Helicobacter pylori lipopolysaccharide activates Rac1 and transcription of NADPH oxidase Nox1 and its organizer NOXO1 in guinea pig gastric mucosal cells


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expression of Nox1 and NOXO1 mRNA and activates Rac1; i.e., it converts Rac to the GTP-bound form. Using a phosphoinositide 3-kinase (PI3K) inhibitor, LY-294002, and an adenosine receptor encoding constitutively active Rac1, we suggest that H. pylori LPS-stimulated \(O_2^\cdot\) production in gastric mucosal cells is controlled by two distinct mechanisms: 1) transcriptional upregulation of Nox1 and NOXO1 and 2) activation of Rac1.

**MATERIALS AND METHODS**

Preparation of primary cultures of gastric mucosal cells under LPS-free conditions. Specific pathogen-free male Hartley guinea pigs were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and treated in accordance with the guidelines of the National Institutes of Health. The present study also was approved by the Animal Care Committee of the University of Tokushima. Gastric mucosal cells were isolated from fundic glands and prepared under LPS-free conditions (21). Cells were cultured for 2 days in RPMI 1640 medium supplemented with 2 mM glutamine, 10% (vol/vol) fetal bovine serum (FBS), 0.1 mg/ml streptomycin, and 100 U/ml penicillin in 5% CO\(_2\)-95% air at 37°C. Growing cells were composed of pit cells (>90%), granule-free pit cell precursors (5%), parietal cells (5%), and fibroblasts (<1%) (38, 39), and pit cells were confirmed to be responsible for O\(_2^\cdot\) generation (39). After being washed with RPMI 1640 medium, the cells maintained in the medium supplemented with 10% FBS were used for experiments. Viability of the cells treated with various compounds or transfected with adenoviral vectors was maintained throughout the experiments, which were based on lack of lactate dehydrogenase release into reaction medium, continued Trypan blue exclusion, and adherence to the culture plates. For measurement of O\(_2^\cdot\) production after washing with HBSS, these cells were incubated in HBSS containing 80 μM cytochrome c. The rate of O\(_2^\cdot\) release was determined by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c and expressed in nanomoles per milligram of protein per hour (39).

**Purification of H. pylori LPS and lipid A.** A clinical strain of type 1 mureinase factor A. A clinical strain of type 1 H. pylori was used in this study (indicated as H. pylori 1 in Ref. 21). H. pylori LPS and lipid A were purified from this strain, and the endotoxin activities of LPS and lipid A were determined to be 109 and 22.1 endotoxin units (EU)/μg, respectively, using the Limulus amoebocyte lysate assay (21).

**Reverse transcriptase (RT)-PCR.** Total RNA was isolated from the indicated cells with an acid guanidium-thiocyanate-phenol chloroform mixture (38), and cDNA was synthesized with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). The following primer sets were used to amplify the respective guinea pig cDNA products with GeneAmp PCR system (Applied Biosystems): tumor necrosis factor-α (TNF-α), 5’-AAAATGACATGCGGACT-3’ and 5’-GTACATCATCTACTTTCCG-3’; and cyclooxygenase-2 (COX-2), 5’-CCAGGTTTGATACATCTTACCA-3’ and 5’-AAGTTTGATACATCTTACCA-3’. The primer sets for Nox1, gp91phox, Nox4, NOX1, p67phox, NOX1A, p22phox, Rac1, Rac2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were described previously (19). Amplified PCR products were ligated into a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. The PCR products were confirmed to be human PAK2 and subcloned into pGEX-4T-1 (Amersham Pharmacia). The glutathione S-transferase (GST)-fused protein was expressed in Escherichia coli JM109 cells and purified by glutathione-Sepharose-4B (Amersham Pharmacia) (2).

