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Helicobacter pylori induces IL-1β protein through the inflammasome activation in differentiated macrophagic cells

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ABSTRACT

More than 50% of people in the world are infected with *Helicobacter pylori* (*H. pylori*), which induces various gastric diseases. Especially, epidemiological studies have shown that *H. pylori* infection is a major risk factor for gastric cancer. It has been reported that the levels of interleukin (IL)-1β are upregulated in gastric tissues of patients with *H. pylori* infection. In this study, we investigated the induction mechanism of IL-1β during *H. pylori* infection. We found that IL-1β mRNA and protein were induced in phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 cells after *H. pylori* infection. This IL-1β production was inhibited by a caspase-1 inhibitor and a ROS inhibitor. Furthermore, K^+ efflux and Ca^{2+} signaling were also involved in this process. These data suggest that NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) and its complex, known as NLRP3 inflammasome, are involved in IL-1β production during *H. pylori* infection because it is reported that NLRP3 inflammasome is activated by ROS, K^+ efflux and/or Ca^{2+} signaling. These findings may provide therapeutic strategy for the control of gastric cancer in *H. pylori*-infected patients.

Helicobacter pylori (*H. pylori*) is a spiral-shaped, microaerophilic, gram-negative bacterium that establishes persistent infection in the stomach and causes gastric inflammation, which contributes to the progression of various gastric diseases such as chronic gastritis, gastric ulcers, and gastric cancer (4, 24, 26, 27). Many investigators have reported the relevance of cytokine production to *H. pylori* infection (23, 34). The inflammatory cytokines induced by *H. pylori* infection are associated with a marked infiltration of immune cells such as neutrophils, lymphocytes, monocytes/macrophages, and plasma cells in the gastric mucosa, which leads to *H. pylori*-associated gastroduodenal diseases (25). Among these cytokines induced, IL-1β is known to be one of the key cytokines during *H. pylori* infection because the levels of IL-1β are closely associated with gastric inflammation and carcinogenesis (5, 13). In addition, it has been described that *IL1B* polymorphisms are associated with the production levels of IL-1β during *H. pylori* infection (6), whereas IL-1β has a crucial role in gastric carcinogenesis *in vivo* (30, 33). According to these evidences, it is an important issue to clarify the induction mechanism of IL-1β

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caused by bacterial and host factor(s). Indeed, recent several studies reported the signaling pathways for IL-1β production (16, 19, 29). However, the detailed mechanism of *H. pylori*-mediated IL-1β induction remains fully clarified.

Host innate immune system acts as a front line of host defense against the infection of pathogens. To protect from invasion of these pathogens, host cells need to detect pathogen associated molecular patterns (PAMPs) by using pattern recognition receptors (PRRs). PRR-mediated signalings result in the activation of nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs) and the subsequent induction of various inflammatory cytokines such as IL-1β, IL-6 and TNF, and interferons (IFNs). These innate immune signalings and cytokine inductions contribute to not only inducing the early host response against pathogens but also linking to adaptive immune system in the late phase of infection. The coordinated responses by innate and adaptive immune systems are essential for efficient elimination of invading pathogens.

IL-1β production generally consists of at least two steps; induction of *IL1B* mRNA and maturation of proIL-1β protein. The latter step is known to be mediated through the activation of inflammasome. The inflammasome is innate immune system receptors/sensors that regulate the activation of caspase-1, which is a crucial enzyme for cleavage of proIL-1β protein (8). Several families of PRRs are known to be important components in the inflammasome complex, including NOD-like receptors (NLRs) such as NLRP3 and NLRC4. Which NLRs forms inflammasome and/or which molecule(s) trigger inflammasome response during *H. pylori* infection is an interesting issue.

In this study, we examined whether IL-1β is induced from human cells in response to *H. pylori* infection. We used a human leukemia cell line, THP-1 cells, which are differentiated into macrophages by using phorbol-12-myristate-13-acetate (PMA) and infected with *H. pylori*. At 8 or 24 h after infection, we analyzed the induction levels of IL-1β mRNA or proteins, respectively. Furthermore, we used several inhibitors to evaluate the inflammasome signalings.

