fig. 3B, the integral value of CL-response of neutrophils stimulated by HP-NAP without endotoxin removal (unfiltered) was higher than that of control. HP-NAP with endotoxin removal (filtered) also increased the CL-response of neutrophils in comparison with unstimulated control (Fig. 3B). The result indicates that recombinant HP-NAP expressed in *E. coli* induces ROS production in neutrophils similar to HP-NAP isolated from *H. pylori*.

Increased release of myeloperoxidase from neutrophils stimulated by HP-NAP

We next investigated whether HP-NAP could induce MPO release from human neutrophils using TMB assay. Both HP-NAP without endotoxin removal (unfiltered) and HP-NAP with endotoxin removal (filtered) induced significant increases in MPO release from neutrophils (Fig. 4A). However, there was no significant difference of MPO activity in the extracellular media from

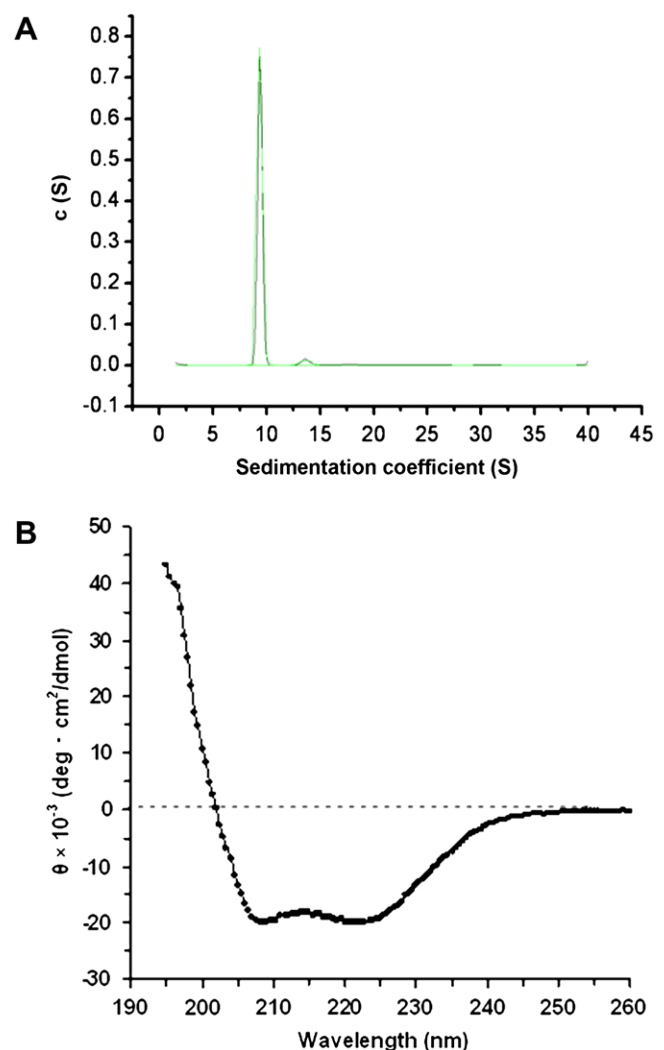

Fig. 2. Physical characteristics of recombinant HP-NAP expressed in *E. coli*. (A) Sedimentation coefficient of recombinant HP-NAP. The sedimentation analysis of HP-NAP was determined by analytical ultracentrifugation. The sedimentation coefficient distribution c(S) is shown as a function of S. The c(S) distribution was analyzed by using the software program SEDFIT. (B) Far UV circular dichroism spectrum of recombinant HP-NAP. Secondary structure of recombinant HP-NAP in D-PBS, pH 7.2, were determined by circular dichroism spectroscopy in the far UV spectral region (195–260 nm).