**RESULTS**

**H. pylori LPS-induced O\(_2^\cdot\) production and Nox1 mRNA expression in gastric mucosal cells.** Guinea pig gastric mucosal cells cultured under LPS-free conditions spontaneously produced O\(_2^\cdot\) at a rate of ~10 nmol·mg protein\(^{-1}·\)h\(^{-1}\), while they secreted 10 times greater amounts of O\(_2^\cdot\) when treated with H. pylori LPS at 180 ng/ml or higher (21). As shown in Fig. 1A, after exposure to 180 ng/ml of H. pylori LPS, the rate of O\(_2^\cdot\) production began to increase within 4 h and reached a maximum at 16 h. In response to H. pylori LPS, TNF-α and Cox-2 mRNA were expressed within 2 h, and these expressions were observed continuously up to 24 h (Fig. 1B). The presence of a...
Fig. 1. Effects of *Helicobacter pylori* (*H. pylori*) lipopolysaccharide (LPS) on \( \text{O}_2^- \) production and NADPH oxidase 1 (Nox1) mRNA expression. A: after guinea pig gastric mucosal cells cultured under LPS-free conditions were left untreated or were treated with 5 \( \mu \)M diphenylene iodonium (DPI) for 30 min, they were then exposed to 180 \( \mu \)g/ml [21 endotoxin units (EU)/ml] of *H. pylori* LPS in RPMI 1640 medium containing 10% fetal bovine serum (FBS) for the indicated times. \( \text{O}_2^- \) generation by these cells was assayed by the cytochrome \( c \) method. B: after cells were untreated or treated with 180 \( \mu \)g/ml *H. pylori* LPS for the indicated times in the absence or presence of either 5 \( \mu \)M DPI or 200 \( \mu \)M cycloheximide was present during the 16-h treatment. C: after pretreatment with 75 \( \mu \)M actinomycin D or 5 \( \mu \)g/ml cycloheximide for 30 min, these cells were exposed to 180 \( \mu \)g/ml of *H. pylori* LPS for 16 h. Actinomycin D or cycloheximide was present during the 16-h treatment. D: after gastric mucosal cells were left untreated or were treated with 180 \( \mu \)g/ml *H. pylori* LPS for 4 h, expression of the Nox1 mRNA in the cells was measured using RT-PCR using Caco-2 cells as a positive control. E: expression of gp91phox and Nox1 mRNA in gastric mucosal cells exposed to 180 \( \mu \)g/ml *H. pylori* LPS for 4 h was assayed using RT-PCR. Human and guinea pig peripheral blood leukocytes (PBL) and guinea pig kidney were used as the corresponding positive controls. The number of cycles is indicated in D and E. Values are means ± SD; \( n = 8 \), *P < 0.001 compared with untreated and *H. pylori* LPS-treated cells (ANOVA and Scheffé’s test) (A and C, respectively). These experiments were repeated 3 times with similar results. *Hp, H. pylori*; GMCs, gastric mucosal cells.

flavoprotein inhibitor, diphenylene iodonium (DPI), blocked the LPS-triggered increase in \( \text{O}_2^- \) production (Fig. 1A). At the same time, DPI inhibited the LPS-induced expression of TNF-\( \alpha \) and Cox-2 mRNA at 8 h or later (Fig. 1B). At these time points, gastric mucosal cell oxidase was fully activated (Fig. 1A). Inclusion of SOD and catalase did not affect the LPS-stimulated expression of the mRNA within 4 h, while it blocked this expression in the late phase similarly to the way DPI did (Fig. 1B). These results suggest that reactive oxygen species (ROS) produced by the *H. pylori* LPS-treated cells also may enhance the inflammatory responses of gastric mucosal cells to *H. pylori* infection.

*H. pylori* LPS-triggered \( \text{O}_2^- \) production was completely canceled in the presence of actinomycin D as well as cycloheximide (Fig. 1C), suggesting a regulation at the transcriptional level. To test this possibility, we first examined whether *H. pylori* LPS stimulated expression of mRNA for Nox1 or other \( \text{O}_2^- \)-producing enzymes. Using the sequence of the guinea pig Nox1 cDNA that we had obtained (GenBank accession no. AB099629), we performed RT-PCR and found that quiescent cells maintained under LPS-free conditions expressed a small amount of the Nox1 mRNA, which was amplified by PCR at 30 cycles but not at 20 cycles (Fig. 1D). Treatment with *H. pylori* LPS for 4 h increased the Nox1 mRNA level (Fig. 1D). On the other hand, the gp91phox and Nox4 mRNA were not detected in guinea pig gastric mucosal cells even after treatment with *H. pylori* LPS (Fig. 1E), as well as in the quiescent cells (data not shown). Thus the \( \text{O}_2^- \) production induced by LPS appears to involve the enhanced expression of Nox1.