MATERIALS AND METHODS

Cells, bacteria, and regents. THP-1 (RCB1189) cells were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT, Japan. THP-1 cells were cultured in RPMI 1640 medium (SIGMA) supplemented with 10% heat-inactivated fetal bovine serum (FBS: GIBCO). To induce differentiation of THP-1 cells into macrophages, THP-1 cells were stimulated with 1 μM PMA (P1585; SIGMA) for 3 h, then washed one time with PBS, and plated at 2 days prior to the experiments. An *H. pylori* (WT, NCTC11637) strain used has been reported previously (10). The inhibitors used were as follows: caspase-1 inhibitor (Ac-YVAD-CMK; Enzo Life Sciences, Farmingdale, NY, USA) used the final concentration at 50 μM, ROS inhibitor (N-acetyl-L-cysteine; Enzo Life Sciences) at 5 mM, P2X7 inhibitor (oxidized ATP; SIGMA) at 300 μM, inhibition of potassium efflux (KCl; Wako) at 40 mM, Ca^{2+} chelator (BAPTA/AM; AAT Bioquest, Sunnyvale, CA, USA) at 10 μM, PLC inhibitor (U73122; Cayman CHEMICAL, Ann Arbor, MI, USA) at 10 μM.

Bacteria culture and H. pylori infection. H. pylori was passaged on Trypticase Soy Agar II with 5% Sheep Blood (BD Biosciences) by incubation in microaerophilic condition (an atmosphere consisting of 8% O_2 and 6% CO_2) for 2 days at 37°C. For *H. pylori* infection with cells, bacteria were cultured in Brucella broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% FBS under the same conditions for 20–24 h at 37°C with shaking at 90–120 rpm. Bacteria were centrifuged at 6,000 rpm for one minute, resuspended in the appropriate cell culture medium, and then added to the cells at a multiplicity of infection (m.o.i.) = 100.

Quantitative-RT-PCR. PMA-differentiated THP-1 cells (2.5×10^5 cells/well, 24-well plate) were infected with *H. pylori* for 8 h. Total RNA was isolated with ISOGEN (NIPPON GENE, Tokyo, Japan), and were treated with DNaseI (Invitrogen). cDNA was synthesized with the ReverTra Ace Moloney murine leukemia virus reverse transcriptase with point mutations (TOYOBO, Osaka, Japan). Quantitative RT-PCR was analyzed with SYBR Premix Ex Taq reagent mixture (TAKARA, Shiga, Japan) and a StepOnePlus real-time PCR system (Applied Biosystems). Data were normalized to the expression of *ACTB* for each sample. Primers for quantitative RT-PCR were designed as follows; *IL1B*, 5'-GCCGCG TCAGTTGTTGTGGC-3' and 5'-TGAGTCCCG GAGCGTGCAGT-3'; *ACTB*, 5'-CATGTACGTTGC TATCCAGGC-3' and 5'-CTCCTTAATGTCACG CAT-3'.

ELISA. The culture supernatant of PMA-differentiated THP-1 cells (2.5×10^5 cells/well, 24-well plate)

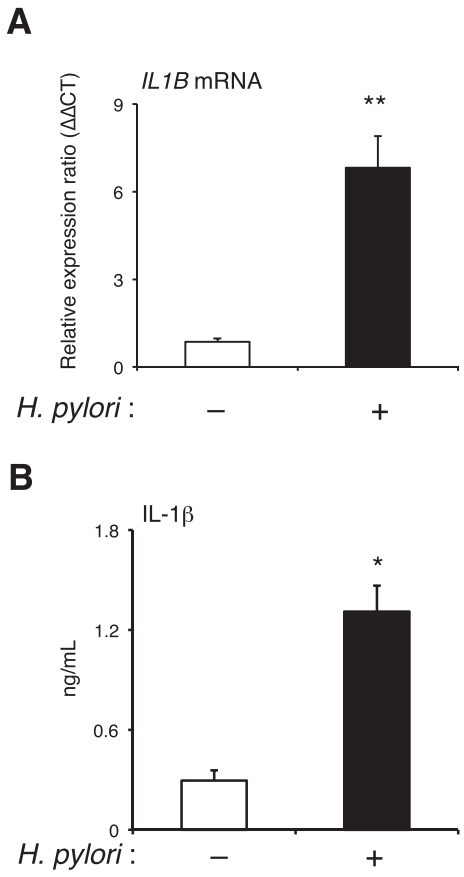

Fig. 1 IL-1β mRNA and protein were induced in response to *H. pylori* infection. PMA-differentiated THP-1 cells were infected with *H. pylori* (100 m.o.i.). At 8 or 24 h after *H. pylori* infection, the induction levels of IL-1β mRNA (A) or protein (B) were measured by quantitative RT-PCR or ELISA, respectively. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ** $P < 0.01$, * $P < 0.05$.