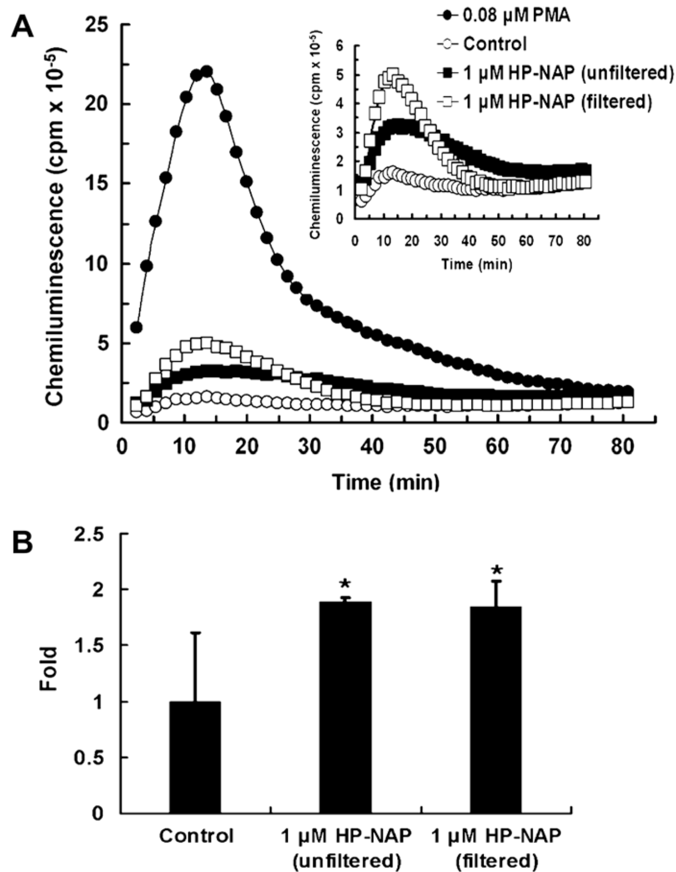

Fig. 3. Production of ROS from human neutrophils stimulated by HP-NAP. (A) The pattern of luminol-dependent chemiluminescence responses of neutrophils stimulated by HP-NAP. Human neutrophils (1×10^5 cells) were treated with 1 μM HP-NAP without endotoxin removal (unfiltered) (■), 1 μM HP-NAP with endotoxin removal (filtered) (□), 0.08 μM PMA (●) as a positive stimulus, and D-PBS as a negative control (○) at 37 °C. The content of ROS generated from neutrophils was measured continuously by using a luminol-dependent chemiluminescence (CL) assay as described in Materials and methods. The inset shows CL-responses of neutrophils treated with 1 μM HP-NAP and D-PBS. The result was represented as the profile of one experiment in triplicate. (B) The integral value of luminol-dependent chemiluminescence responses of neutrophils stimulated by HP-NAP. The integral CL signals were used to compare the responses of neutrophils treated with 1 μM HP-NAP without endotoxin removal (unfiltered), 1 μM HP-NAP with endotoxin removal (filtered), and D-PBS as a negative control. The values shown here are the fold changes for treated cells relative to control, which was set to 1. Data were represented as the mean ± S.D. of one experiment in triplicate (★, $p < 0.05$ vs. control). Similar results were obtained in two independent experiments.

neutrophils stimulated by HP-NAP without (unfiltered) and with (filtered) endotoxin removal (Fig. 4A). The result indicates that HP-NAP can promote MPO release from human neutrophils, which is independent of endotoxin. Although most endotoxin was removed from purified HP-NAP, residual endotoxin might stimulate neutrophils to release MPO. In order to rule out that endotoxin can activate neutrophils directly, LPS was used to examine its effect on MPO release from human neutrophils. As shown in Fig. 4B, the release of MPO from neutrophils stimulated by LPS was similar to that from untreated control. Thus, endotoxin cannot stimulate neutrophils to release MPO directly *in vitro*. Taken together, recombinant HP-NAP expressed in *E. coli* can induce degranulation of neutrophils with the release of MPO.

Discussion

The present study shows that recombinant HP-NAP induces the release of myeloperoxidase (MPO) from human neutrophils in addition to stimulating the production of reactive oxygen species (ROS). These results indicate that HP-NAP not only involves respiratory burst but also results in neutrophil degranulation. The increased amount of MPO released from HP-NAP-stimulated neutrophils might contribute to the increased amount of MPO found in the gastric mucosa in *H. pylori*-infected patients. In this study, HP-NAP is identified for the first time as a bacterial factor of *H. pylori* to induce MPO release from human neutrophils.