Induction of NOXO1 mRNA expression by *H. pylori* LPS. We next investigated the expression of mRNA for Nox-activating proteins such as NOXO1 and NOX1 in gastric mucosal cells untreated or treated with *H. pylori* LPS. For this purpose, we had cloned the guinea pig NOXO1 cDNA (GenBank accession no. AB105906) (19), the deduced amino acid sequence of which shows 72% identity with that of human NOXO1 (GenBank accession no. AF539796). Northern blot analysis performed with a probe derived from the cDNA revealed that *H. pylori* LPS stimulated the expression of NOXO1 mRNA, with a peak at 4 h (Fig. 2A), and a similar time course was observed in the upregulation of Nox1 mRNA (Fig. 2A). Lipid A of *H. pylori*, which is also capable of enhancing \( \text{O}_2^- \) generation by gastric mucosal cells (21), stimulated the expression of NOXO1 and Nox1 mRNA (Fig. 2B). The expression of these two mRNA was blocked by polymixin B (Fig. 2B), an agent that interacts with the lipid A moiety of LPS and inhibits the *H. pylori* LPS- or lipid A-triggered elevation of \( \text{O}_2^- \) generation (21).

In contrast to the NOXO1 mRNA, the p47phox mRNA does not seem to exist in quiescent guinea pig gastric mucosal cells or in cells stimulated with *H. pylori* LPS or its lipid A. Although we had previously obtained a very weak signal of a 47-kDa protein immunoreactive to an antibody against human...
p47phox in guinea pig gastric pit cells (39), the carefully performed Northern blot analysis (Fig. 2A) and RT-PCR (Fig. 2B) in the present study revealed that neither quiescent nor stimulated cells express p47phox mRNA.

We also estimated the level of the mRNA for Nox activators, i.e., NOXA1 and p67phox, in gastric mucosal cells. To do this, we had cloned the cDNA for guinea pig NOXA1 (GenBank accession no. AB105907) and p67phox (GenBank accession no. AB105909) (19) and performed RT-PCR and Northern blot analysis on the basis of the cDNA sequences. As shown in Fig. 2, A and B, the NOXA1 and p67phox mRNA were expressed in quiescent cells, whereas neither H. pylori LPS nor lipid A increased these mRNA levels. This may be inconsistent with our previous finding that in guinea pig gastric mucosal cells, the amount of a 67-kDa protein that cross-reacted with an antibody against human p67phox increased in parallel with elevation of O2/H2O2 generation after treatment with H. pylori LPS (21). To explore this inconsistency, we developed a novel polyclonal antibody against human recombinant p67phox that recognized the guinea pig p67phox with a molecular mass of 63 kDa, and the amount was not affected by H. pylori LPS (data not shown). On the basis of these findings, we concluded that H. pylori LPS did not stimulate the induction of p67phox. In addition, the mRNA for p22phox, a possible partner of Nox1 (37), was expressed in quiescent gastric mucosal cells, and the amount of the mRNA did not change even after the addition of H. pylori LPS (Fig. 2B). Taken together, gastric mucosal cells constitutively express the mRNA for NOXA1, p67phox, and p22phox, and the treatment with H. pylori LPS specifically induces the transcription of the Nox1 and NOXO1 genes, which is likely involved in the induction of O2 generation.

Effects of LY-294002 on H. pylori LPS-triggered O2 generation. Activation of PI3K plays a crucial role in the activation of the phagocyte oxidase in response to a chemoattractant, N-formylmethionyl-leucyl-phenylalanine (fMLP) (10, 18, 32). In this study, we tested whether a PI3K inhibitor, LY-294002, suppressed H. pylori LPS-triggered elevation of O2 secretion from gastric mucosal cells. Pretreatment with LY-294002 for 30 min dose dependently blocked the LPS-stimulated upregulation of O2 secretion from gastric mucosal cells. Pretreatment with LY-294002 for 30 min dose dependently blocked the LPS-stimulated upregulation of O2 secretion, with an IC50 of 7 μM (Fig. 3A). It should be noted that at 0.5 to 50 μM concentrations, LY-294002 did not affect the LPS-stimulated expression of Nox1 and NOXO1 mRNA (Fig. 3B). Phosphorylated lipid products generated by PI3K act as second messengers to activate protein kinases, including Akt (18). We also confirmed that H. pylori LPS phosphorylated Akt at Ser473 and that pretreatment with LY-294002 completely blocked the phosphorylation in H. pylori LPS-treated cells (Fig. 3C). These results suggest that in addition to the expression of Nox1 and NOXO1, a LY-294002-sensitive event appears to be involved in the H. pylori LPS-enhanced O2 generation.