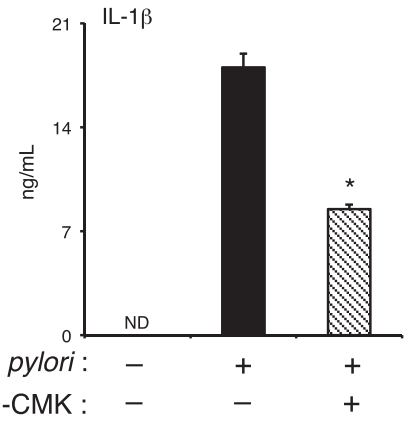

Fig. 2 IL-1β production was dependent on caspase-1. PMA-differentiated THP-1 cells treated with Ac-YVAD-CMK, were infected with *H. pylori* (100 m.o.i.) for 24 h. The induction levels of IL-1β protein were measured by ELISA. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ND, not detected. * $P < 0.05$.

was collected after 24 h of *H. pylori* infection. The levels of IL-1β protein were measured by IL-1β ELISA kit (R&D systems) according to manufacturer's protocol.

Statistical analysis. Values are shown as mean ± SD. Statistical significance between two samples was determined with Student's *t*-test.

RESULTS

IL-1β is produced during H. pylori infection in a caspase-1 dependent manner

To first examine whether IL-1β was secreted by *H. pylori* infection, PMA-differentiated THP-1 cells were infected with *H. pylori* and analyzed. As shown in Fig. 1A, IL-1β mRNA was induced at 8 h after infection with *H. pylori*. Next we studied the protein levels of IL-1β. IL-1β production was also observed at 24 h after infection (Fig. 1B). For the production of IL-1β, an intracellular cysteine protease, caspase-1, is required for processing the inactive precursors into mature form through the inflammasome complex (28). To evaluate the involvement of caspase-1 for the production of IL-1β, we used Ac-YVAD-CMK, a caspase-1 inhibitor. *H. pylori*-mediated IL-1β production was inhibited by treatment with Ac-YVAD-CMK (Fig. 2). These data indicated that in PMA-differentiated THP-1 cells, *H. pylori*-mediated IL-1β production is dependent on caspase-1, possibly through the activation of inflammasome.

H. pylori-mediated IL-1β production is dependent on reactive oxygen species, extracellular ATP and intracellular potassium

The inflammasome complex mainly consists of three members, NLRs, apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1. In many bacteria infection, the generation of reactive oxygen species (ROS) is a key signal for the activation of inflammasome (8). We examine whether ROS are important for the inflammasome activation during *H. pylori* infection. Treatment with a ROS inhibitor N-acetyl-L-cysteine significantly suppressed *H. pylori*-mediated IL-1β production (Fig. 3), suggesting that ROS signaling is involved in this process. In order to know upstream signals for the generation of ROS, we next studied the dependency of extracellular ATP and intracellular potassium (K^+)

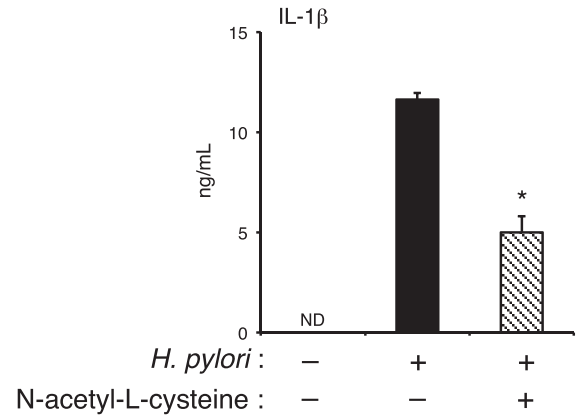

Fig. 3 ROS inhibitor suppressed *H. pylori*-mediated IL-1β production. PMA-differentiated THP-1 cells treated with N-acetyl-L-cysteine, were infected with *H. pylori* (100 m.o.i.) for 24 h. The induction levels of IL-1β protein were measured by ELISA. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ND, not detected. * $P < 0.05$.