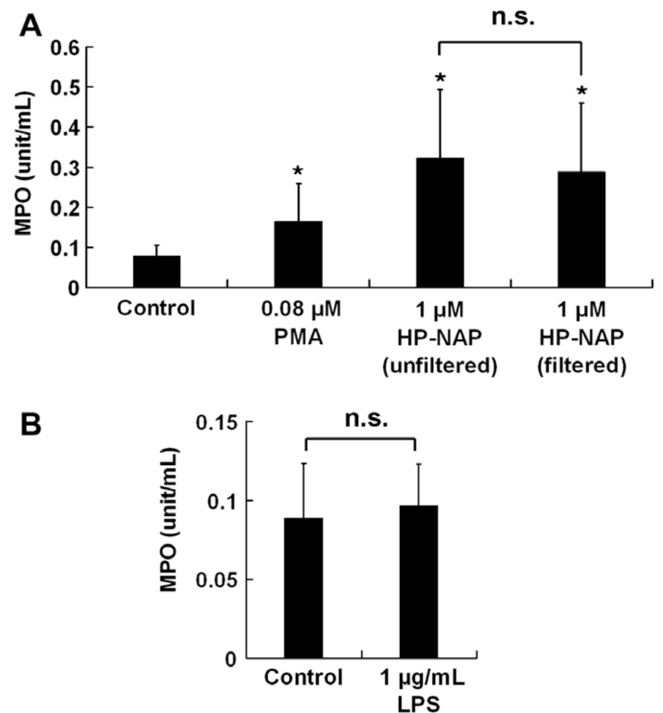

Fig. 4. Effects of HP-NAP and LPS on MPO release from human neutrophils. (A) The release of MPO from neutrophils stimulated by HP-NAP. Human neutrophils (1.5×10^5 cells) were exposed to 1 μM HP-NAP without endotoxin removal (unfiltered), 1 μM HP-NAP with endotoxin removal (filtered), 0.08 μM PMA as a positive stimulus, and D-PBS as a negative control at 37 °C for 30 min. The release of MPO was determined by TMB assay as described in Materials and methods. Data were represented as means ± SD of eight independent experiments in triplicate (★, $p < 0.05$ vs. control; n.s., not significant). (B) The release of MPO from neutrophils stimulated by LPS. Human neutrophils (1.5×10^5 cells) were exposed to 1 μg/mL LPS and D-PBS as a negative control at 37 °C for 30 min. The release of MPO was determined as described in (A). Data were represented as means ± SD of six independent experiments in triplicate (n.s., not significant).

For investigating the effect of HP-NAP on immune cells, endotoxin contamination in purified HP-NAP expressed in *E. coli* should not be neglected. One group has utilized polymixin B-coated magnetic beads to remove LPS from purified His-tagged HP-NAP expressed in *E. coli* [21]. However, the efficiency of endotoxin removal was not indicated. We have applied a syringe filter with positively-charged, hydrophilic Mustang E membrane to remove endotoxin from purified HP-NAP. The recovery of HP-NAP varied from 80% to 98.4%, whereas the filtration resulted in more than 97.6% of the amount of endotoxin removal from HP-NAP. The residual amount of endotoxin present in purified HP-NAP ranged from 0.4 to 14 EU/mg protein. The high efficiency of endotoxin removal and good recovery of HP-NAP indicate that this filtration step is a simple and efficient way to remove endotoxin from recombinant HP-NAP.

We have removed endotoxin form HP-NAP prior to evaluating its effect on ROS production and MPO release from neutrophils. However, one might argue that residual endotoxin present in purified HP-NAP may stimulate neutrophils. Previous evidence showed that direct LPS stimulation had no significant effect on chemiluminescence generation in neutrophils [22]. In addition, plasma factors

was required for LPS-inducd ROS production in neutrophils [23,24]. Thus, residual endotoxin present in purified HP-NAP could not affect ROS production in neutrophils. In terms of MPO release, it has been reported that LPS alone induced small but not significant increments in MPO release from neutrophils [25]. Also, endotoxin resulted in little or no MPO release from neutrophils in the absence of plasma [26]. Our finding that no significant difference in MPO release from human neutrophils stimulated by HP-NAP with and without endotoxin removal (Fig. 4A) supports the idea that residual endotoxin will neither induce nor prime neutrophils to release MPO.

In summary, HP-NAP can promote human neutrophils to release MPO. MPO generally serves as a maker of neutrophil activation and has been considered as an important pathophysiological factor in oxidative stress. Future studies are needed to define the basis of HP-NAP-induced MPO release from neutrophils in *H. pylori*-associated gastritis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.072.

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