Activation of Rac1 by stimulation with H. pylori LPS. It is known that PI3K inhibitors suppress activation of Rac in neutrophils (2), which is likely involved in the blockade of...
activation of the phagocyte NADPH oxidase. On the other hand, it remains unclear whether Rac plays a role in the activation of the other NADPH oxidases, including Nox1. To elucidate the role of Rac in the 

*H. pylori* LPS-induced generation of $O_2^−$, we first examined the expression of Rac isoforms in guinea pig gastric mucosal cells. As shown in Fig. 4A, gastric mucosal cells expressed only Rac1 mRNA between the two Rac isoforms thus far identified in guinea pigs (25), in contrast to the predominant expression of Rac2 in human neutrophils (17).

We next tested whether *H. pylori* LPS activated endogenous Rac in gastric mucosal cells by a performing pull-down assay using the Rac-binding domain of the protein kinase PAK2 (PAK2-RBD). PAK2 binds to GTP-bound Rac with high
affinity, whereas the affinity for the GDP-bound form is undetectably low (2, 41). As shown in Fig. 4B, Rac1 in guinea pig gastric mucosal cells stimulated by H. pylori LPS bound to GST-fused PAK2-RBD but not to GST alone. This activation occurred within 5 min and continued for up to 16 h (Fig. 4C). H. pylori LPS dose dependently activated Rac1 (Fig. 4D), and its minimum effective concentration of 180 ng/ml was similar to that required for the upregulation of O$_2^-$ production (21). Furthermore, LY-294002 blocked the LPS-stimulated activation of Rac1 (Fig. 4E) in association with the inhibition of O$_2^-$ generation (Fig. 3A), suggesting the involvement of Rac1 activation, an event sensitive to LY-294002, in the LPS-induced O$_2^-$ generation by Nox1.

Restoration of LY-294002-inhibited O$_2^-$ generation by expression of a constitutively active Rac1. To confirm the role of Rac in H. pylori LPS-stimulated O$_2^-$ generation, we transduced an adenoviral vector encoding a constitutively active form of Rac1 (G12V) to gastric mucosal cells and tested whether it affected the inhibition of O$_2^-$ production using LY-294002. As shown in Fig. 5, the active Rac1 (G12V) dose dependently restored O$_2^-$ production even in the presence of LY-294002. On the other hand, an inactive form of Rac1 (T17N) failed to do so. The findings indicate that expression of Rac1 plays an essential role in LPS-stimulated O$_2^-$ generation. The role appears to be specific for Rac because a constitutively active form of Cdc42 (G12V), another Rho family small GTPase, had no effect (Fig. 5). Thus expression of the active Rac was required for the induction of O$_2^-$ generation when LPS-stimulated cells were treated with LY-294002, an agent that did not affect the expression of Nox1 and NOXO1 (Fig. 3B). On the other hand, the constitutively active Rac1 failed to stimulate O$_2^-$ generation by the quiescent cells, alternatively supporting the importance of the expression of Nox1 and NOXO1 (Fig. 5).

**DISCUSSION**

In the present study, we have shown that H. pylori LPS induced the transcription of the Nox1 and NOXO1 genes and activated the GTPase Rac1 in gastric mucosal cells, and also that the two events played crucial roles in O$_2^-$ production induced by the bacterial component. The property of the Nox1 gene as an inducible one also has been demonstrated in other types of cells. Interferon-γ, 1α,25-dihydroxyvitamin D$_3$, or *Salmonella enteritidis* flagellin stimulates the Nox1 transcript expression in colon cancer cell lines (13, 19), and platelet-derived growth factor or angiotensin II also upregulates the expression level of Nox1 mRNA in rat vascular smooth muscle cells (28, 35).

Activation of Nox1 as well as of gp91$^{{phox}}$ requires both a Nox organizer and a Nox activator; the organizers include p47$^{{phox}}$ and NOXO1, and the activators include p67$^{{phox}}$ and NOXA1 (4, 6, 14, 37). Gastric mucosal cells constitutively express the two Nox activators (Fig. 2). Hence, expression of a Nox organizer is required for the oxidase activation in these cells. Indeed, H. pylori LPS induces the transcription of the NOXO1 gene (Fig. 2). To the best of our knowledge, the present study is the first to show that NOXO1 is regulated at the transcriptional level. At present, no specific antibodies for guinea pig Nox1 and NOXO1 are available for immunoblotting; therefore, the levels of these two proteins and the kinetics of their synthesis were not determined. However, the inhibition of the LPS-stimulated O$_2^-$ production with cycloheximide indirectly supports that synthesis of Nox1 and NOXO1 proteins accompany the expression of their mRNA.