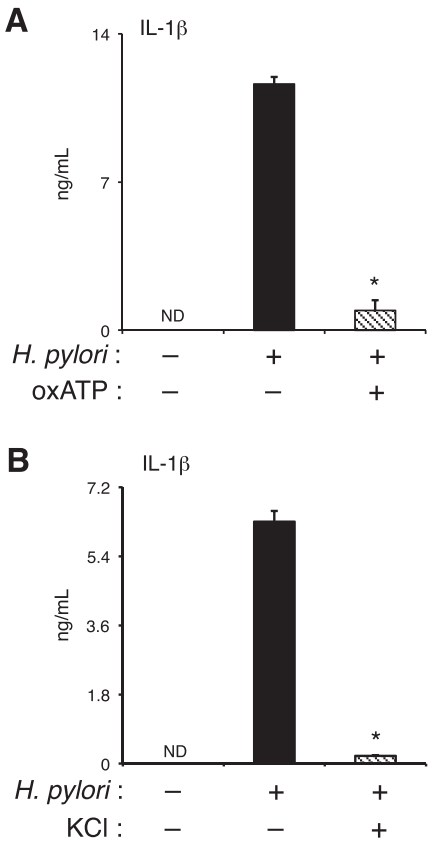

Fig. 4 Extracellular ATP and K^+ efflux were also involved in *H. pylori*-mediated IL-1β production. PMA-differentiated THP-1 cells treated with oxidized ATP (oxATP) (A) or KCl (B), were infected with *H. pylori* (100 m.o.i.) for 24 h. The induction levels of IL-1β protein were measured by ELISA. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ND, not detected. * $P < 0.05$.

signalings. As shown in Fig. 4A and 4B, *H. pylori*-mediated IL-1β was inhibited by treatment with oxidized ATP (oxATP) and KCl, respectively. These data suggest that extracellular ATP and intracellular K^+ signalings generate ROS, which activates inflammasome complex for the production of IL-1β in response to *H. pylori* infection.

Intracellular calcium signaling pathway also activates H. pylori-mediated inflammasome

Recently, intracellular calcium (Ca^{2+}) concentration triggers the inflammasome and subsequent producing ROS (8). We next investigated the involvement of Ca^{2+} signaling during *H. pylori* infection. Treatment with a Ca^{2+} cheator, BAPTA/AM, partially repressed *H. pylori*-mediated IL-1β production (Fig. 5). In addition, we used U73122, an inhibitor of phospholipase C (PLC), because PLC plays an important role in Ca^{2+} signaling. As shown in Fig. 6, IL-1β secretion during *H. pylori* infection was dependent on PLC. These data suggest that Ca^{2+} signaling also modulates the production of IL-1β during *H. pylori* infection.

DISCUSSION

It is well known that in *H. pylori*-infected patients, the levels of IL-1β are associated with the increased risk of gastric cancer development. Therefore, it is an important issue to reveal the detail mechanism for IL-1β induction in response to *H. pylori* infection. In this study, we firstly show that *IL1B* mRNA is induced after infection with *H. pylori*. In general, *IL1B* transcription is mediated by transcription factor NF-κB through PRR signalings such as TLR4 (8). In *H. pylori* infection, it is reported that TLR4-mediated NF-κB activation was observed in gastrointestinal epithelial cells and neutrophils (1, 31), whereas CagA, one of virulence factor, which is closely related to gastric cancer (22), also activates NF-κB (17). In relation to this, there are several reports about the relevance of *IL1B* mRNA levels to CagA positivity in *H. pylori*-infected patients (23, 32). Considering these reports, we could easily speculate that NF-κB activation mediated by TLR4 signaling and/or CagA regulates *IL1B* mRNA induction, although further analyses are required to clarify the contribution of CagA to NF-κB activation.

We also show that the protein levels of IL-1β are induced after *H. pylori* infection in PMA-differenti-

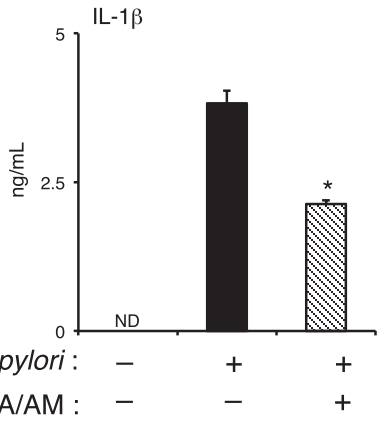

Fig. 5 Intracelluar Ca^{2+} signaling regulated the production of IL-1β during *H. pylori* infection. PMA-differentiated THP-1 cells treated with BAPTA/AM, were infected with *H. pylori* (100 m.o.i.) for 24 h. The induction levels of IL-1β protein were measured by ELISA. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ND, not detected. * $P < 0.05$.