For activation of the phagocyte oxidase containing gp91$^{{phox}}$, two switches are required to be turned on at the same time: conformational change of p47$^{{phox}}$ and activation of Rac. The conformational change of p47$^{{phox}}$ allows its SH3 domains to bind to p22$^{{phox}}$ for the oxidase activation; the SH3 domains are normally masked via intramolecular interaction with the auto-inhibitory region. Because this region is absent in NOXO1 (4, 6, 14, 37), NOXO1 may exist in a constitutively active form. Indeed, NOXO1 is capable of binding via its SH3 domains to p22$^{{phox}}$ without any conformational changes (37). p22$^{{phox}}$ is constitutively expressed in gastric mucosal cells. Therefore, once NOXO1 is synthesized with Nox1 in the LPS-treated gastric mucosal cells, Nox1 assembled with p22$^{{phox}}$ is expected to constitutively interact with NOXO1, thereby entering into a quasi-activated state. Cheng and Lambeth (6) also have shown the colocalization of NOXO1 and Nox1 in HEK-293 cells overproducing these proteins.

Several lines of evidence demonstrated in the present study suggest that activation of Rac serves as a switch for the activation of Nox1 similarly to that for gp91$^{{phox}}$. First, Rac1 activation by H. pylori LPS occurred much earlier than the increase in O$_2^-$ production; however, Rac1 was still in an active state (Fig. 4B) when gastric mucosal cells increased O$_2^-$ production (Fig. 1A). Second, LY-294002 inhibited the LPS-induced activation of Rac1 in a similar dose-dependent manner because it blocked O$_2^-$ generation (Figs. 3 and 4). Finally, expression of a constitutively active Rac1 completely restored O$_2^-$ production in the presence of LY-294002 (Fig. 5). For the restoration, Cdc42 failed to replace Rac (Fig. 5), although Cdc42 is highly similar to Rac in its amino acid sequence. In agreement with this finding, the Nox activators p67$^{{phox}}$ (24)
and NOXA1 (37) are capable of binding to GTP-bound Rac but not to GTP-bound Cdc42.

The kinetics of activation of Rac2 in fMLP-stimulated neutrophils coincides with rapid and transient generation of $O_2^-$ in these cells (2). The activity of the small GTPase is regulated by guanine-nucleotide exchange factor (GEF) (40) and GTPase-activating protein (GAP) (30). A recent study (12) suggested that the neutrophil NADPH oxidase might be regulated by GAP, including Breakpoint cluster region protein, p50RhoGAP, and p190RhoGAP. Recently, it was shown that ROS production in growth factor-stimulated cells is mediated by the sequential activation of PI3K, a Rac-GEF (BpIX), and Rac1 (33). In gastric mucosal cells, $H.\ pylori$ LPS activated Rac1 within 5 min, while rapid activation was not linked to enhanced $O_2^-$ production. The continuous activation of Rac1 and the slower induction of Nox1 and NOXO1 may result in the full activation of the oxidase. The Rac1 activation in the intervening period may exhibit distinct functions besides activation of Nox1 and the suppression of spontaneous apoptosis after the maturation of pit cells (38). With regard to activation of Nox1, studies to identify GEF and GAP as proteins that are involved in Nox1 activation in guinea pig gastric mucosal cells are underway.

Gastric mucosal cells maintained under LPS-free conditions spontaneously produced $-10 \ \text{nmol} \ O_2^-/\text{mg} \ \text{of protein/h}$. DPI and/or LY-294002 partially reduced the production by 40–50%, indicating that the primary cultures may contain a small number of activated cells. However, these cells still have Nox-1 and Rac1-dependent $O_2^-$-producing capability. At present, the source of the activity is unknown. However, basal $O_2^-$ generation plays a crucial role in the stimulation of cell growth and the suppression of spontaneous apoptosis after the maturation of pit cells (38). With regard to activation of Nox1, colonic epithelial cells (T84 cells) preferentially use the Toll-like receptor (TLR)5, rather than TLR4, against $S.\ enteritidis$ infection in vitro (19), whereas TLR4 and its adaptor protein (MD-2) are crucial against $H.\ pylori$ infection in gastric epithelial cells (21). Thus gastric and colonic epithelial cells may use different TLR members to discern pathogenicities among bacteria, depending on their environment and to activate Nox1 appropriately for host defense. Although the amounts of $O_2^-$ produced by the gastric and colonic epithelial cells are not enough to directly kill $H.\ pylori$ and $S.\ enteritidis$, respectively, at least in vitro (19, 39), Nox1 may be one of the key molecules representing the initial trigger for host innate and inflammatory responses against microbial pathogens.

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