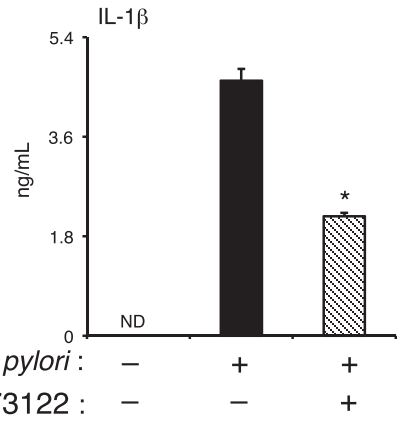

Fig. 6 PLC inhibitor suppressed *H. pylori*-mediated IL-1β production. PMA-differentiated THP-1 cells treated with U73122, were infected with *H. pylori* (100 m.o.i.) for 24 h. The induction levels of IL-1β protein were measured by ELISA. Data are from one representative of at least two independent experiments (mean and SD of triplicate samples). ND, not detected. * $P < 0.05$.

ated THP-1 cells. Consistent with our observation, a previous report demonstrated that human monocytes and macrophages secreted IL-1β in response to *H. pylori* infection (7). We next analyzed the induction mechanism of IL-1β. As mention above, it is well known that IL-1β production is mediated by inflammasome activation. In this study, we show that *H. pylori*-mediated IL-1β production is dependent on caspase-1, which is a crucial enzyme for the activation of inflammasome. To determine which signalings are involved in *H. pylori*-mediated caspase-1 activation, we used several representative inhibitors and evaluated IL-1β production. Consequently, we also reveal that ROS, extracellular ATP, intracellular K^+ and Ca^{2+} signalings are involved in the production of IL-1β, suggesting that NLRP3 inflammasome plays an important role in this process in human macrophages. In this regard, two recent studies by other groups using murine bone marrow-derived dendritic cells described the involvement of NLRP3 in *H. pylori*-induced IL-1β secretion, which is consistent with our observation.

At least a few models have been proposed for the activation of NLRP3 inflammasome. One model proposes a key role for ROS production, which is important for the priming step rather than the regulation of complex assembly, and other models implicate phagolysosomal damage and K^+ efflux (28). K^+ efflux is caused by external ATP, considered as a danger signal, which causes the opening of the P2X7 receptor and the subsequent recruitment of the channel pannexin-1 leading to the release of intracellular K^+ (15). It has also been reported that the bacterial toxin nigericin derived from *Streptomyces hygroscopicus* activates NLRP3 by causing K^+ efflux in a pannexin-1-dependent manner (21). In our study, IL-1β production is dependent on ROS, K^+ efflux and extracellular ATP, suggesting that some factors derived from *H. pylori* affect this process. Indeed, Semper *et al.* show that cag pathogenicity island (cagPAI) and vacuolating cytotoxin A (VacA) are involved in the process of inflammasome activation (29). Further investigations are needed to clarify the detail mechanism for the NLRP3 activation and involvement of other bacterial factors.

We also demonstrate that intracellular Ca^{2+} signaling also regulates IL-1β production in response to *H. pylori* infection. Recent accumulating evidences show that intracellular Ca^{2+} signaling also plays a crucial role in NLRP3 activation (12, 18). In this regard, there are interesting data that intracellular Ca^{2+} concentration is increased through *cagA* and *picB/cagE* genes after *H. pylori* infection and this Ca^{2+} upregulation is regulated by a PLC-dependent mechanism (20). In addition, another group reported that CagA was interacted with PLCγ1 (3). CagA is known to be an effector protein that is translocated directly from bacteria into the host cells via the effector translocator system, known as a type IV secretion system (T4SS), and to interact with many host proteins, which leads to interfere with cellular signaling pathways that regulate cell growth, morphology, and motility (2, 9, 26, 27). Considering these reports, one could speculate that CagA activates not only

IL1B mRNA induction but also inflammasome assembly for IL-1β production, although further analyses are needed to clarify the involvement of CagA and detail mechanism for the activation of NLRP3 inflammasome during *H. pylori* infection. In order to rule out the involvement of other virulence factors derived from *H. pylori*, reconstitution assay of the NLRP3 inflammasome might solve this point.

This study revealed that *H. pylori* infection induces IL-1β mRNA and protein in human macrophages, possibly through the NLRP3 inflammasome activation. It is reported that IL-1β increased in the stomach during *H. pylori* infection is strongly associated with the generation of gastric carcinoma (30, 33). In addition, recent evidences show that *H. pylori* and IL-1β are closely related with not only gastric carcinoma but also diabetes and Alzheimer's disease (11, 14). Thus our findings may have clinical implications for *H. pylori*-associated diseases, and further research is desired.

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